# Structural studies on angiogenic proteins

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# STRUCTURAL STUDIES ON ANGIOGENIC PROTEINS

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*To my parents*

*with all my love*

## ABSTRACT

Angiogenesis is the process of formation of blood vessels from pre-existing blood vessels. It occurs in a series of orchestrated network of cooperative interactions. This process plays a vital role in reproduction, growth and healing. The hypothesis made by Judah Folkman in 1971 forms the groundwork for the entire angiogenesis research. This hypothesis stated that blood vessel formation is integral to tumour progression. Equilibrium between positive and negative regulators of angiogenesis is the key determinant to the state of the capillary networks. Malignant transformations are characterised by the onset of angiogenesis, which occur when this balance tips in favour of the inducers of the process. During the last decade numerous angiogenic genes and gene products have been isolated and purified from both normal and neoplastic tissues. These regulatory molecules induce or inhibit blood vessel formation by both paracrine and autocrine mechanisms. Comprehensive pre-clinical research activities have translated the various advances in basic angiogenesis research into possible clinical therapies by using a number of *in vitro*, *in vivo* and transgenic animal models.

Biomedical research at present is going through an era of Structural Genomics and hence the elucidation of protein structures has become fundamental to the understanding of complex molecular mechanisms. Protein crystallography is an essential tool for solving the three-dimensional structure of proteins. It enables a detailed insight into the function of proteins by virtue of their three-dimensional structure.

One of the main themes of the research carried out in our laboratory is concerned with the structure determination of proteins involved in the process of angiogenesis and inflammation. The work described in this thesis involved the use of X-ray crystallography for structural studies on some important angiogenic molecules: Placenta Growth Factors (**PlGF-1** and **PlGF-2**), Vascular Endothelial Growth Factor-B (**VEGF-B**) and Matrix Metalloproteinase-1 (**MMP-1**) and the Tissue Inhibitor of Metalloproteases-1 (**TIMP-1**).

Placenta Growth Factor (PlGF) is a member of the cysteine-knot family of growth factors. PlGF exists in three different forms with all the three isoforms differing from one another in the number of residues in the mature protein and their ability to bind heparan sulphate proteoglycans (HSPGs). PlGF-1 shares around 42% amino acid sequence identity with another growth factor, Vascular Endothelial Growth Factor-A (VEGF-A). In this study, the crystal structure of recombinant PlGF-1 was elucidated at both 2.0Å and 1.55Å resolution. This study provides a structural basis for altered specificity of this molecule based on biochemical and mutagenesis data based on both the native structure of PlGF-1 as well as modelling studies on PlGF-1 with its tyrosine-kinase receptor, Vascular Endothelial Growth Factor Receptor-1 (VEGFR-1). Also discussed in this thesis are some preliminary structural results obtained for PlGF-2 using Circular Dichroism.

Vascular Endothelial Growth Factor-B (VEGF-B) is another important member of the cysteine-knot family of growth factors. The molecule exists as two splice variants: VEGF-$B_{167}$ and VEGF-$B_{186}$. VEGF-B is a functional homologue of PlGF based on its binding specificity to VEGFR-1. However VEGF-B seems to be more similar to VEGF-A at the primary sequence level. Recombinant VEGF-$B_{10-108}$ (truncated form of VEGF-$B_{167}$) was crystallised using 2,4-dimethyl pentane diol (MPD) as a precipitant by the hanging-drop vapour-diffusion method. X-ray diffraction data have been collected to 3.50 Å resolution at 100 K using synchrotron radiation. The crystals belong to the hexagonal space group P6(4) with unit-cell parameters a = b = 121.62, c = 40.04.

The Matrix Metalloproteinases (MMPs) is a large family of zinc endopeptidases (26 members) characterised by the presence of a structural zinc ion. MMP activity is regulated by tissue-inhibitor of metalloproteinases (TIMPs). Preliminary diffraction studies on the crystals of the nTIMP-1•MMP-1:CD complex have been discussed. The crystals belong to C2 space group and diffract to 2.54 Å resolution.

## ABBREVIATIONS

| | |
|---|---|
| ADP | Atomic Displacement Parameter |
| BAECs | Bovine Aortic Endothelial Cells |
| BDNF | Brain-derived Neurotrophic Factor |
| CAM assay | Chorioallantoic membrane assay |
| CCD | Charge-Coupled Detector |
| CCP4 | Collaborative Computational Project, Number 4 |
| CD | Circular Dichroism |
| CNS | Crystallography and NMR System |
| DM | Density Modification |
| EBI | European Bio-informatics Institute |
| ECs | Endothelial Cells |
| ECM | Extra-cellular Matrix |
| EPMR | Evolutionary Program for Molecular Replacement |
| FFFEAR | Fast Fourier Feature Recognition |
| FFT | Fast Fourier Transform |
| FGF | Fibroblast Growth Factor |
| Flt-1 | *fms*-like tyrosine kinase-1 |
| FOM | Figure of Merit |
| HSPG | Heparan Sulphate Proteoglycan |
| HUVECs | Human Umbilical Vein Endothelial Cells |
| IF-γ | Interferon-γ |
| IL | Interleukin |
| KDR | Kinase-insert Domain-containing Receptor |
| LLG | Log Likelihood Gain |
| mAbs | Monoclonal Antibodies |
| MAD | Multi-wavelength Anomalous Dispersion |
| MDL | Molecular Dimensions Limited |
| MIR | Multiple Isomorphous Replacement |
| MIRAS | Multiple Isomorphous Replacement Anomalous Scattering |
| MMP | Matrix Metalloproteinase |
| MMP-1: CD | Matrix Metalloproteinase: Catalytic Domain |

| | |
|---|---|
| MPD | 2,4-dimethyl pentane-diol |
| NCS | Non-crystallographic Symmetry |
| NGF | Nerve Growth Factor |
| NMR | Nuclear Magnetic Resonance |
| NP-1 | Neuropilin-1 |
| NP-2 | Neuropilin-2 |
| N-TIMP-1 | N-terminal domain of TIMP-1 |
| OB-fold | Oligonucleotide-binding fold |
| OD | Optical Density |
| PCNA | Proliferating Cell Nuclear Antigen |
| PDB | Protein Data Bank |
| PDGF | Platelet-derived Growth Factor |
| PlGF | Placenta Growth Factor |
| rmsd | Root mean square deviation |
| SAD | Single Anomalous Dispersion |
| SC | Shape Complimentarity |
| SDS-PAGE | Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| sFlt-1 | soluble-Flt-1 |
| SIR | Single Isomorphous Replacement |
| SIRAS | Single Isomorphous Replacement Anomalous Scattering |
| SRS | Synchrotron Radiation Source |
| TGF-β | Transforming Growth Factor-β |
| TIMP | Tissue Inhibitor of Metalloprotein |
| TNF-α | Tumour Necrosis Factor-α |
| TSP-1 | Thrombospondin-1 |
| VEGF | Vascular Endothelial Growth Factor |
| VEGFR | Vascular Endothelial Growth Factor Receptor |
| VPF | Vascular Permeability Factor |

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# CHAPTER-1

# MACROMOLECULAR X-RAY CRYSTALLOGRAPHY

## 1. INTRODUCTION

Macromolecular crystallography is a science that deals with the detailed study of the three-dimensional structure of macromolecules that form crystals using X-ray diffraction techniques. This structural information is of great importance because it provides a platform to elucidate the molecular mechanism of biological reactions mediated by macromolecules such as proteins and enzymes.

### 1.1 PRINCIPLES OF X-RAY DIFFRACTION

W. L. Bragg in 1913 was the first to elucidate the crystal structures of NaCl, KCl, KBr and KI using X-ray analysis (Bragg, 1913). This came as the final proof to establish the wave nature of X-rays. The electromagnetic wave nature of X-rays allows us to draw an analogy between the formation of an image from X-ray diffraction and the formation of an image using light rays.

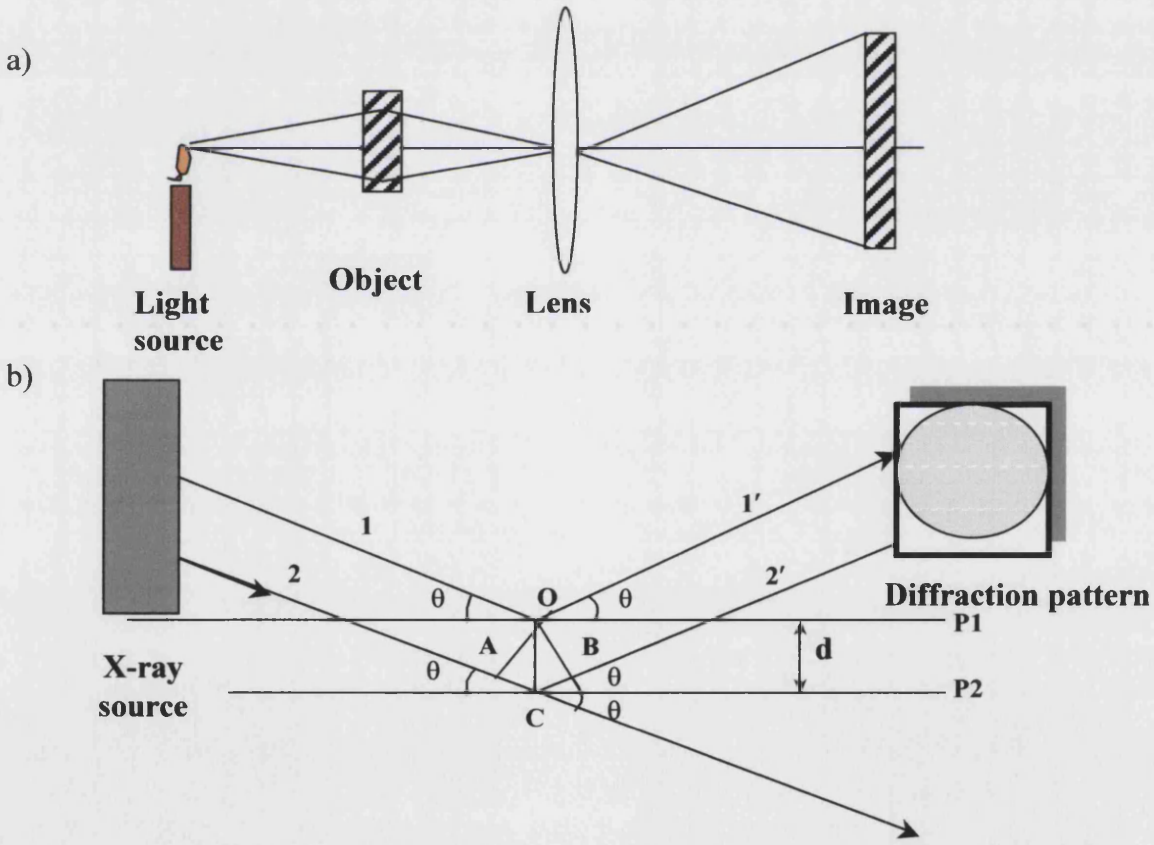


***Fig 1.1*** *A schematic illustration of the the analogy between (a) scattering and (b) diffraction. (Rays 1 and 2 represent the incident beam whereas Rays 1′ and 2′ represent diffracted beam making an angle θ with P1 and P2. . P1 and P2 are a pair of parallel planes with an interplanar spacing of d*

In the case of light rays (*Fig 1.1a*), the scattered rays may be combined using an objective lens to reconstitute the image of an object. This is the principle on which a light microscope works. However, since there is no lens capable of focussing X-rays, it is not possible to carry out a direct recombination of the scattered X-rays. In case of X-rays, a finely collimated beam is used to record the diffraction pattern, say for a protein crystal, on an image plate, a photographic film or a CCD detector.

**Bragg's Law**

The scattering of X-rays by a protein crystal follows the *Bragg's Law*. The crystal planes are illuminated at an angle $\theta$ and X-rays are scattered at an angle of reflection also equal to $\theta$. Constructive interference between rays scattered from successive planes in the crystal will take place only if the path difference between the rays is equivalent to an integral number of wavelengths.

In *Fig1.1b*, the separation between two successive planes of atoms is *d*. The path difference between the two rays is $AB+BC = 2dsin\theta$. Hence, for constructive interference:

$$2d\sin\theta = n\lambda \quad \text{(Bragg's Law)}$$

where, $\lambda$ is the wavelength of the X-rays and n is any integer. This basic equation predicts the position of any diffracted ray in space. The closer the separation between the planes the larger is the diffraction angle.

### 1.1.1 X-RAY DIFFRACTION BY A CRYSTAL

A *crystal* is a regular arrangement of molecule(s), repeated translationally, on a three-dimensional lattice. This repeating motif is *the unit cell,* the basic block from which the whole volume of the crystal can be built. The edges of the unit cell are conventionally defined by three vectors **a**, **b** and **c** with axial lengths *a, b and c* respectively. The angles between these vectors are denoted by $\alpha$, $\beta$ and $\gamma$ as shown in *Fig 1.2*. A unit cell may contain one or more *asymmetric units*. An asymmetric unit is the fundamental repeating motif (made of one molecule or multiple copies of the same molecule), which is

related to all the other identical motifs in the unit cell governed by symmetry rules. This format allows for compact packing of the molecules within the crystal.

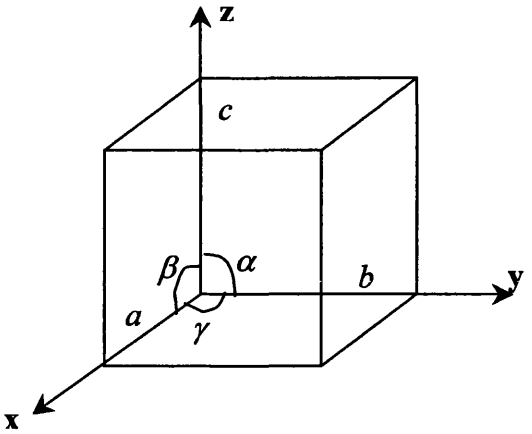


***Fig 1.2*** *The unit cell: a, b and c represent the unit cell axes in x, y and z direction and α, β and γ describe the angles between these axes.*

There are four basic types of symmetry operations possible for a crystal lattice: Rotation, Reflection, Inversion and Translation. Based on the highest rotational symmetry element present, crystals can be grouped into 7 crystal systems and these can be further divided into 14 space lattices called the *Bravais Lattices* (Blundell and Johnson, 1976), depending upon the type of lattice (primitive or non-primitive) chosen to describe the unit cell (*Table 1.1*). A combination of these symmetry elements gives rise to 230 space groups, of which only 65 are permitted for proteins.

***Table 1.1*** *The fourteen Bravais Lattices*

| Name | Bravais Lattice types | Lattice |
|---|---|---|
| Triclinic | P | $a \neq b \neq c \quad \alpha \neq \beta \neq \gamma$ |
| Monoclinic | P, C | $a \neq b \neq c \quad \alpha = \gamma = 90^\circ \neq \beta$ |
| Orthorhombic | P, C, I, F | $a \neq b \neq c \quad \alpha = \beta = \gamma = 90^\circ$ |
| Tetragonal | P, I | $a = b \neq c \quad \alpha = \beta = \gamma = 90^\circ$ |
| Trigonal | P | $a = b \neq c \quad \alpha = \beta = 90^\circ, \gamma = 120^\circ$ |
| | or R | $a = b = c \quad \alpha = \beta = \gamma < 120^\circ, \neq 90^\circ$ |
| Hexagonal | P | $a = b \neq c \quad \alpha = \beta = 90^\circ, \gamma = 120^\circ$ |
| Cubic | P, I, F | $a = b = c \quad \alpha = \beta = \gamma = 90^\circ$ |

### 1.1.2 THE CONCEPT OF RESOLUTION

The resolution of a crystal structure refers to the minimum interplanar spacing of the reflections included in structure determination. The unit of resolution is Angstrom (Å = $10^{-8}$ cm). Resolution is an important term in defining the quality of the structure of a macromolecule. The main features of the macromolecule that can be viewed at different resolutions are as follows (Blundell and Johnson, 1976; Acharya and Rees, 1995):

| | |
|---|---|
| 6.0 Å resolution | Outline of the molecule, secondary structure features such as helices and strands, can be identified. |
| 3.0 Å resolution | Course of the polypeptide chain can be traced and topology of the folding can be established. With the aid of amino acid sequence, it is possible to place the side chains within the electron density map. |
| 2.0 Å resolution | Main chain conformations can be established with great accuracy. Details of side chain conformations can be interpreted easily without amino acid sequence data. Bound water molecules, metal ions and cofactors can also be identified. |
| 1.5 Å resolution | Individual atoms are almost resolved. It is possible to make out the solvent structure. |
| 1.0 Å resolution | Hydrogen atoms may become visible. It is also possible to see multiple conformations for some side chains. |

### 1.1.3 FROM REFLECTIONS TO A 3-D STRUCTURE

In X-ray diffraction the dimensions of the scattering objects are of the same order of magnitude as the wavelength of the X-rays. The diffraction pattern appears for example, on X-ray film or on a detector screen, as an array of discrete spots. The spots where X-rays impinge are also called reflections because they emerge from the crystal as if reflected from planes of an atom.

The darker the spot stronger is the intensity of the X-ray reaching that spot. The position of each spot gives the direction in which the X-ray was diffracted by the crystal. The array of points which form the vertices of the unit cells make up the Real space lattice. Because of the inverse relationship between the lattice points and the spots or reflections on the diffraction pattern is also known as the Reciprocal space lattice (Rhodes, 1993).

The diffraction pattern of a single molecule is called the *molecular transform* of that molecule. Since a crystal is basically a three-dimensional lattice where a molecule is repeated at each of its lattice points, the *Fourier transform* of the crystal is nothing but the product of the diffraction pattern of the molecule with that of the lattice. So, if a spot appears to be dark then it is because of the underlying molecular transform at that point and vice-versa.

Just like each vertex in the unit cell is assigned a set of three coordinates x, y, z, each individual reflection in the reciprocal space lattice is designated *h, k, l*. These are termed as the Miller indices. These indices characterise the crystal lattice in terms of a set of planes. They are a symbolic vector representation for the orientation of an atomic plane in a crystal lattice and are defined as the reciprocals of the fractional intercepts, which the plane makes with the crystallographic axes.

### 1.1.3.1 TREATING REFLECTIONS AS WAVES

The reflections are treated as waves because of the electromagnetic nature of the X-rays. Recombination of these waves produces the image of the molecules in the unit cell. The periodic function of a simple wave can be represented by the following equation:

$$f(x) = F\cos 2\Pi (hx + \alpha)$$

where, f(x) is the vertical height of the wave at any horizontal position x along the wave, F is a constant, which specifies the amplitude of the wave and $\alpha$ is the phase of the wave. For each f(x) there exists another function F(h) which is the Fourier transform of the f(x) and the units of h are reciprocals of the units of x.

The sum of each simple periodic function is known as the *Fourier series* and the Fourier series that describes a diffracted X-ray is known as the *Structure Factor Equation.* Hence, for each reflection the sum of all the waves that describe that reflection is represented in terms of *Structure Factor*, $F_{hkl}$. Each reflection in the diffraction pattern carries information from each atom in the unit cell. So the structure factor for a diffracted ray can be written as:

$$\mathbf{F_{hkl} = f1 + f2 + f3 + \ldots\ldots\ldots\ldots fn}$$

where, 1, 2, 3.....n each represents an atom within the unit cell. Each single term in the above series is called an *atomic structure factor,* $f_{hkl}$, which represents the contribution of a single atom within the unit cell to the reflection *hkl.*

$$\mathbf{F_{hkl} = fj \exp 2\Pi i (hxj + kyj + lzj)}$$

where, fj is the *atomic scattering factor* of the atom j.

Atomic scattering factor is the integration of the individual contributions over the volume of an atom (which is taken as a single sphere of electron density). Since each element will have different number of electrons, the diffraction of X-rays by each element differs. The phase of a wave depends on the atomic coordinates x, y, z of the atom and is implicit in the exponential formula of the structure factor. Thus for a unit cell with n number of atoms, $F_{hkl}$ will be the sum of all the $f_{hkl}$ values for individual atoms.

$$\mathbf{F_{hkl} = \sum_{j=1}^{n} fj \exp 2\Pi i (hxj + kyj + lzj)}$$

The same equation, if considered in terms of contributions of the varying electron density from each atom in the unit cell can be rewritten as:

$$\mathbf{F_{hkl} = \int_{V} \rho (x,y,z) \exp 2\Pi i (hxj + kyj + lzj)\, dV}$$

where, V is the volume of the unit cell and ρ is the electron density of an element centred at x, y, z. Each element contributes to $F_{hkl}$ with a phase determined by its coordinates (x, y, z) (Blundell and Johnson, 1976; Rhodes, 1993). When the scattered X-rays impinge on the detector or the photographic film, all the phase information is lost and what is recorded is only the energy

or in other words the intensity of each diffracted ray. Intensity and Structure Factor are related to each other by the following equation:

$$I_{(hkl)} = F_{(hkl)}^2$$

### 1.1.3.2. UNDERSTANDING THE DIFFRACTION PATTERN

The geometric construction by Ewald (Ewald, 1921) is a most useful aid to visualise the reciprocal space and the diffraction geometry (*Fig 1.3*).

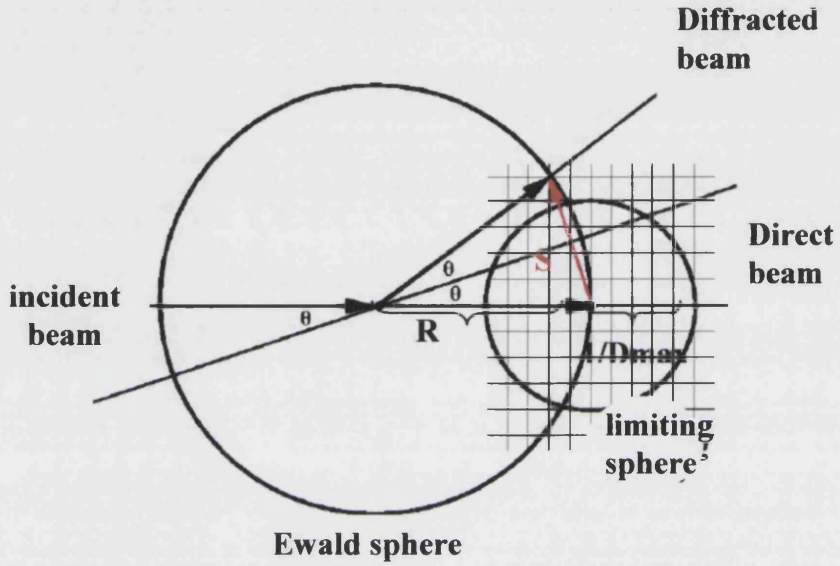


***Fig 1.3*** *The Ewald sphere construction*

The above geometric construction shares the properties of Bragg's law. The angle between the incoming and outgoing beams is always two times theta. If the angle of the lattice plane with respect to the incoming beam is changed then the outgoing beam will change its direction but its endpoint will always be on the circle. The vector connecting the points of exit, of the diffracted beam and the direct beam, from the circle depends on the radius of the circle, **R** and the angle θ.

$$\mathbf{S = 2R\sin\theta}$$

$$\mathbf{S = 2\sin\theta/\lambda}\ (R = 1/\lambda)$$

where, **S** is the reciprocal space vector. When compared with Bragg's Law, the length of the vector **S** corresponds to 1/d. If the center of the circle represents the origin of the real space, the point of its exit from the circle, the

origin of the reciprocal space then a given reflection will only diffract if its reciprocal lattice point intersects the circle. This circle when extrapolated into three-dimensional space represents the Ewald sphere.

### 1.1.3.3. MOLECULAR TOPOLOGY: THE ELECTRON DENSITY

The X-ray beams are actually diffracted by the electron cloud that surrounds each atom. Diffraction by each electron in the atom should in essence reveal the electron density of the atom. Since the crystal lattice is an ordered array of molecules, the electron density can also be represented in terms of a periodic function. Since, $F_{hkl}$ is a Fourier transform of ρ (x,y,z) and Fourier transforms are *reversible*, electron density in essence is the *Fourier transform of the structure factors*, which represents the hazy shape of the molecule (Blundell and Johnson, 1976; Rhodes, 1993).

$$\rho(x,y,z) = 1/V \sum_h \sum_k \sum_l F_{hkl} \exp[-2\Pi i (hx + ky + lz)$$

### 1.1.3.4. THE PHASE PROBLEM

The $F_{hkl}$ in the above equation represents both the amplitude as well as the phase for a given reflection, hkl. So in order to calculate the electron density we need to know both the amplitude as well as the phase of the recorded reflections. But as the phase information is lost during diffraction data collection, it is not possible to elucidate a structure directly from the diffraction images. This fundamental hurdle in X-ray crystallography is termed as the *phase problem*. However, there are four principle methods used to determine the approximate phase angles of the protein structure factors:

- Multiple/Single Isomorphous Replacement (MIR/SIR) method: In this method a heavy atom is introduced into a protein structure and used as a marker atom to provide the initial phases.
- Multiple/Single Anomalous Dispersion (MAD/SAD) method: Phases are obtained from anomalous scatterers whose absorption frequency is close to the experimental wavelength.
- Combination of Isomorphous Replacement and Anomalous Scattering (MIRAS/SIRAS) method.

- Molecular Replacement (MR) method: This method calculates the initial phases from a close structural homologue, if available.

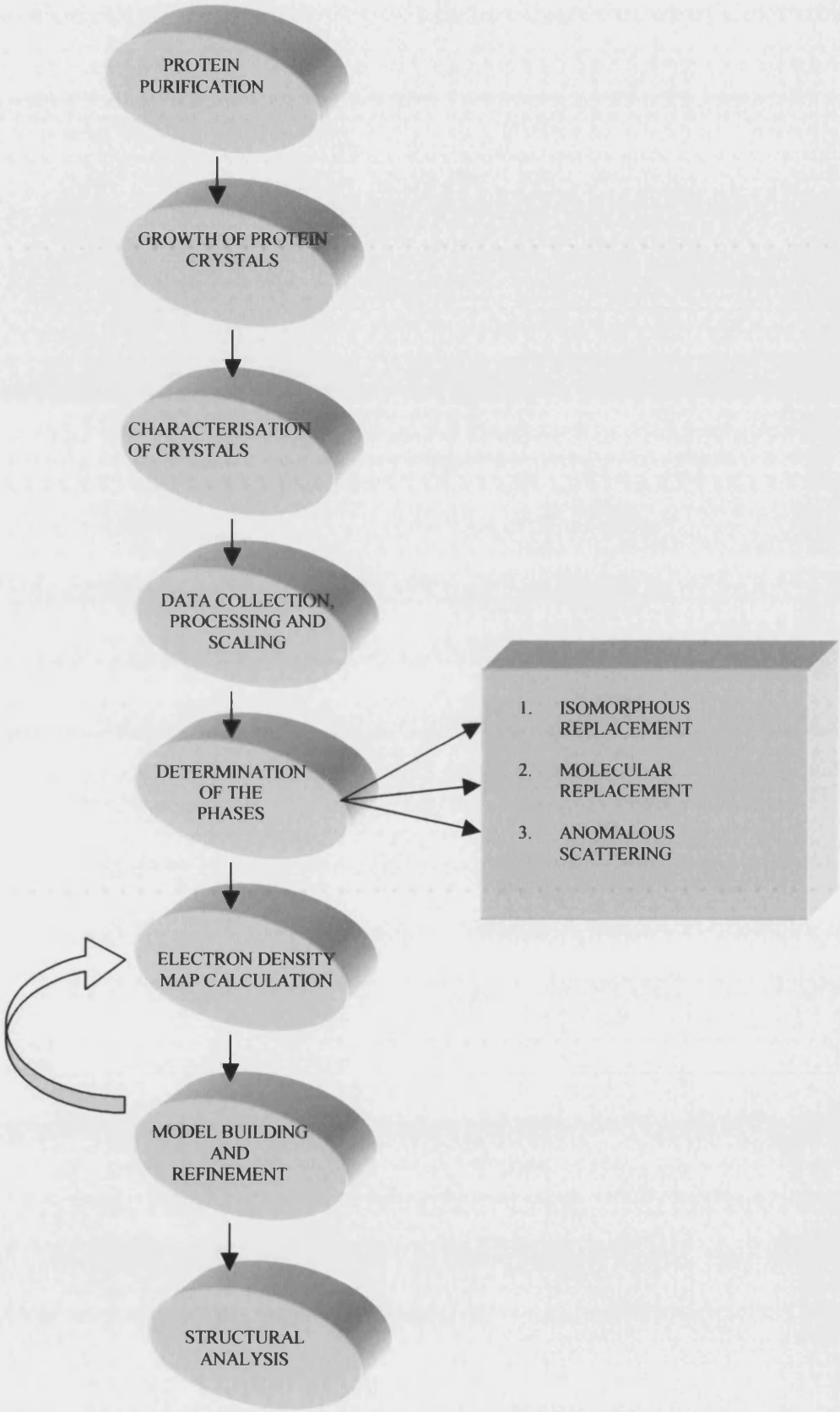


***Fig 1.4*** *A flow diagram representation of the stages involved in structure determination*

### 1.2.1. CRYSTALLISATION OF PROTEINS

The order and symmetry within crystals is a reflection of their ability to diffract X-rays. Macromolecular crystals differ from small molecule crystals in being rather small in size with volumes not exceeding 10 $mm^3$ and are also very fragile. Water molecules occupy about 30 - 80% of the crystal volume and only a small portion of the protein surface is involved in contacts with neighbouring protein molecules. The integrity of the crystal depends on the almost faultless repetition of the protein-protein interactions.

Crystallogenesis is a multi-parametric process and there is absolutely no definitive theory or a principle that can guarantee crystal growth. The idea however, is to bring a supersaturated solution to a lower, more stable energy state by reduction of solute concentration. The basic principles of protein crystallisation are the same in most respects as those for small molecules like salts or amino acids (*Fig 1.5*). This metastable protein solution might be achieved by a gradual change of various factors affecting crystallisation like precipitant or protein concentration, change of pH or temperature. The crystalline state when reached is usually thermodynamically most stable and of far lower solubility than the closest amorphous state. When the solution is in a metastable state, the crystal nuclei exist in a state of equilibrium of formation and dissolution. This continues till appropriate molecular aggregates or nuclears slowly reach a critical size from which the growth of the crystal proceeds as a spontaneous process (Arakawa and Timasheff, 1985).

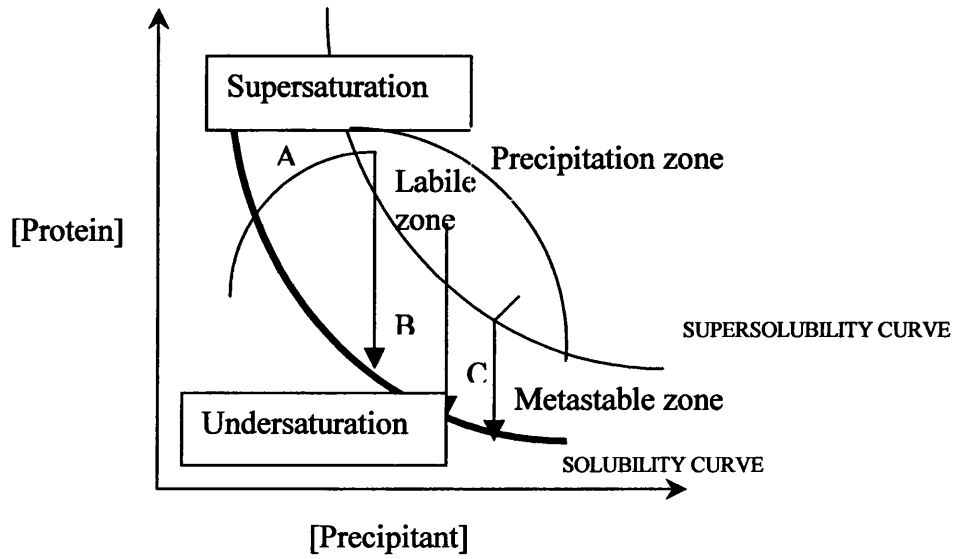


*Fig 1.5 The solubility curve diagram for crystallisation of proteins*

### 1.2.1.1. FACTORS AFFECTING CRYSTALLISATION

Identification of the right condition, required to produce good diffraction quality crystals, can be a difficult task in view of the large number of conditions that need to be screened. The two most essential stipulations surrounding protein crystallisation are the purity and the availability of the protein sample. Even small amounts of impurities represent a high enough concentration in small volumes that are used during crystallisation experiments. This leads to contamination of the crystal lattice and hence poor quality crystals or more often than not, hinders crystal growth. Protein crystallographers also need to grow crystals of a substantial size, as intensity of the X-ray diffraction pattern from a crystal is roughly proportional to the crystal volume. Higher concentration of the protein means that more protein is available for crystal growth and therefore the probability of obtaining large crystals increases (McPherson, 1992; Ducruix and Giege, 1999). Factors affecting solubility of proteins are as follows:

- ***Ionic Strength***- The ionic strength of the solvent changes the interactions of the ions with the water molecules and thus modifies the solubility. At low concentrations solubility of the proteins tend to increase as it increases the possibilities of favourable interactions with the water molecules. Proteins are known to be more soluble in the presence of small amounts of electrolytes than in pure water. This phenomenon is known as 'salting in'. As the ionic strength increases the ions added begin to compete with each other and the surrounding protein molecules for the surrounding water. The resulting removal of water molecules from the solute begins to decrease the solubility. This phenomenon is known as 'salting out'. These phenomena indicate that one may decrease the solubility of the protein dissolved in a medium of low ionic strength by increasing or decreasing the concentration of ions in an attempt to induce crystallisation of proteins.

- ***pH and Counter Ions***- The net charge on proteins can be modified by adding or withdrawing protons i.e. by changing the pH or by specifically binding ions to the polar groups of the protein. The more the net

charge a protein contains the more soluble it is. The point of least solubility is when the protein has a net charge of zero. This is the *isoelectric point*. The solubility in the absence of electrolytes changes with pH and shifts the conditions for salting out. The solubility reaches a minimum in the region of isoelectric point and increase on the addition of either acid or base at this point.

- ***Temperature***- Temperature coefficient of solubility varies from protein to protein, and with conditions such as ionic strength and presence of organic solvents. Variation of temperature at high ionic strength and pH affects the solubility of proteins in the absence of electrolytes. At high ionic strength most proteins are less soluble at 25°C than at 4°C. The solubility sometimes decreases ten-fold on warming up a protein in ammonium sulphate solution. The negative temperature coefficient of solubility of proteins is characteristic only of high ionic strength media. At low ionic strength media the solubility increases with increasing temperature. In pure water the temperature coefficient is positive for most proteins.

- ***Organic Solvents***- The addition of organic solvents also produces marked changes in the solubility of proteins in aqueous solutions. This is because of decrease in the dielectric constant of mixtures of water and organic solvents when compared to the dielectric constant of water alone. This decrease increases the attraction between unlike charges on the protein molecules and lowers the solubility. Generally the protein solubility decreases with decrease of temperature when substantial amounts of organic solvent are present. Organic solvents often denature proteins and this could be minimised by crystallizations at low temperature (Blundell and Johnson, 1976).

### 1.2.1.2 METHODS FOR PROTEIN CRYSTALLISATION

There are different methods that can be employed to set up crystallisations.

*1.* ***Batch Method***- The traditional approach to crystallisation is the batch method, which involves adding precipitant or changing the pH until the

protein comes to the limit of its solubility. The major limitation of batch experiments is that the precipitant conditions remain static and it also consumes a lot of the protein sample. The only change in the time course of the experiment is the decrease in the protein concentration.

2. ***Dialysis***- Dialysis procedures have also been quite successful where a range of precipitant conditions are automatically screened but the protein concentration remains constant. Dialysis methods are not economical on the protein but they do allow more flexibility, as amorphous precipitates in failed attempts to produce crystals can be re-dissolved with appropriate solution placed outside of the chamber, and another condition tested with the same sample.

3. ***Vapour Diffusion***- Vapour diffusion has been the most successful method used to date. In this method, evaporation of the protein solution is controlled by equilibration against a reservoir a slightly higher concentration. As the two solutions tend towards equilibrium, the solvent is transferred from the protein solution to the reservoir through the vapour phase. This makes the protein solution more concentrated and thus crystals tend to form by achieving supersaturation in the drop. The protein drops can either be suspended from siliconised cover slips used to seal the reservoir contained in a vessel (*hanging drop; Fig 1.6a*), placed on a support above the reservoir (*sitting drop; Fig 1.6b*) or by placing the drop sandwiched between the support and the coverslip (*sandwich drop; Fig 1.6c*). Vapour diffusion has the benefit of automatically screening a range of precipitant concentrations. It allows for optimising a crystallisation so that the precipitant concentration slowly reaches the nucleation region of the solubility phase diagram and thereafter increases just fast enough to compensate for the loss of protein to the crystals. This requires not only optimising the end point of the experiment but also the rate of equilibration. The simplest way to vary the rate of equilibration is to vary the surface to volume ratio (the size) of the drop. In general, larger drops equilibrate more slowly than the smaller drops (Blundell and Johnson, 1976).

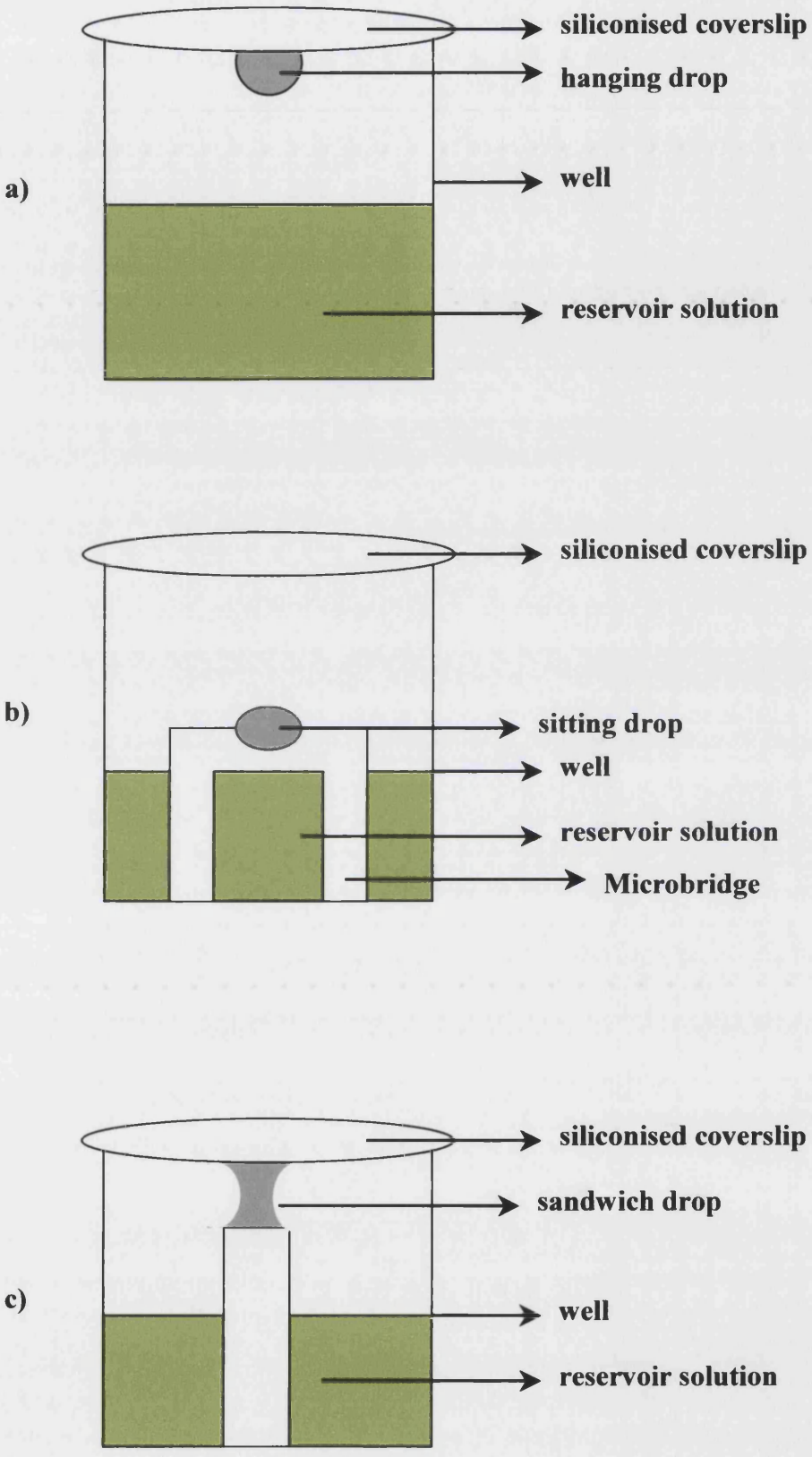


***Fig 1.6*** *The different physical techniques of protein crystallisation involving the vapour diffusion method.*

*4.* ***Gel Crystallisations***- In recent years, the application of gels in crystallisation has been the focus of ever increasing attention. The gels mainly used for bio-molecular crystallisations are hydrogels like silica gel and agarose (Robert *et al.*, 1999). There are several gel properties related to crystal growth that are making them a popular alternative to the conventional solution techniques. Some of these advantages are as follows:

*a)* In a gel network, there is no convection as a result of which the mass transfer from solution to the crystal proceeds only by diffusion

*b)* Crystals grow uninterrupted at their nucleation site and do not sediment like they do in free solution.

*c)* Nucleation occurs homogeneously in the whole volume and they usually appear at the same time. Gels have also known to show reduced heterogeneous and secondary nucleation thereby preventing a shower of tiny crystals.

All the current methods of protein crystallisation like the batch method and the vapour diffusion method can be used to grow crystals either outside or inside the gel instead of free solutions.

*5.* ***Seeding***- Seeding is more of a technique for optimising a screened condition than a method on its own. A seed (usually a tiny crystal or part of a crystal) acts as a template for the assembly of macromolecules. The addition of a seed to an already equilibrated drop redistributes the protein separated between the solution phase and the aggregate phase. This disturbs the equilibrium and thus slows down the rate of crystal growth. Seeding helps to partition nucleation phase from crystal growth. The most important stage of seeding is the pre-seeding stage. In this stage, the protein solution to be seeded is pre-equilibrated with the crystallisation solution in such a way that spontaneous nucleation is avoided before the introduction of the seed. Seeding can be done using four different methods. These methods are:

*a)* Streak seeding: This is usually used as an analytical method to determine the conditions, eg the supersaturation threshold, that would be most suitable for seeding to succeed.

*b)* Microseeding: In this method, microscopic crystal fragments

are introduced into a protein solution.

*c)* Macroseeding: In this method, a single crystal is introduced into a pre-equilibrated protein solution.

*d)* Epitaxial nucleation: Impurities like the cellulose fibres sometimes provide surfaces that are suitable for bio-macromolecular crystal growth.

### 1.2.2. DIFFRACTION DATA COLLECTION

Protein molecules crystallise with large unit cell when compared to small molecules, and a large number of reflections have to be measured at any given resolution. Therefore, an efficient method of data collection is required to make the measurement of intensities as precise as possible and at the same time minimize the exposure time of the crystal to the X-ray beam.

***1.2.2.1 X-ray Sources and Detectors***- X-ray sources are of two types: Laboratory sources and Synchrotron radiation sources. The X-rays are produced in laboratories either by sealed tube sources or by a copper-rotating anode (fixed wavelength of 1.5418Å for Cu-Kα radiation). Although they are very convenient and reliable, they produce about 100-1000 times less flux than the sources at the synchrotron. Synchrotron sources, on the other hand, produce electromagnetic radiation by an electron storage ring. The X-rays are produced when the electrons are radially accelerated by electric fields in a linear accelerator to reach a final energy of 1GeV to 8GeV (Helliwell, 1997). The electrons within the storage ring are constrained to move in a circular path using magnetic fields. The X-rays are produced when the electrons change direction in the magnetic devices. The current of the beam decays with time in the storage ring because of loss of electrons. Hence the beam is regularly dumped and the storage ring is refilled with fresh beam, usually at about 300mA. These X-rays are very intense and hence very well suited for very small crystals. Even crystals that diffract very weakly at the laboratory sources are able to give data with better signal-to-noise ratio at the synchrotron sources. This gain in intensity translates into a very short exposure time and therefore fast data collection. Protein crystallography has benefited to a great

extent from the synchrotron sources because of the highly monochromatic and tunable nature of the beam as well as the good detector systems like the present day CCD systems. The ability to choose wavelengths to collect diffraction data at the absorption edge of a heavy metal has made isomorphous replacement and anomalous dispersion experiments, a routine, everyday research tool for phasing. The national Synchrotron Radiation Source (SRS) is situated in Daresbury, UK (http://www.srs.ac.uk/srs). The SRS has a 2GeV electron storage ring that offers a variety of experimental facilities with provision for simultaneous, multi-user experiments requiring a range of radiation from infrared rays to X-rays.

***1.2.2.2 Crystal Mounting***- The type of data collection governs the method employed for crystal mounting. For room temperature data collection, the crystal is drawn into a thin-walled glass or quartz capillary tube by suction. The excess mother liquor is then removed using thin strips of filter paper. Usually a very small quantity of mother liquor is left surrounding the crystal before both the ends of the tube is sealed off with wax, as shown in *Fig 1.7a*, to prevent the crystal from drying up. Also the surface tension of the mother liquor surrounding the crystal prevents it from slipping within the capillary. It is important not to let the crystal dry up because it affects their diffraction quality. On the other hand, it is also important that the crystal is dry enough so that it does not move during data collection.

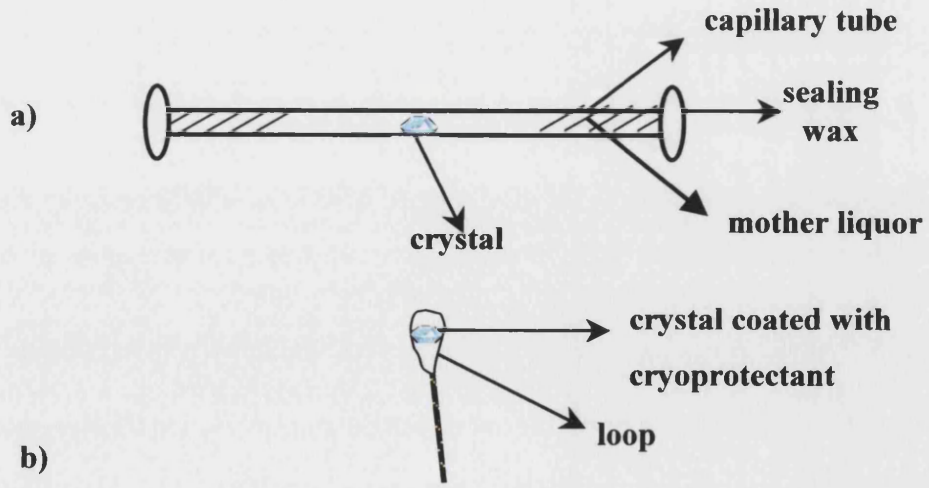


***Fig 1.7*** *Crystal mounting methods: a) Capillary tube method and b) loop method*

Data collection of biological macromolecules these days is usually performed at temperatures ranging between 80-100 K. Cryo-techniques have become a pivotal tool in crystallography because of the higher quality of data collected at these temperatures in comparison to those collected at room temperature. The technique usually used for cryogenic data collection is the *loop mounting method* (Teng, 1990; Rudman, 1976). In order to collect data at freezing temperatures it is necessary to prepare a *cryoprotectant* or a *cryosolution*, which is usually made up of the mother liquor and an anti-freeze [e.g. Glycerol, MPD (2, 4-dimethyl pentane diol)]. The crystal is dipped in the cryoprotectant and mounted on to a loop (*Fig 1.7b*) made up of a very thin fibre (e.g. nylon or mohair) where it is held by means of the surface tension of the liquid. The crystal is then immediately placed under a direct stream of a cryogen or plunged into a cryogen at around 100 K. The cryogen commonly used is liquid nitrogen. The liquid nitrogen solidifies the thin film of the cryosolution in the loop and thus holds the crystal in place. The anti-freeze in the solution prevents ice formation around the crystal during data collection (Garman and Schneider, 1997). Data collection at such low temperatures prolongs the life of a crystal and reduces radiation damage and thus, in most cases, makes it possible to collect an entire dataset from a single crystal.

***1.2.2.3 Steps in Data Collection*** - The process of data collection is not just a mere technique but is a scientific process that needs important decisions to be input by the experimenter in order to collect data of high quality and as complete as possible. The completeness of the data in terms of quantity depends a lot on the geometry of the crystal lattice and the detector set up. The qualitative completeness of the data depends on the strategies employed during the process of data collection. Factors influencing quality of data (Dauter, 1997) are as follows:

- ***Oscillation (rotation) Method:*** As understood from the Ewald sphere construction, *Bragg's Law* is fulfilled only if the reciprocal lattice point lies on the surface of the Ewald sphere. This gives rise to a diffracted beam. For any given orientation of the crystal, only a few reflections will lie on the surface of the Ewald sphere. So in order to collect a complete dataset, in terms of the number of recorded reflections, the crystal is rotated about a single axis.

That is, successive reciprocal lattice points are moved to the surface of the Ewald sphere (the radius of the sphere is constant because of the use of monochromatic X-rays) to observe diffraction from a different set of reflections. The reciprocal lattice points are arranged in planes and each plane of reflection projects on to the detector as an ellipse. The ellipses from a family of parallel planes form a lune. These lunes are more pronounced if the reciprocal lattice planes are perpendicular to the X-ray beam. Hence, the axis along which the crystal is rotated during data collection is perpendicular to the beam. Since the axes perpendicular to the rotation angle are limiting to the data collected, the crystal is always aligned along its longest dimensions (Dauter, 1997).

- ***Crystal Mosaicity (partial and fully recorded reflections):*** Mosaicity is a measure of the angular range over which a given reflection satisfies to the trigonometric condition of *Bragg's law* ($n\lambda = 2d\sin\theta$). The Ewald sphere represents a situation where the reciprocal lattice points are dimensionless, i.e. perfect points. However, in the real case scenario, the crystals are made of molecules, all of which do not share the same orientation. And because the X-rays are monochromated to a defined narrow wavelength window, the Ewald sphere gets broadened, causing diffraction by a particular reflection to spread over a range of crystal rotation and period of time. This causes the reflections to start diffracting during one exposure and finish in the next time interval. As a result these reflections are termed *partial reflections* as their intensity gets divided over two or more images. *Full reflections* are those reflections whose intensities get recorded on a single image. The higher the mosaicity more are the number of partial reflections recorded, as the lunes are wider which tend to overlap. When the mosaic spread equals the oscillation range, there are only partial reflections recorded and is it not possible to distinguish the individual lunes on the diffraction images.

- ***Oscillation:*** The optimal rotation angle is usually decided upon after the diffraction quality of the crystal is assessed from the first image. The degree of oscillation chosen depends on several factors like mosaic spread, the crystal-to-detector distance and the unit cell dimensions. It is important to select the rotation range per single exposure in such a way so as to avoid

overlapping of the lunes. The total rotation range depends on the crystal system as discerned after indexing the first diffraction image as well as the crystal orientation. It is one of the most important factors that govern data completeness. Since a 360° of data is always at the expense of the expensive beam time at the synchrotron sources, the strategy employed is to reach high completeness as soon as possible with all the unique reflections measured at least once. However, with the modern day fast data collection techniques it is possible to collect a highly redundant data to decrease the errors in intensity measurements.

- ***Crystal-to-detector Distance***: This helps in deciding upon the maximum obtainable resolution for collecting data from a crystal. The distance also affects the spot size in the diffraction pattern. Thus the distance set should be such that the reflection profiles are resolvable and are not overlapping. In cases where one of the unit cell dimensions is large, data collected at the maximum diffraction resolution would have more number of partially recorded reflections as compared to the fully recorded reflections. This would decrease data completeness. Hence, it is always better to trade off resolution in favour of data completeness.

- ***Wavelength***: The wavelength of the laboratory sources is usually fixed at the value characteristic for the metal of which the rotating anode is made of (1.5418Å for Cu-Kα radiation). However, at the synchrotron wavelength can be chosen according to the nature of the experiments, e.g. different wavelengths can be chosen according to the anomalous scatterer or heavy atom for a MAD or MIR experiment. For native data collection, any wavelength will do, especially as the beam intensity at the synchrotrons is quite high. Usually shorter wavelength is used because it is favourable for high-resolution data collection and reduces radiation damage by crystals.

- ***Exposure Time***: This factor controls the intensity of each reflection. Longer exposure times is useful in recording the high-resolution weak reflections. However, this leads to saturation of some spots (or reflections). These are referred to as *overloads*. This problem can be overcome by collecting low-resolution data with shorter exposures.

*1.2.2.4 Preliminary Characterisation of the Crystal-* A crystal form of a protein can be initially characterised from the diffraction pattern. Most importantly the unit cell dimensions and the space group in which the protein has crystallised can be inferred from the inter-spot spacing and the symmetry of the diffraction pattern, and possibly the systematic absences of some reflections. The ratio of the volume of the unit cell to the molecular mass of the protein contained in the cell gives the solvent content of the crystal (Matthews, 1968). If the molecular mass is known, then the number of protein molecules in the asymmetric unit can be deduced from the unit cell dimensions and the space group. The crystallographic space group can sometimes provide information on the symmetry present in the quaternary structure of an oligomeric protein (Louie, 1995).

*1.2.2.5 Data Integration and Reduction-* Data processing essentially means to index each reflection in the diffraction pattern and in turn all the diffraction patterns in the dataset. There are several software packages that use different algorithms, which are applied with the aim of reducing raw data into a set of indices for each reflection and the corresponding measured intensities of each spot in the diffraction pattern. The commonly used packages are the MOSFLM (CCP4, 1994) and the HKL package (Otwinowski and Minor, 1997). The HKL package comprises of DENZO, which helps in the analysis of each image mathematically, the XdisplayF, which helps to visualise the each image/diffraction pattern and SCALEPACK, which helps in scaling the processed data.

Data processing involves autoindexing of the spots to determine the unit cell dimensions and the space group of the crystal lattice. The program writes out a list of probable lattice types on the basis of the symmetry rules in the International Tables for X-ray Crystallography in the International Union of Crystallography, Vol I (Henry and Lonsdale,1952). The space group used as the input for the rest of data processing is the one that has highest symmetry with least percentage of distortion. Once data is processed, the program SCALEPACK is used to average and merge the processed data taking into account the errors, which appear due to air and crystal absorption, crystal decay etc. The output of the scaling process is a list of reflections with

systematic absences characteristic of the space group determined earlier during autoindexing. The quality of the processed and scaled data can be assessed by three values:

- ***Percent completeness***: Higher the percentage of complete data, more is the information held by the processed data.
- $R_{sym}$: This is the estimate of the disagreement between the measured and the observed intensities. The value of $R_{sym}$ depends on data multiplicity. The lower the $R_{sym}$ (the disagreement) higher is the data precision although this value increases with higher multiplicity. This value is called $R_{merge}$ when more than one dataset are scaled together.

$$R_{merge} = \sum_{hkl} \sum_{i} |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \langle I_{hkl} \rangle.$$

The removal of outliers from the merging process improves the $R_{merge}$ value. The percentage of rejected outliers should not be more than 1% because this will decrease data multiplicity and thus adversely affect data quality. Also weak reflections should not be removed from the merging process as the weaker intensities also contain some information and their removal can introduce intensity distribution bias. An overall $R_{merge}$ of up to 10-12% is within the accepted range for a 2.0Å dataset.

- $I/\sigma I$: $\sigma I$ is the error in measuring the intensity of a reflection. Basically, if the value of $I/\sigma I$ is 1.0, the intensities can provide some reliable information. However, the acceptable resolution limit for scaling data is usually where the value of $I/\sigma I$ is above 2.0.

### 1.2.3. DETERMINATION OF PHASES - STRUCTURE SOLUTION

We need both amplitudes and the phases to calculate an electron density map. $F_{hkl}$ is a vector quantity that comprises of the amplitude, $|F_{hkl}|$ and its phase, $\alpha_{hkl}$. Thus, the electron density equation can be written as:

$$\rho(x, y, z) = 1/V \sum_{h} \sum_{k} \sum_{l} |F_{hkl}| \exp[-2\Pi i (hx + ky + lz - \alpha_{hkl})$$

What is known from this equation is the frequency of the wave and the

amplitude of the wave, which is the square root of the measured intensity, $I_{hkl}$ of the reflection *hkl*. The information that we cannot access from the experimental data is the phase or the angle at which the X-ray is diffracted. Thus we are faced with what is known as the "phase problem" (Blundell and Johnson, 1976).

There are various methods to evaluate or approximate the phases required to reconstruct a three-dimensional structural image from experimental diffraction data. All of them are somewhat indirect since there is no direct method to obtain phases from diffraction measurements. Determination of phases is a very important step in macromolecular crystallography since an erroneous phase will lead to an inaccurate electron density map and thus result in wrongly determined structure. There are three major methods used for obtaining the phase information:

### 1.2.3.1 ISOMORPHOUS REPLACEMENT

Max Perutz and his co-workers pioneered the use of Isomorphous Replacement method (Green *et al.*, 1954). This classical method is usually the pre-dominant method for initial phasing when a close structural homologue of the protein in question is unavailable. A number of heavy atoms have been used quite successfully in crystal structure analyses. The historic example of horse haemoglobin made use of mercury (Hg) and silver (Ag) compounds bound to a sulphydryl group on the surface of the molecule. Since then this method has proven to be a milestone in protein phasing.

The screening of suitable heavy atoms is a rate-limiting step next to crystallisation. Stabilising solutions like the mother liquor in which the crystals can be maintained for long periods of time expedite the search for heavy atoms. The stabilising solution is of a similar condition as the reservoir in which the native crystals grew but with higher precipitant concentration. The stabilising solution, because it lacks in protein, should compensate for its absence and not affect the quality of crystal diffraction (Rould, 1997). The chances of obtaining an isomorphous derivative increases if a single stabilising solution is used for native as well as derivative data collection. The

term isomorphous derivative means a crystal where a few heavy atoms replace some of the water molecules without changing the unit cell dimensions or the structure of the protein itself.

Protein crystals are usually derivatised by direct soaking of the crystals in the heavy atom solution. Binding of the heavy atom to a specific position on the protein surface is driven by entropy within the crystal. As a result, usually the binding of heavy atoms lacks specificity or the sites may only be partially occupied. A good heavy atom derivative depends on the amino acid sequence, the spatial arrangement of the side chains in the tertiary structure of the protein, the heavy atom compound, pH as well as the concentration of the buffer used. Strategy for screening for derivatives involves different stages. The search for derivatives should coincide with availability of beam time at the synchrotron radiation sources and lots of good crystals (Rould, 1997; Blundell and Johnson, 1976). The heavy atom solution used for soaking contains very low concentration of the heavy compound. The right concentration, which does not destroy the crystal, is usually found by a trial and error method, starting with the highest possible concentration and smallest crystals. A good heavy atom solution is the one in which crystals when soaked do not show loss of birefringence and do not exhibit decrease in diffraction quality or increase in mosaicity. A good isomorphous derivative will show significant isomorphous differences between the native and the derivative dataset without changing the native crystal lattice (a change of up to 5% is normally within acceptable limits).

An initiative undertaken by Islam *et al.* (1998) resulted in a computer-based archive of experimental as well as derived information. This is the Heavy Atom Databank (HAD). It provides a wealth of information on crystal structures solved so far with the use of heavy atoms and the coordinates of the heavy atoms. HAD is an assembly of information based on the analyses of the various successes and failures faced by protein crystallographers, in preparation of heavy atom derivatives (http://www.bmm.icnet.uk/had/)

- **Isomorphous Replacement Equation**

The structure factor equation representing the 'n' number of atoms of the native or parent crystal, P, is

$$F_P(hkl) = \sum_{n} f_P \exp 2\Pi i\,(hx_P + ky_P + lz_P)$$

Similarly, the structure factor equation for the 'm' number of heavy atoms, H, can be represented as:

$$F_H(hkl) = \sum_{m} f_H \exp 2\Pi i\,(hx_H + ky_H + lz_H)$$

The derivative will contain the heavy atoms in addition to all the light atoms in the native crystal. Hence, the structure factor equation for an isomorphous derivative ($\mathbf{F_{PH}}$) will be equal to the sum of the structure factor of the protein ($\mathbf{F_P}$) and the heavy atom ($\mathbf{F_H}$).

$$\mathbf{F_{PH} = F_P + F_H}$$

The contribution of heavy atoms to the overall intensity is not proportionate and hence they change the scattered intensity quite significantly when introduced into a crystal lattice. This allows for the change to be easily measured (Ke, 1997).

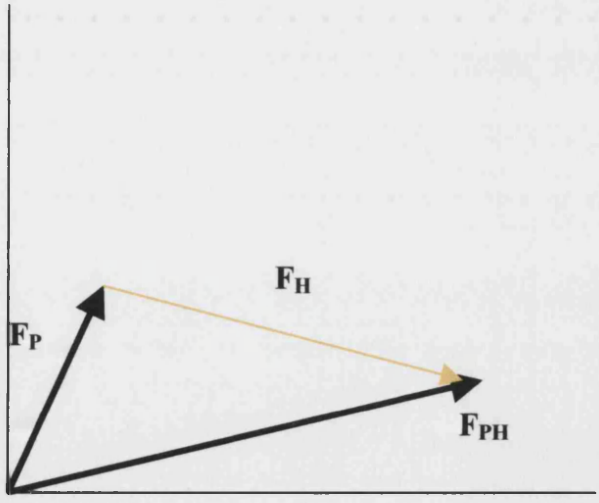


***Fig 1.8*** *Vectorial representation of structure factor for the derivative, protein and the heavy atom*

- **Single/Multiple Isomorphous Replacement (SIR/MIR) method**

Vectorial representation of the structure factors (*Fig 1.8*) helps one to visualise the above equation as a triangle where the lengths of all the three vectors defining the three sides of the triangle are known. Since we also know the orientation of $\mathbf{F_H}$ all that remains is to calculate the phases for $\mathbf{F_P}$ using a simple equation:

$$\mathbf{F^2_{PH} = F^2_P + F^2_H + FPF_H \cos\theta}$$

From the above equation it is obvious that there are two possible phases for $\mathbf{F_P}$ depending upon how the triangle is drawn as illustrated in *Fig 1.9*.

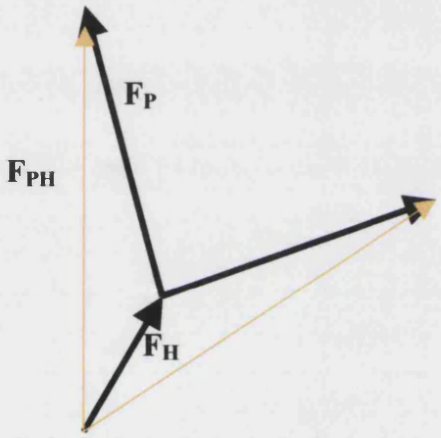


***Fig 1.9*** *Two possible ways to calculate phases for the protein depending on the way the triangle is constructed*

The ambiguity in the choice of the right phase can be overcome by the use of a second heavy atom derivative that binds to the protein at sites different from the first one. The information derived from the second/third derivative will be consistent with all the observations for only one of the two phases. The more the number of derivatives lesser is the phase ambiguity. The steps involved in structure solution using the MIR method can be summed up with following flow-chart (*Fig 1.10*).

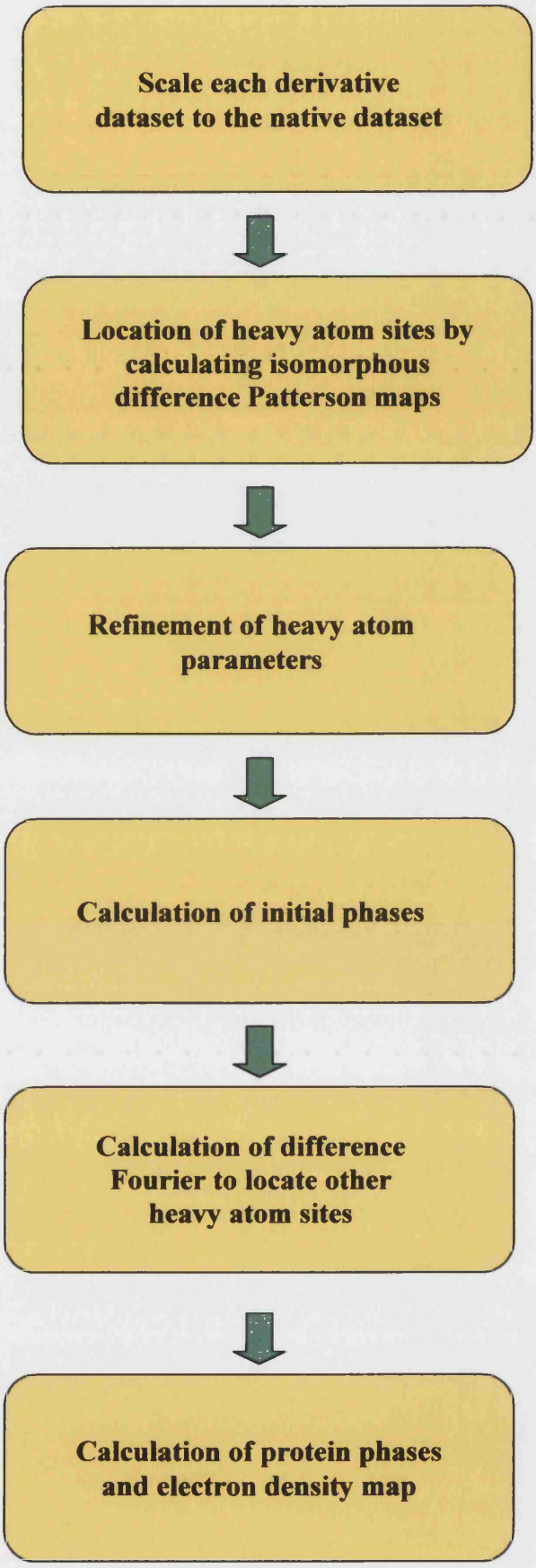


***Fig 1.10*** *Steps involved in Multiple Isomorphous Replacement method.*

- **Patterson Function**

Phase information from the heavy atoms can be obtained by determining their positions. The Patterson function (Patterson, 1934) is a very valuable tool towards this end. Patterson function is a Fourier transform of the measured intensities. The resultant Patterson map, calculated using the experimental intensities, represents vectors between the scattering objects within the unit cell.

In a protein with **N** number of atoms, there will be **N** number of self-vectors and **N (N-1)** number of cross-vectors. The self-vectors appear as large peaks. They are also known as the origin peaks because a vector between an atom in one cell and the same atom in a neighbouring cell will always fall on the origin. Since the density in the Patterson map corresponds to the square of the number of electrons present in the scattering atom, heavy atoms tend to dominate the map by virtue of having more number of electrons than the lighter atoms usually found in a protein structure. The Patterson map is a collection of set of both self and cross vectors and hence densities depend on the number of atoms present as well. As a result there is a lot of overlapping of both self and cross peaks. The self vectors can be removed by subtracting the average intensity from $I_{(hkl)}$ before Fourier transformation. But despite this the Patterson map remains non-interpretable. However, only heavy atom peaks dominate a Patterson map calculated from isomorphous difference amplitudes. Such a Patterson map is an invaluable tool for determining the initial phases for the macromolecule (Blundell and Johnson, 1976).

Patterson-space unit cells are of the same dimensions as real-space cells. The dimensions of Patterson vector space are called (u, v, w) and these correspond to the x, y and z dimensions of real space. Patterson maps are centrosymmetric as they contain equal peaks corresponding to a vector between atom 1 and atom 2 and a vector from atom 2 to atom 1. The height of the Patterson peak is proportional to the product of the heights of these two peaks in the electron density map.

Heavy atom sites in a second derivative can be located by calculating a *difference Fourier* transform using weighted phases calculated from the first derivative ($\mathbf{F_{PH2}} - \mathbf{F_{obs}}$). Also, a *double difference Fourier* can be calculated using phases calculated from the known positions of a derivative to locate further sites of the same heavy atom in that derivative ($\mathbf{F_{Phi}} - (\mathbf{F_P} + \mathbf{F_H})$).

- **Harker vectors and Harker sections (Harker, 1956)**

The peaks in a difference Patterson map represent vectors between all pairs of heavy atom sites within the unit cell of a crystal. The vectors between symmetry-related atoms are known as the Harker vectors. The Patterson peaks generated by symmetry operations are found on Harker sections. These harker sections tell us the location of the atoms relative to the cell axes. Inter-atomic peaks not related by symmetry fall on non-harker sections.

- **Refinement of heavy atom parameters**

The basic idea behind refinement of heavy atom parameters is to minimise the error between the calculated value of $|F_{PH}|$ and the measured value to produce a set of parameters that best fitted the measurements. There are several programs that can be used to bring to convergence these two values. Perhaps the most popular by far is MLPHARE (Collaborative Computational Project, 1994) and SHARP (de La Fortelle and Bricogne, 1997). The MLPHARE program uses the maximum likelihood method of refinement to arrive at a set of parameters that maximises the likelihood of the measurements being correct by evaluating all possible phases during refinement.

- **Statistics**

One of the difficulties usually faced is, not knowing, if the heavy atom positions found are correct. There are several indicators that can be used to monitor the progress of refinement and to adjudge a heavy atom derivative as valid. These quantities are as follows:

1) ***Real Occupancy***

2) ***$R_{cullis}$***: Reduction in $R_{cullis}$ (Cullis *et al.*, 1961) as a function of resolution is the most useful signal for a useful derivative. It is the ratio of the lack of closure to the isomorphous difference. The lack of closure, $\epsilon_j$, is graphically illustrated in *Fig 1.11*. Isomorphous differences are the differences

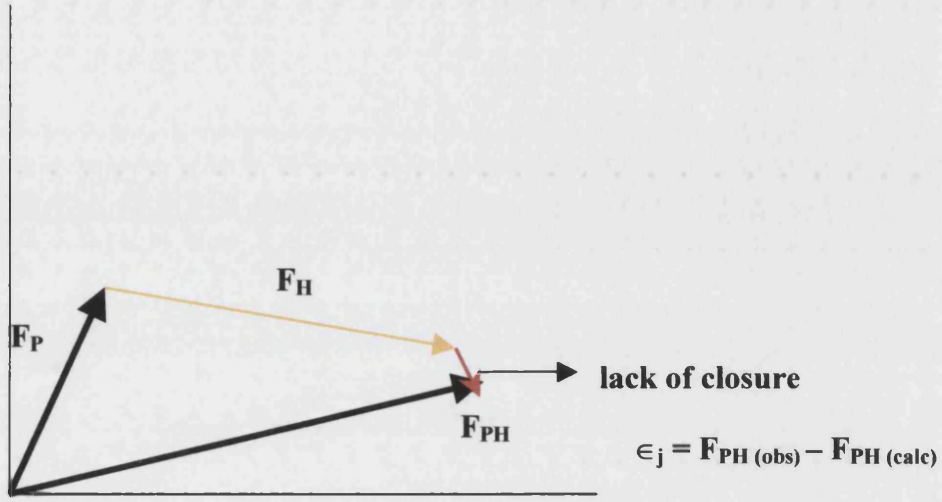


***Fig 1.11*** A *vectorial representation of the lack of closure.*

in the intensities of the reflections (acentric or centric) between the native and the derivative dataset. $R_{cullis}$ (centrics) < 0.6 is considered to be very good whereas if $R_{cullis}$ is between 0.6 and 0.9, the derivative is only just usable. A site is considered to be probably incorrect if its addition does not reduce the Cullis R-factor ($R_{cullis}$).

$$R_{cullis} = \frac{\sum \left| \left| F_{PH} \pm F_P \right| - F_{H\ (calc)} \right|}{\sum \left| F_{PH} - F_P \right|}$$

3) ***Phasing power***: This value should increase with resolution. A phasing power of up to 0.5 is usable and if it is more that 1.5 then it is considered an excellent derivative.

**PHASING POWER = $|F_H|$ / probability-weighted lack of closure**

4) ***Figure of Merit***: the higher the figure of merit the better would be the phases derived from the derivative (Please refer to Appendix B (Page 176) for the definition of Figure of Merit).

### 1.2.3.2 ANOMALOUS SCATTERING/DISPERSION METHODS

Anomalous Scattering/Dispersion is another technique used in macromolecular crystallography to determine phases. These phasing methods are commonly known as Multiple/Single Anomalous Dispersion (MAD/SAD) methods. Like the isomorphous replacement method, it also exploits the abrupt changes to the scattering power (diffraction) of heavy atoms near their absorption edges (Hendrickson *et al.*, 1985). The light atoms in the unit cell do not have absorption edges near the wavelength of the X-rays used in crystallography. The changes in diffraction result in differences between the reflections measured at different wavelengths.

The principle behind anomalous scattering breaches the Friedel's Law, which states that members of a Friedel pair have equal amplitudes but opposite phases, where the Friedel pair are Bragg's reflections related by an inversion through the origin. This method is useful when the protein has a metal ligand attached to it like in metalloproteins.

$$|F_{hkl}| = |F_{\overline{hkl}}| \qquad \varphi_{hkl} = -\varphi_{\overline{hkl}} \quad \text{(Friedel's Law)}$$

When X-rays of high energy are incident on the atoms in a protein molecule, some photons are scattered normally. Whereas some photons are absorbed and re-emitted at a lower energy when the wavelength of the X-rays is strongly coupled to the absorption energy edge of the scattering object. Hence, the normal atomic scattering coefficient $f_0$ of the scattered photon gains an imaginary component to its phase, $f''$, or in other words the photon scatters with a phase delay. Since $f''$ is always positive both the amplitude and the phase relationship do not hold true when some atoms in the crystal lattice scatter anomalously. The scattering factor for the atom can be written as:

$$\mathbf{f = f_0 + \Delta f' + i\,\Delta f''}$$

where, $\Delta f'$ is the dispersion component of the anomalous scattering and $\Delta f''$ is the imaginary absorption component. Both $\Delta f'$ and $\Delta f''$ are sensitive to wavelength especially near the absorption edge. The absorption coefficients

for the light atoms are small enough to be ignored. However, for the anomalous scatterers, these coefficients cannot be ignored, especially as the

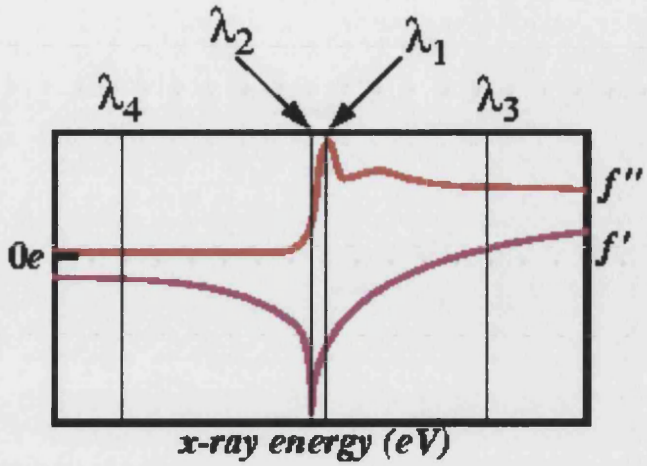


***Fig 1.12*** *Schematic representation of hypothetical values of f′ and f″ as a function of X-ray energy*

absorption edge for these elements is close to the wavelength of X-rays commonly used. By applying mirror symmetry on these complex structure factors $F_P$, $F_H$ and $F_{PH}$ for the reflection (-h-k-l) through the real axis so that they superimpose on those for the reflection (hkl), it will be seen that $|F_{PH+}|$ and $|F_{PH-}|$ are unequal, *i.e.* Friedel's relation no longer holds.

It is obvious from *Fig 1.12* that the change in both the dispersion coefficient (real part) and the absorption coefficient (imaginary part) is dependent on wavelength. This is indicative of the fact that the diffraction images collected at different wavelengths especially near the absorptional edge of the anomalous scatterer present would show differences. These anomalous differences are equivalent to isomorphous differences and are thus used to calculate the initial phases. The advantage of these changes in the diffraction pattern at different wavelengths is that they are perfectly isomorphous changes as the data is collected from the same crystal at all wavelengths. Hence, the data is not subject to errors on account of non-isomorphism. The tunable nature of wavelength at the synchrotron radiation sources is of much importance in such experiments. Some commonly available anomalous scatterers are Sulphur, Iron and Zinc. In the modern times, molecular biology techniques are also being used to aid MAD/SAD

experiments. For example by substituting sulphur in methionine residues with selenium (selenomethionine derivative), replacing the N-terminal amino acid of the protein with a heavy atom modified amino acid, or iodination of the tyrosyl side chains. The steps involved in a MAD/SAD data collection experiment can be summed up in the following flow-chart (*Fig 1.13*).

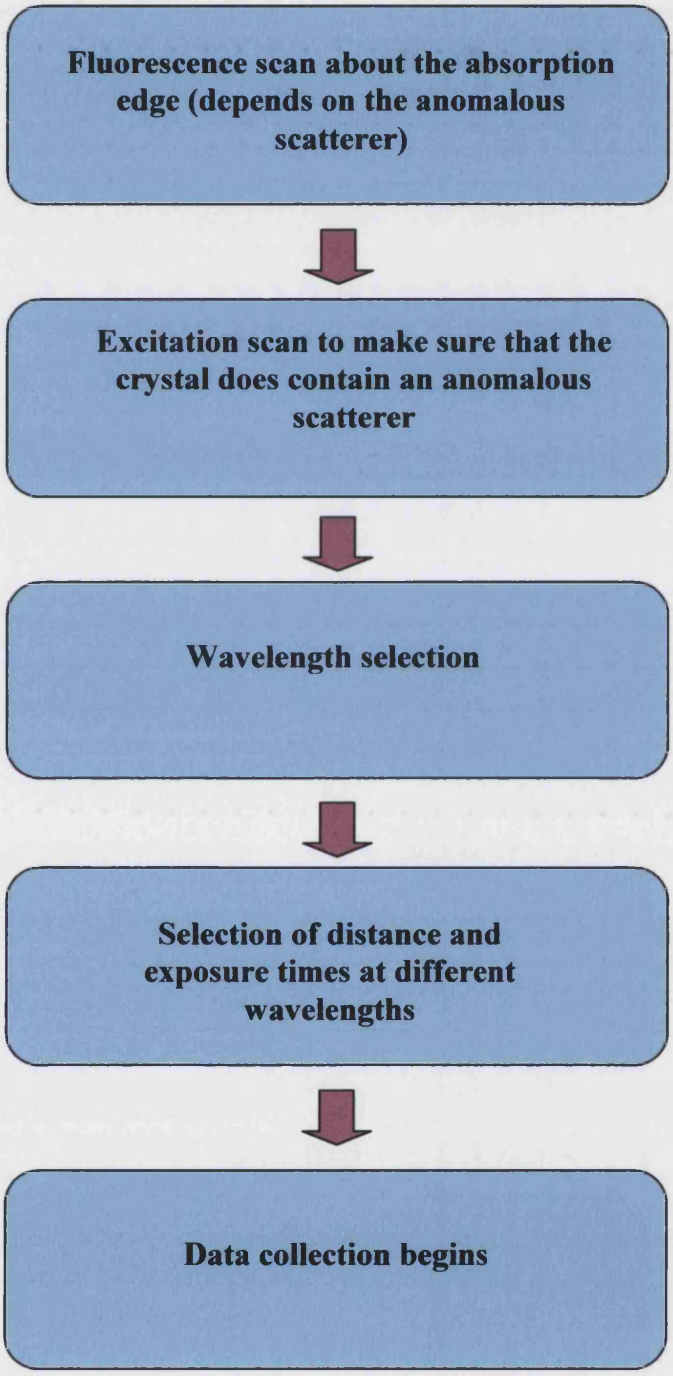


***Fig 1.13*** *Steps involved in MAD/SAD data collection.*

### 1.2.3.3 MOLECULAR REPLACEMENT

Molecular Replacement method is a technique that determines the orientation and the position of a molecule in the unit cell using a previously solved structure as the *search model* (Rossmann and Blow, 1962). The method works mainly by computing three rotation angles to correctly orient the model and then by searching for the translation vector, to place the search model in the unit cell of the unknown protein. The search model can come from various places. It can be a structural homologue, NMR model, a mutant of the native protein or the structure of the same protein but solved in a different space group.

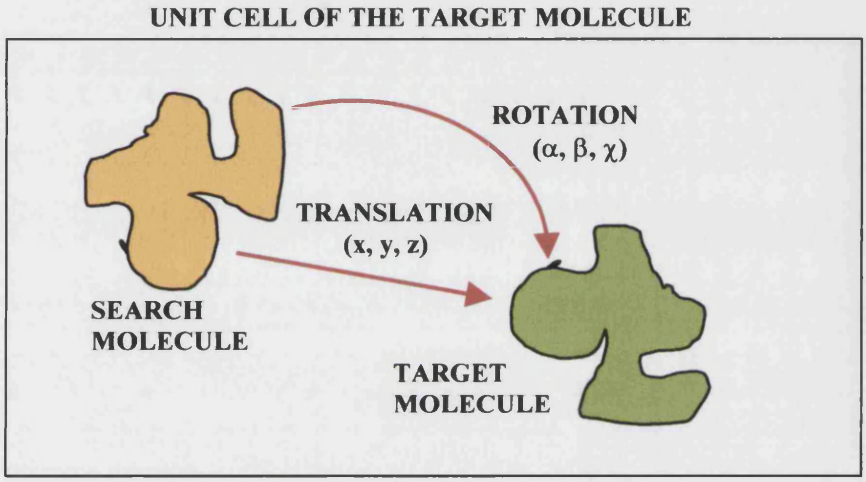


***Fig 1.14*** *A schematic illustration of the Molecular replacement method.*

Traditionally the molecular replacement methods were based on Patterson function properties. A Patterson function can be computed without phase information. When the Patterson map calculated from the observed intensities is rotated against itself, i.e. when the search and the target functions are the same, the resulting function is known as the *self-rotation* function. This helps in computing the rotational non-crystallographic symmetry (NCS). On the other hand, when the Patterson map calculated from the observed intensities is compared with that computed from the atomic coordinates of the structural homologue, the rotation function describes the cross-Patterson vectors. So, if the search model is properly oriented and placed in the unit cell then the observed and the calculated Patterson maps should have similar features. This is the cross rotation function that exploits the intra-molecular vectors of the Patterson function, as they are independent of the position of the

atoms within the unit cell. The translation function, as opposed to cross-rotation function, exploits the inter-molecular Patterson vectors, which depend on both the orientation as well as the position of the molecule within the unit cell. The overlap between the observed and the calculated Patterson map is quantified by multiplying the corresponding peaks in both the maps, which is maximum when the two Patterson maps overlap best. The success of molecular replacement depends on various parameters:

- ***Sequence homology***: The general rule of thumb for molecular replacement to be successful is for the search model to share at least 35-40% sequence identity with the unknown protein.
- ***Quality of the search model***: A good and well-refined search model up to at least 2.0-2.5Å resolution means that the phases calculated from the model will be closer to the true phases (observed phases).
- ***Resolution***: Both high and low-resolution limits affect structure solution.
- ***Radius of integration***: This is chosen in such a way so as to try and include only the intra-molecular vectors. The radius should be smaller than the smallest of the unit cell dimensions and the volume of the sphere defined by this radius should not be more than the volume of the asymmetric unit. Usually 75% of the longest cell dimension is considered an optimal value.
- ***Model cell***: The norm is to try and choose the search model box size in such a way so as to reduce interference by inter-molecular vectors between search models related by unit cell translations.

Once the search model is correctly oriented, it needs to be placed within the unit cell relative to the symmetry of the crystal. A P1 (triclinic) cell has no symmetry elements and hence the search model can be placed anywhere within the unit cell whereas for other space groups, it is imperative to chose the position of model relative to the symmetry axes in the crystal.

Doing a rigid body refinement can optimise the solution so obtained and the *R-factor* or the *Reliability index* can be used as a measure to know if the structure solution is correct or not. The R-factor is an estimate of error

between the observed and the calculated structure factors. The correct solution will give the lowest R-factor. The next step would be to check for crystal packing on the graphics to ensure that there are no clashes between symmetry related molecules.

### 1.2.4. PHASE IMPROVEMENT

Most of the solvent content in the protein crystals is disordered. A continuous disordered solvent channel surrounds the protein molecules within the crystal lattice. The electron density maps hence phased from MIR or anomalous scattering methods are not sufficient to determine the correct structure. The process of density modification is applied in such cases to convert an uninterpretable, poor quality map into an interpretable one. The method involves features like solvent flattening, histogram matching, and if some features of the molecule are known from X-ray analysis, then they can also be incorporated (Zhang *et al.*, 1997). This process is also used sometimes as a starting point to reduce model bias when using phases from a molecular replacement model. This is especially powerful in cases with high non-crystallographic symmetry. NCS averaging reduces model bias and improves the phases by increasing signal-to-noise ratio by a factor of $\sqrt{N}$, where N is the number of copies of the molecule in the asymmetric unit. The phase improvement calculation is a cyclic process and involves modification of the phases in order to obtain best agreement with the known constraints on the electron density and the observed structure factor data.

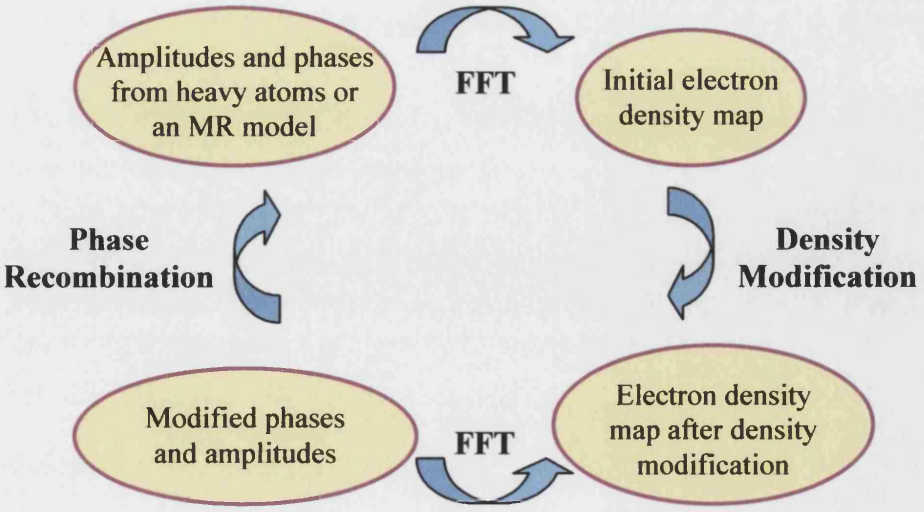


***Fig 1.15*** *A diagrammatic representation of phase improvement protocol*

### 1.2.5. CRYSTALLOGRAPHIC REFINEMENT, MODEL BUILDING AND STRUCTURE VALIDATION

The process of refinement is absolutely necessary because:

1) Manual fitting will inevitably contain errors.

2) Quality of the electro density map is insufficient for better interpretation.

Crystallographic refinement is carried out in a cyclic process of gradual improvement of the model. Refinement is a process by which the model is changed during model building at the graphics display in such a way that the structure factors calculated from the built model get closer to the observed amplitudes. It is a process by which the continuous electron density is represented by discrete atoms. Iterative cycles of refinement optimise the atomic model, allow for improved phases and thus help compute electron density maps that show finer details of the molecular structure. During refinement the parameters of the model get adjusted to the information content of the data. The higher the resolution of the data, more are the parameters that need to be refined. A model that is under parameterised is not as informative as it could be, as it does not represent the data properly. On the other hand, it is not good to over parameterise the model also as it might lead to modelling of abnormalities and noise. The observation to parameter ratio is usually not high enough to introduce and refine each parameter one-by-one. Hence, restraints and constraints are usually used to increase the number of observations. These restraints and constraints reduce the number of parameters to be refined either by forcing a parameter to have a certain value or by setting up relationships between parameters. Although the atomic positions of the structure are unknown, the relationship between them is used as weighted information that effectively increases the observations.

The target function for crystallographic refinement can be expressed by the following expression:

$$E = E_{chem} + w_{xray} E_{xray}$$

where, $E_{chem}$ is an empirical function of potential energy of all atomic positions. It contains information about describing all covalent and non-

covalent interactions between atoms. $E_{xray}$ is the difference between the observed and calculated diffraction data, while the term $w_{xray}$ is the weight that balances the forces arising from each term. The target function is a mathematical score that describes how best a model fits the data. It can be a *least squares* (LS) target wherein the target function is the weighted sum of the squares of the deviations between observed and calculated quantities or it can be a *maximum likelihood* target. This target function can be of three types:

- MLF: Target based on amplitudes
- MLI: Target based on squared amplitudes or intensities
- MLHL: Target based on amplitudes and prior phase information encoded by Hendrickson-Lattman coefficients.

The basic principle behind maximum likelihood function is that the best model will be most consistent with the observations. Consistency can be defined by the following ratio:

$$\textbf{Consistency} = \frac{\mathbf{M_{probability}}}{\mathbf{M_{possibility}}}$$

The ratio represents the number of models in an exhaustive set of equally likely models ($M_{probability}$) that produce an observed value to the total number of possible models ($M_{possibility}$). To apply maximum likelihood it is important to account for the different types of errors in the model such as coordinate errors, missing atoms, and errors in temperature factors (B-factors).

In order to improve the model and subsequently refine (minimise) the target function we use a mathematical algorithm or in other words, an optimisation method. These techniques provide the model to overcome the barriers of being trapped in false/local minima during minimisation by providing the system with the necessary potential energy. There are different algorithms used like *Simulated annealing* (used by CNS; Brünger *et al.*, 1998; Brünger and Rice, 1997), *Full-matrix* (used by SHELXL; Sheldrick and Schneider, 1997), *Conjugate gradient method* (a second derivative target function in which information is derived from the history of the refinement run). This method identifies the nearest minimum in target function by increasing the radius of convergence.

Annealing is a physical process in which a solid is heated to temperatures wherein all the molecules of the solid are randomly arranged in a liquid-viscous phase, and then it is cooled down slowly so that the molecules transcend from their liquid phase into solid phase, re-arranging themselves in the lowest energy state possible. During crystallographic refinement, this process can be simulated, by defining the target energy function as the resting energy of the system. The simulated annealing algorithm used for refinement follows the *torsion angle molecular dynamics* (fixed bond lengths and bond angles). This is based on the observations that the deviations from ideal values are usually small for X-ray crystal structures.

The torsion angle molecular dynamics is coupled with temperature during simulated annealing. The annealing schedule generally followed during the course of refinement is a slow-cooling protocol with a starting temperature of around 5000K and a drop of 25K every 25 seconds. The sampling of several conformations at higher temperatures allows for better results and avoids trapping of structures in false minima. The choice of temperature depends on the quality of the model and resolution of the data.

Each atom can be described by at least 6 parameters. These are the scattering factor for the atom type, the three positional coordinates (x, y, z), the B-factor and the occupancy. B-factors are atomic displacement factors and are very closely associated with coordinates of the model. They represent the distribution of positions occupied by an atom over a period of time (dynamic disorder) as well as variations in the position of an atom between different unit cells (static disorder). Static disorder arises because the observations from the diffraction data represent an averaged structure wherein each conformation for a particular portion of the structure is given a weighting factor according to the fraction of unit cells containing that particular conformation. Hence, large B-factors are indicative of errors in the model coordinates. The method employed for refinement of B-factors depends on the resolution and completeness of the data. Atoms with different atomic displacement parameters (ADPs) contribute differently to diffraction intensities. At a resolution of about 2.5Å or better, B-factors are refined isotropically, i.e.

refinement is based on enforcing similarity of correlation coefficients between pairs of displacement parameters. When the mean atomic displacement parameter is large, then the distribution of these parameters will be wide and so their contribution toward high-resolution intensities will be weak. On the other hand, when the mean atomic displacement parameter is small then most atoms would contribute towards diffraction intensities over the whole resolution range. Atomic resolution (1.5Å or better) means a higher observations-to-parameter ratio and better signal-to-noise ratio. Also the observed intensities at atomic resolution are sufficiently accurate to be able to determine the positional parameters of the model with accuracy. This allows for refinement of the full atomic parameters of the model.

Gross errors in the model cannot be corrected by these refinement protocols. Visual inspection of electron density maps calculated from a given set of phases (either initial phases at the beginning of refinement or at a later stage) is necessary. These Fourier maps (usually $2|F_{obs}|-|F_{calc}|$ and $|F_{obs}|-|F_{calc}|$ maps) can be used to build the structure of the molecule in question with the aid of the known amino acid sequence of the molecule. They aid in identification of the errors in the model. The $|F_{obs}|-|F_{calc}|$ maps highlight the areas in the map that reflect the greatest difference between the observed and the calculated phases. Such unexplained features in the Fourier maps clearly indicate whether or not a model has been accurately refined and complete. These maps help in identifying solvent molecules as well as ligands and ions that are either an integral part of the structure or sometimes get incorporated into the crystal lattice from the crystallisation buffer. Modelling of solvent in the difference densities depends on the peak height (~3-4$\sigma$ above threshold) as well as geometric considerations (the interactions of the water molecule with protein residues). At high resolutions the difference maps also highlight alternative conformations for the side chains of some of the amino acid residues. These residues are usually found on the solvent exposed surface of the protein structure and maybe significant for the protein's biological function.

The quality of the map depends on the accuracy or the inaccuracy of

the phases determined as well as on the resolution. Model building is done on a graphics workstation with the program O (Jones *et al.*, 1991), which allows for the manual fitting of the amino acids into the electron density map. There are now a number of automatic fitting and tracing programs like ESSENS, ffear and warpNtrace, but they all require a high enough resolution (~2.0Å) to be successful. Highly disordered regions of the structure usually occur because of model bias. As a result the Fourier maps around that area are not quite interpretable. Such problems can be dealt (to a certain extent) by calculating *omit maps*. The coordinates for atoms in the disordered region of the structure are left out from Fourier calculation. As a result the density seen for that region in the omit map is completely unbiased and solely based on observed phases.

The primary measure of a refining structure is given by the residual index or the R-factor. As the model improves the measured Fs ($F_{obs}$) and the calculated Fs ($F_{calc}$) converge and the value of the R-factor drops. This can be represented by the following equation:

$$R_{cryst} = \frac{\Sigma \| |F_{obs}| - |F_{calc}| \|}{\Sigma |F_{obs}|}$$

where, $F_{obs}$ is derived from the measured reflection intensity and $F_{calc}$ is the corresponding structure factor obtained from the current model. This conventional parameter is known as the crystallographic R ($R_{cryst}$).

There is another parameter, which states the quality of a structure. It is the $R_{free}$ value. This concept was developed by Brünger (1992a) in order to avoid over-fitting of the model. This cross-validation parameter is a more objective indicator of model quality than the $R_{cryst}$. Setting apart a set of reflections, usually 5-10% of the total reflections, and refining the structure against the remaining data reduces model bias. As the model improves, with iterative model building and refinement cycles, both the $R_{cryst}$ and the $R_{free}$ values drop. Decreasing R-factor values are complimented by another parameter called the *Figure of Merit* (FOM). This value indicates the fraction of the model that represents true structure factors.

In addition to the R-factors, the quality of the model can be assessed by means of *Ramachandran plots* (Ramachandran and Sasisekharan, 1963). These plots underline the stereochemistry, conformation (planarity of the peptide bonds, bond lengths, bond angles etc) and the backbone conformational angles ($\phi$, $\psi$) of the current model based on the expected values for simple organic molecules. These plots give information about the outliers (residues with unusual properties) in the protein model. These outliers can be because of inaccuracy in the modelling process or they may be a genuine feature of the structure e.g. *cis-peptides*. The program PROCHECK (Collaborative Computational Project, 1994) is a useful validation program and can be used to assess the quality of the structure at various stages of refinement.

Another statistical value that helps in assessing the quality of the structure is the deviation from ideal geometry given by root mean square deviation (*r.m.s.d.*) value. If the refinement is successful in imposing the restraints, based on fixed bond lengths and bond angles, then the value for r.m.s.d. will be quite low. For very well refined structures this value is usually between 0.005 – 0.010. This value is usually output by the refinement program CNS after the annealing schedule.

### 1.2.6. STRUCTURE ANALYSIS AND PDB DEPOSITION

Once the structure is complete the next step is to deposit the coordinates with the Protein Data Bank (PDB) at either European Bioinformatics Institute (EBI; http://www.ebi.ac.uk/) or with the Research Collaboratory for Structural Bioinformatics (RCSB; http://www.rcsb.org/). The PDB is a vast global repository of all the three-dimensional crystal structures elucidated so far. The PDB helps in providing access to structural information (for proteins, nucleic acids, ligands and other biological macromolecules) to scientists all over the world.

Understanding of the functional aspects of the protein comes from

understanding the three-dimensional structure of the protein. Hence, it is necessary to analyse the structural data obtained. The final coordinates of the refined model not only reveal the detailed architecture of the polypeptide chain folds but also give an insight into how proteins evolved, the basis on which they interact with each other and how they bind with other molecules. There are several programs (CCP4 supported) available that are used in structure analysis (http://www.ccp4.ac.uk/html/INDEX.html). Some of these programs are DSSP (for assigning secondary structure elements in the structure) CONTACTS (for analysing Hydrogen-bonding interactions), SC (analysis of the shape complimentarity between two interacting protein surfaces), AREAIMOL (to calculate the solvent accessible area in a protein structure), CAVENV (to visualise cavities in the protein structure), PROSA (to determine the native structural fold of the protein).

## 1.3 CIRCULAR DICHROISM

Light can be represented as a transverse electromagnetic wave that is composed of fluctuating electric and magnetic fields, mutually perpendicular to each other. The magnetic field component remains same whereas the electric field component is constantly changing as it propagates (*Fig 1.16*). Light is linearly polarised if it is made up of waves that fluctuate in only one specific plane. In a linearly polarised light, the direction is constant and magnitude is modulated. However, if the waves are 90 degrees out of phase (i.e. one is at maximum when the other is at a zero), the resulting light is circularly polarised. Here the magnitude is constant and the direction is modulated.

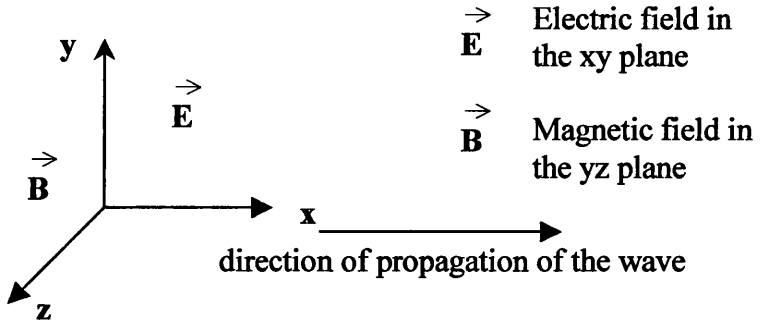


*Fig 1.16 Vectorial representation of the electric and magnetic field components of light.*

Dichroism is a phenomenon that is observed when an optically active matter absorbs right and left handed circularly polarised light slightly differently. This arises due to structural asymmetry and this difference is measured by Circular Dichroism (CD) spectroscopy.

### 1.3.1. CIRCULAR DICHROISM ON PROTEINS

The absence of a regular structure results in zero CD intensity whereas a regular structure can produce positive as well as a negative CD signal. The CD intensity is a result of peptide bond (Far-UV spectral region), disulphide bonds and amino acids such as tyrosine, tryptophan and phenylalanine (Near-UV spectral region) that act as chromophores in the proteins at different wavelengths. Far-UV experiments are carried out in the region of 190-250nm whereas the Near-UV experiments are carried out in the region of 250-350nm. Choice of buffer is very important for Far-UV experiments e.g. reagents like DTT, histidine, imidazole, EDTA should not be used as buffer as they absorb radiation in the far-UV region. Far-UV experiments can determine the approximate fraction of each secondary structure element (α-helix, β-sheets, and random coils and turns) present in the protein structure. On the other hand, the Near-UV CD signals are used to analyse whether or not the protein has folded into a well-defined structure. It is also sensitive to structural changes upon protein-protein interaction, denaturation effects of changes in the protein's environment and so forth. The protein sample used should be free of contaminants. Concentration of the sample must always be checked prior to a CD experiment. Usually 200μl of the protein solution with a concentration in the range of 50μg/ml to 1mg/ml is required for obtaining a Far-UV spectrum whereas a Near-UV spectrum would require 1ml solution at a concentration of about 2.0mg/ml. The higher the concentration better is the absorbance and thus a good CD signal can be recorded.

There are several programs available to analyse the CD spectra of a protein (http://www.cryst.bbk.ac.uk/cdweb/html/home.html/). In general, the CD spectral analysis is 97% accurate for α-helices and only ~70% for β-sheets and 50% for β-turns.

# CHAPTER-2

# ANGIOGENESIS

## 2.1 INTRODUCTION

Angiogenesis is the process of formation of vascular sprouts from pre-existing microvasculature. Physiological angiogenesis occurs only during foetal development, a woman's endometrial cycle, wound healing and muscular growth. The process of vascularisation is also essential for the progression of several diseases. Pathological conditions pertaining to this process can be a function of either uncontrolled angiogenesis as seen in psoriasis, rheumatoid arthritis, atherosclerosis, and many cancers (Folkman 1971; Folkman 1995; Norrby 1997) or dysfunctional angiogenesis that can be associated with ischemia of the heart, poorly healing wounds and fractures, ulcers and infertility (Arnold *et al.*, 1987; Folkman 1998).

An angiogenic stimulus triggers off a highly regulated sequence of events leading to the formation of blood vessels. A vessel wall is composed of an endothelial cell lining, a basement membrane, and a layer of cells called pericytes that partially surrounds the endothelium. The construction of a vascular network requires different sequential steps usually starting with the disappearance of the pericytes. The proteases released from the endothelial-lining breakdown the basement membrane resulting in the binding of the released angiogenic factors to their receptors on the endothelial cells (ECs). This leads to their proliferation and subsequent migration into the interstitial space. The migrating endothelial cells reach the site of stimulus and reorganise themselves as a lumen. The fusion of these newly formed vessels with the generation of a new basement membrane initiates blood flow.

Overall, angiogenesis is the result of an extensive interplay between oncogenes and suppressor genes, stimulatory and inhibitory molecules, proteases and endogenous inhibitors and environmental factors such as the oxygen level (Suri *et al.*, 1996). Angiogenesis is such a complex phenomenon that a clear-cut distinction between angiogenic factors as being either inducers or inhibitors is an oversimplification. Some angiogenic factors act as both direct and indirect inducers of the process while some function contextually, sometimes as inducers and sometimes as inhibitors. In other words, angiogenesis is a process tightly regulated by environmental and genetic

angiogenic regulators that display spatially and temporally co-ordinated activities.

During physiological angiogenesis, there exists a dynamic balance between the positive and the negative regulators of the process whereas during pathological circumstances the rate of blood vessel formation exceeds that of its inhibition. It has been experimentally established that the loss of control of termination and stabilisation of the blood vessels is due to the up-regulation of the positive regulators as well as due to the exhaustion of the endogenous inhibitors of the process of blood vessel formation.

## 2.2 TUMOUR ANGIOGENESIS

More often than not, tumour growth starts with uncontrolled cell proliferation. The rapidly dividing tumour mass soon reaches a steady state due to lack of nutrients and oxygen. The tumour may remain dormant for several months or years yet when the circumstances are beneficial it can switch to an angiogenic phenotype. This event can be triggered off by environmental stress factors such as hypoxia, low pH and pressure generated by proliferating endothelial cells (ECs), immune responses, and genetic factors such as activation of oncogenes or deletion of tumour-suppressor genes that control the production of factors regulating the process of angiogenesis (*Fig 2.1*).

Some tumours and metastases in particular do not initiate in an avascular manner (Pezzella, 1997; Holash *et al.*, 1999). They rather grow by homing in on a host vessel and start off as a vascular tumour. Soon after tumour co-option the host vessels begin to die as a result of apoptosis and the tumours become secondarily avascular and hypoxic. This leads to a definitive increase of tumour derived Vascular Endothelial Growth Factor (VEGF). The induced VEGF leads to a new burst of angiogenic sprouting from the regressing blood vessels. Thus the onset of a robust new angiogenesis allows the tumour to survive and grow further.

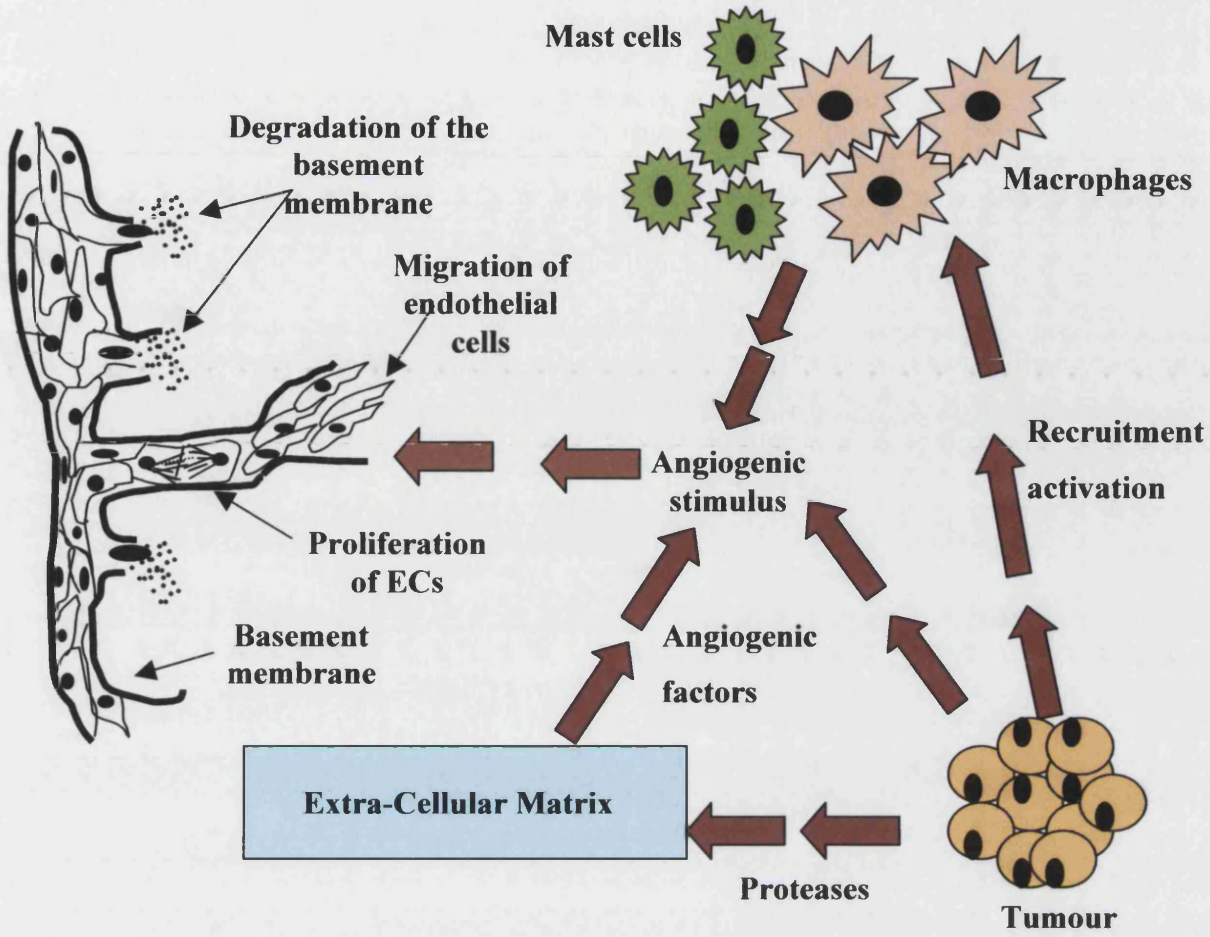


***Fig 2.1*** *A schematic of events that can trigger off the angiogenic switch in dormant tumours*

## 2.3 MODELS TO STUDY THE PROCESS OF ANGIOGENESIS

Considerable insight in the molecular and cellular biology of angiogenesis has been obtained by *in vitro* and *in vivo* studies. However, comparison between different studies is rather difficult because of the great diversity in the models being used. Also, any given compound that is angiogenic *in vitro* may not show any response *in vivo*, where it might be activated or inactivated by other molecules and thereby show results opposite to that of the *in vitro* model. Likewise, substances with no obvious effects, chemotactic and/or mitogenic, *in vitro* may play a crucial role *in vivo* in angiogenesis. Hence, both *in vitro* and *in vivo* models must be employed in order to evaluate the efficacy and potency of various angiogenic and/or anti-angiogenic compounds.

### 2.3.1 *in vitro* MODELS

Most steps in the angiogenic cascade can be understood by using endothelial cells to analyse processes such as proliferation, migration and differentiation. It is easier to control the spatial and temporal concentrations of the various angiogenic mediators involved while using *in vitro* models to study angiogenesis.

***Chemotaxis*** can be examined in a Boyden Chamber. The chamber consists of two wells, an upper well and a lower well, separated by a membrane filter. The chemotactic solution is placed in the lower well and the cells in the upper well and after a period of incubation, the cells on the lower surface of the membrane are counted which gives an indication of the number of cells that have migrated towards the chemotactic stimulus (Fan *et al.*, 1993).

***Proliferation*** studies are based on cell counting, thymidine incorporation, or immuno-histochemical staining. The increase in the number of cells is calculated by measuring Proliferating Cell Nuclear Antigen (PCNA; Hall *et al.*, 1990) or by measuring apoptosis (by the Tunel Assay; Mori *et al.*, 1994).

***Differentiation*** can be studied by culturing the ECs in different extra-cellular matrix (ECM) components, including two- and three-dimensional fibrin clots, collagen gels and matrigel (Benelli and Albini, 1999).

### 2.3.2 *in vivo* MODELS

Classical *in vivo* angiogenic assays include the Chick Chorioallantoic Assay (CAM), the rabbit cornea assay, sponge implant models, matrigel plugs and conventional tumour models. The first evidence of tumour induced angiogenesis used a syngenic combination of tumour and mouse cornea, without involvement of inflammation (Muthukkaruppan and Auerbach, 1979; Muthukkaruppan *et al.*, 1982).

***CAM assay***: The CAM assay is a relatively simple, inexpensive and most widely used assay for large-scale screening as the early chick embryo lacks a mature immune system (Auerbach *et al.*, 1974). The major disadvantage of the model is the presence of a developed network of blood vessels that makes

it difficult to differentiate between the newly developed capillaries and the already existing ones.

***Rabbit cornea assay***: This assay is more reliable than the CAM assay as it represents an *in vivo* avascular site and thus makes it easier to differentiate between newly developed blood vessels and already existing ones. Computer image analysis after perfusion of the cornea with India ink helps to measure the vascular response. It is not a practical model for large-scale screening as it is technically very demanding and very expensive as well.

***Implantation models***: Subcutaneous implantation of various artificial sponges like gelatin and polyvinyl alcohol (incorporated/injected with the compounds to be evaluated) provides another means to study the process of angiogenesis. However, the implantation of these sponges result in some non-specific immune responses which in turn may result in a vascular response even in the absence of exogenous growth factors in the sponge (Andrade *et al.*, 1987).

***Matrigel***: Matrigel generated from Engelbreth-Holm-Swarm (EHS) murine sarcoma contains basement membrane components (collagens, laminin, and proteoglycans) as well as matrix degrading enzymes/their inhibitors and growth factors. Role of the ECM receptors and enzymes in tumour progression has been well characterised using a matrigel incorporated with tumour cells (Passaniti *et al.*, 1992).

***Transgenic models***: Transgenic models are fast becoming the gold standard for manipulating the angiogenic candidate molecules in a controllable manner. In the transgenic approach, a particular candidate disease gene is chosen and re-engineered using molecular tools, confirmed as the desired gene, and is then introduced into the model so it becomes a part of that model's genome. Transgenic models have allowed us to study the consequences of deficiencies, mutations and conditional expression of the different gene products.

## 2.4 ANGIOGENIC PROTEINS

The field of angiogenesis is very vast and complicated and it is not possible to put across the complexity of the process and detail all that is known about each angiogenic molecule within the realm of this thesis. What follows is a brief description of the properties of the angiogenic proteins that I have studied during my PhD. For a recent and exhaustive review on this subject, please see Iyer and Acharya (2002).

### 2.4.1 CYSTEINE-KNOT SUPERFAMILY OF GROWTH FACTORS

The most important and extensively studied class of angiogenic regulators are the Growth Factors. The growth factors and the cytokines present in the tumour's microenvironment regulate a variety of genes associated with malignant properties of tumour cells such as growth, migration, invasion, and metastatic capacities. Several of the angiogenic polypeptide growth factors belong to the cysteine-knot superfamily of growth factors (*Fig 2.2*). These bioactive proteins exemplify the development of distinct biological functionalities during the process of blood vessel formation. These polypeptides display a common structural architecture despite little sequence homology. The crystal structures of these growth factors demonstrate that all have a similar topology based on a cyclic-knot of cysteines involved in both intra-chain and inter-chain disulphide bonds.

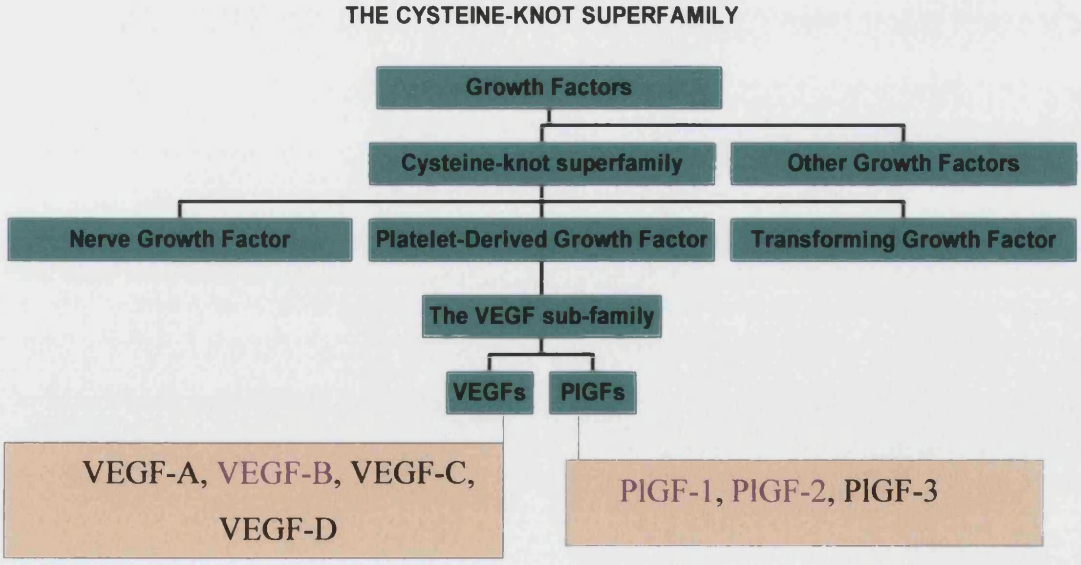


***Fig 2.2*** *Hierarchical organisation of growth factors showing the cysteine-knot superfamily members.*

This cysteine connectivity stabilises the Cα framework of these growth factors for elaboration of the solvent exposed loop regions that form the receptor-binding surface on these polypeptides. The members of this family occur as either homo- or hetero-dimers. Sequence alignment also shows these cysteines to be highly conserved (*Fig 2.3*). These molecules differ on the basis of the number of disulphide bonds, the orientation of the monomers relative to the dimer axis and the extent of buried surface, thus varying the residues that are involved in the dimer formation. The disulphide bonds spatially bring the key residues involved in receptor recognition into close proximity of each other. The cysteine-knot members, although topologically similar, act via distinct cell-surface receptors with which they form complex signalling systems. The parent molecule to the two proteins (Placenta Growth Factor and Vascular Endothelial Growth Factor-B) that I have studied is the Vascular Endothelial Growth Factor-A (VEGF-A). Any study on angiogenesis would be incomplete without the mention of this all-important molecule. VEGF-A shares around 35-40% amino acid sequence identity with PlGF-1 and VEGF-B.

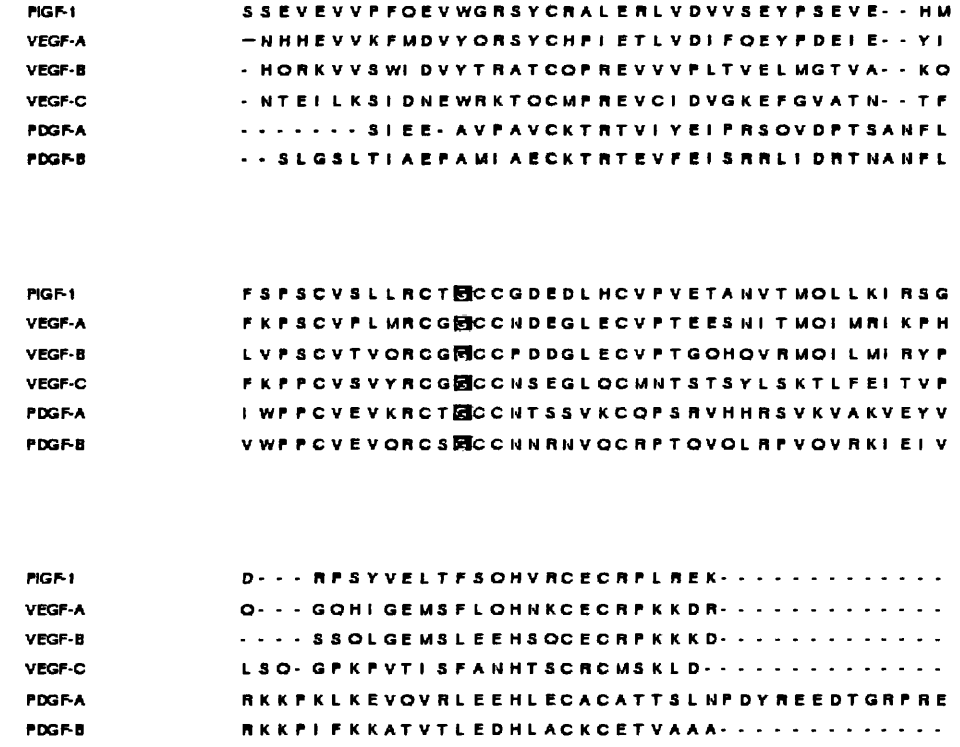


***Fig 2.3*** *Sequence alignment of the members of the cysteine-knot superfamily (PDGF-family) highlighting the conserved cysteines (8 in all). The Glycine (shaded blue) is also conserved in order to accommodate the motif.*

### 2.4.1.1 VASCULAR ENDOTHELIAL GROWTH FACTOR-A (VEGF-A)

Vascular endothelial growth factor (VEGF) is the most potent and pivotal regulator of angiogenesis and vasculogenesis. A highly specific mitogen for vascular endothelial cells, VEGF promotes extravasation of proteins from tumour-associated blood vessels. Disruption of genes encoding VEGF results in severe defects and abnormalities in the development of the cardiovascular system (Ferrara *et al.*, 1996; Ferrara, 2002). VEGF induces cell signalling in different ECs as for example in HUVECs (Human Umbilical Vein Endothelial Cells) and initiates a variety of biological effects. Hypoxia is one of the major up-regulators of VEGF expression and is thought to drive angiogenesis during organogenesis. In developing organs, increasing distance between the migrating cells and the existing blood vessels creates hypoxic conditions. This stimulates the formation of new vasculature towards the VEGF producing cells (Stone *et al.*, 1995; Pierce *et al.*, 1995). Besides hyper-proliferation, blood vessels undergo unregulated and excessive fusion leading to formation of vessels with exceptionally large lumens. On the other hand, limited/reduced VEGF supply to the tissues leads to inhibition of organ development (Folkman and Hanahan, 1991; Drake and Little, 1995; Ferrara *et al.*, 1998).


***Fig 2.4*** *Crystal Structure of Vascular Endothelial Growth Factor – A (PDB code: 2VPF; Muller et al., 1997)*

Crystal structure of VEGF-A (*Fig 2.4*) at 1.93 Å resolution (Muller *et al.*, 1997b) showed that each protomer consists of three intra-chain disulphide bonds and one inter-chain disulphide bond. The monomers are perpendicular to the dimer axis such that there are two disulphide bonds related to each other by the dimer axis. Receptor dimerisation via a VEGF bridge activates signal transduction events that lead to a variety of mitogenic and chemotactic responses that are specific to VEGF-A depending upon where it is expressed.

VEGF is expressed in spatial and temporal association with physiological as well as tumour angiogenesis in vivo (Shweiki *et al.*, 1993; Jakeman *et al.*, 1993; Kim *et al.*, 1993; Millauer *et al.*, 1993). Expression of VEGF induces the formation of vesiculo-vacuolar organelles that channelise the blood borne proteins into the tumours. This forms an extravascular fibrin gel that stimulates ECs and tumour cells to proliferate and migrate and also supports the invasion of stromal cells into the growing tumours (Dvorak *et al.*, 1995). The similarity of mechanism of VEGF induction during physiological as well as tumour angiogenesis explains why VEGF plays a central role in so many types of diverse tumours. A plethora of cytokines, growth factors and other external factors regulate VEGF production and thus indirectly stimulate/inhibit angiogenesis. These include FGF, PDGF, TGF-β, TNF-α, IL-6, IL-10 and IL-13.

Alternative splicing of VEGF mRNA produces 5 isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. These isoforms differ in their heparin and heparan sulphate binding ability. Binding of VEGF to ECM-associated heparan sulphate proteoglycans (HSPGs) releases angiogenic growth factors such as the basic-FGF (Jonca *et al.*, 1997). Heparin and not heparin like molecules enhance the binding of $VEGF_{165}$ to vascular endothelial growth factor receptor-2 (VEGFR-2). HSPGs, like the Glypican, act as a chaperone and restore the binding of $VEGF_{165}$ to VEGFR-2 after oxidative damage. The same however is not true for $VEGF_{121}$ (Gitay-Goren *et al.*, 1992; Weksberg *et al.*, 1996).

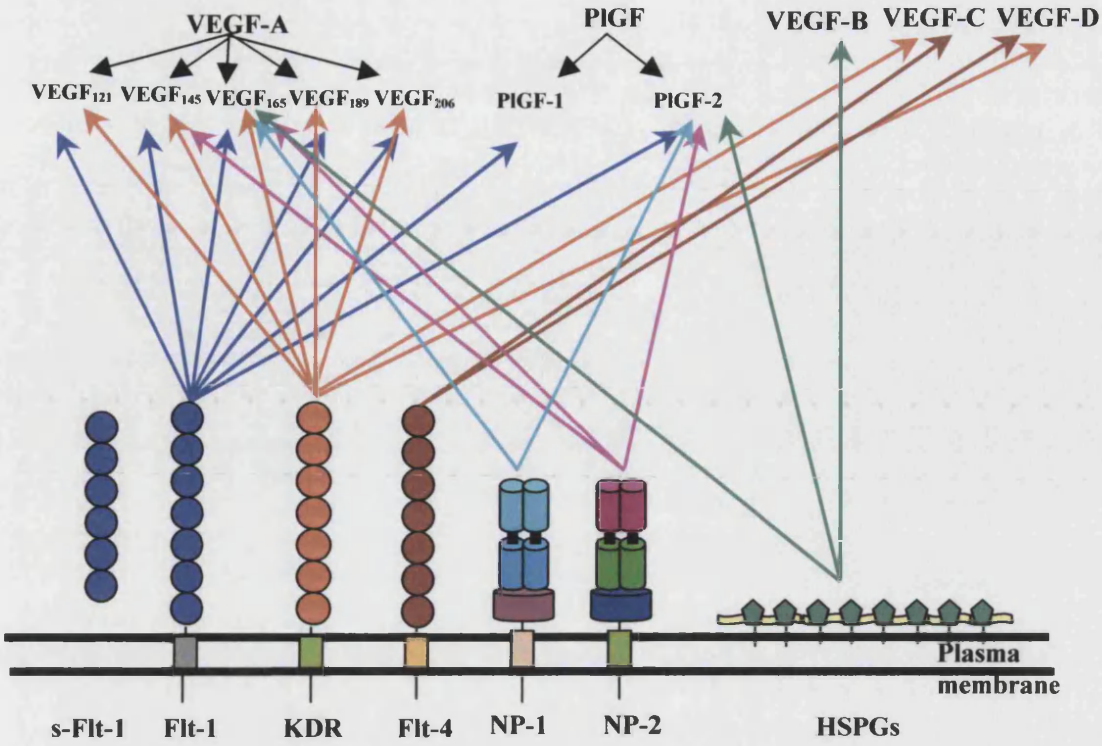


***Fig 2.5*** *A schematic representation of the various receptors characterised for the members of the cysteine-knot family of growth factors. Flt-1, KDR and Flt-4 are tyrosine kinase receptors. s-Flt-1 is the soluble truncated form of Flt-1. NP-1 and NP-2 are neuropilins that belong to the family of semaphoring receptors. HSPGs represent heparan sulphate proteoglycans that act as binding partners for some of the growth factors. The receptors have been coloured individually. The arrows point to the ligands that bind each of these receptors and these have been coloured according to the receptor they represent.*

Apart from binding to cell surface HSPGs/heparin, VEGF binds two receptors (*Fig 2.5*) belonging to the tyrosine kinase receptor family: VEGFR-1/Flt-1 (*fms-like* tyrosine kinase receptor –1) and VEGFR-2/KDR (kinase-insert domain containing receptor). These two receptors are, predominantly, expressed by the ECs, monocytes, trophoblasts and tumorigenic cell types like melanomas. Residues from both the monomers of the active VEGF dimer contribute to each of the two receptor-binding domains, located at opposite ends of the molecule. (Keyt *et al.*, 1996; Muller *et al.*, 1997a). Since VEGF is known to bind both VEGFR-1 as well as VEGFR-2, there is some evidence that the two receptors can heterodimerise via a VEGF bridge (Wiesmann *et al.*, 1997). Activation of VEGFR-2 by VEGF results in a mitogenic response

while that of VEGFR-1 does not induce cell proliferation. Some isoforms of VEGF namely the $VEGF_{145}$ and $VEGF_{165}$ bind another class of receptors called the neuropilins-1 and 2 (Soker *et al.*, 1998). The binding of the VEGF isoforms to these receptors is mediated by the exon 7 of the VEGF gene (Soker *et al.*, 1997) that is absent in $VEGF_{121}$. Gene disruption studies indicate that neuropilins may act as co-receptors of $VEGF_{165}$ because a dysfunctional/ absent neuropilin leads to an impaired development of cardiovascular system leading to the death of mouse embryos. Recently, it was shown that VEGF-A could also strongly induce lymphangiogenic response. Over-expression of VEGF-A results in giant lymphatics that are structurally and functionally abnormal (Nagy *et al.*, 2002).

Inhibition of VEGF signalling inhibits the development of many tumours. Impaired VEGF function abrogates tumour metastasis (Millauer *et al.*, 1996; Skobe *et al.*, 1997). Production of antagonistic VEGF mutants (Siemeister *et al.*, 1998), VEGF receptor inhibitors (Strawn *et al.*, 1996), antisense mRNA expressing constructs (Cheng *et al.*, 1996), inhibitory soluble receptors (Kendall and Thomas, 1993; (Lin *et al.*, 1998), humanised monoclonal antibodies against human VEGF (Presta *et al.*, 1997) are some of the strategies that are being undertaken to treat VEGF induced tumour angiogenesis. VEGF is also being used to develop therapeutics for the treatment of diseases related with dysfunctional angiogenesis (Walder *et al.*, 1996; Magovern *et al.*, 1997; Baumgartner *et al.*, 1998).

### 2.4.1.2 PLACENTA GROWTH FACTOR

The human term placenta seems to be a source of several angiogenic factors. A human cDNA coding for one such angiogenic protein was isolated from a placental cDNA library and named as the Placenta Growth Factor (PlGF). PlGF belongs to the PDGF family of growth factors. The gene encoding this protein is located on chromosome 14 of the human genome (Maglione *et al.*, 1993). DNA sequence analysis of the PlGF gene showed that an alternative splicing mechanism is responsible for the production of the different forms of PlGF. PlGF-mRNA is abundantly expressed in the placental tissue and is also present in very small amounts in heart, lung, thyroid, goitre

and skeletal muscle. No expression of PlGF has been detected in kidney and pancreas (Ziche *et al.*, 1997). The PlGF coding sequence is encoded by seven exons spanning approximately an 8000-kb long DNA interval. The PDGF-like domain exhibited by the PlGF protein is encoded by exons 3 and 4. There are three isoforms of PlGF, namely PlGF-1, PlGF-2 and PlGF-3 (*Fig 2.6*), which arise from alternative splicing of the PlGF-mRNA (Maglione *et al.*, 1993).

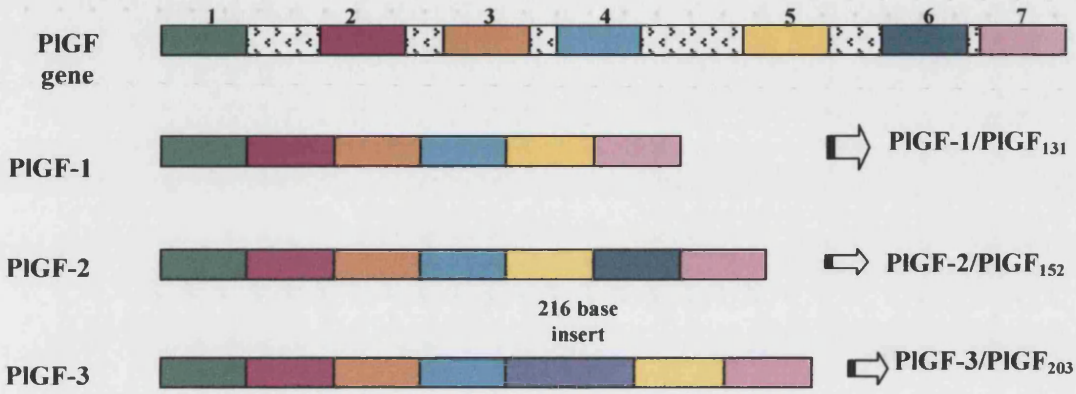


***Fig 2.6*** *A schematic representation of the three spliced variants of the PlGF gene. The gene has 7 exons (each coloured differently) separated by introns (shaded). PlGF-1 and PlGF-3 mRNAs lack exon 6. PlGF-3 mRNA has a 216 base insert between exon 4 and 5. The length of the amino acid sequence indicated for each isoform corresponds to that of the mature protein (Maglione et al., 1993).*

Until recently the biological significance or the relevance of the different forms of PlGF with different composition was not known, but hybridisation studies reveal that PlGF is well conserved in the mammalian, bovine, chicken, amphibian and insect genomes. This suggests that the PlGF gene has specific and indispensable functions. The importance of this protein is emphasised by the fact that a dysfunctional/absent gene in a mouse embryo leads to impaired blood vessel network and subsequently the death of the embryo. PlGF occurs in the placenta, thyroid and lungs. It is important for maintaining the network of blood vessels between the mother and the growing baby. The development and cell-specific regulation of the process of alternative mRNA splicing may have important consequences for physiological and pathological processes. Studies reveal a preferential

expression of PlGF-2 in the placental tissue and cell lines, suggesting that this long form of PlGF may be involved in the growth and maintenance of pregnancy (Maglione *et al.*, 1993; Iyer and Acharya, 2002a).

Purification of PlGF-1 from over-expressing eukaryotic cells and measurement of angiogenic activity of the purified PlGF-1 *in vivo* in the rabbit cornea and CAM assays showed induction of a strong neo-vascularisation process that was blocked by affinity-purified anti-PlGF-1 antibody. In the avascular cornea, PlGF-1 induced angiogenesis in a dose-dependent manner. PlGF-1 has been shown to induce comparable cell growth and migration of endothelial cells from bovine coronary post-capillary venules and from human umbilical veins (HUVECs) *in vitro* (Ziche *et al.*, 1997).

Experiments reveal that PlGF is induced in human keratinocytes during wound healing (Failla *et al.*, 2000). PlGF expression is regulated by key cytokines released during an injury or a wound. It has also been shown that melanoma progression in humans is accompanied by deregulated, constitutive PlGF expression (Graeven *et al.*, 2000). PlGF, however, serves no apparent autocrine role in melanoma proliferation. In case of PlGF-2 it has been established that the recombinant, purified protein is able to stimulate bovine aortic endothelial cells (BAECs) and HUVECs (Hauser and Weich, 1993), but not the ECs from hepatic sinusoids (Sawano *et al.*, 1996). PlGF isoforms have very little mitogenic or permeability-enhancing activity. However, they significantly potentiate the action of low concentrations of VEGF *in vitro* and more strikingly *in vivo* (Park *et al.*, 1994). The credibility of these findings were further cemented by recent experiments with knock out mice (Carmeliet *et al.*, 2001). Synergistic cooperation between PlGF and VEGF (*Fig 2.7*) in pathological conditions is specific. Upregulation of PlGF by ECs leads to displacement of VEGF from VEGFR1. As a result, increased amounts of VEGF are available to bind to the mitogenic response inducing receptor, VEGFR2. These experiments have implicated PlGF in pathological angiogenesis unlike VEGF that is essential for both physiological as well as pathological angiogenesis. This makes PlGF a novel target for therapeutic treatment.

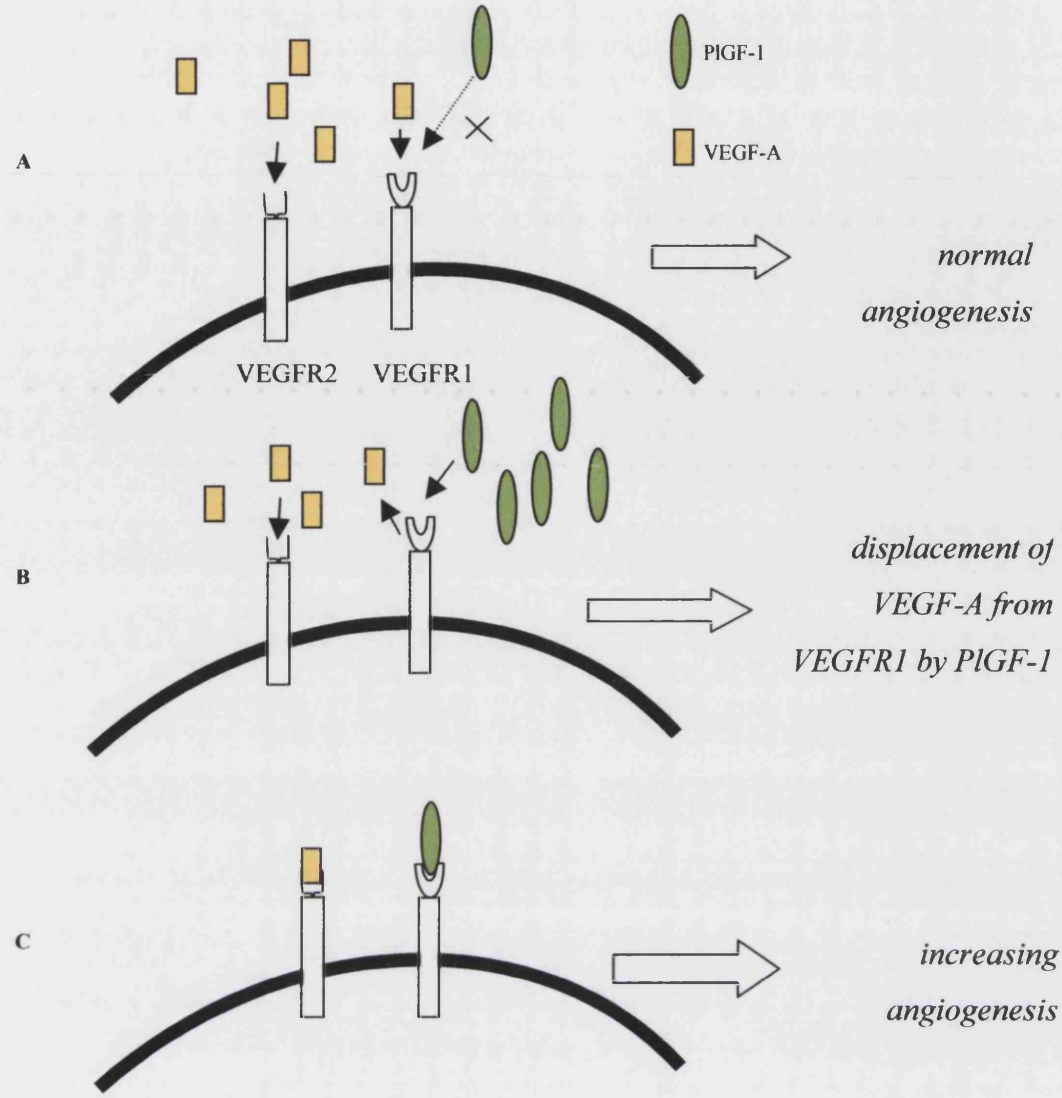


***Fig 2.7*** *Modification of the EC response to VEGF by PlGF-1 during pathological conditions:*
*A) low concentration of PlGF B) increasing concentration of PlGF*

PlGF-1 also forms heterodimers with VEGF-A in tissues where both the molecules are co-expressed (Disalvo *et al.*, 1995; Cao *et al.*, 1996). It acts as an antagonist of VEGF-A activity when both the growth factors are synthesised by the same population of cells. The underlying mechanism was experimentally determined as formation of PlGF-1/VEGF-A heterodimers that are functionally inactive (Eriksson *et al.*, 2002). These results have again shown that despite being weak in comparison to VEGF-A as an angiogenic molecule, PlGF-1 plays an important dual role of regulating VEGF-A, both positively and negatively.

### 2.4.1.3 VASCULAR ENDOTHELIAL GROWTH FACTOR-B (VEGF-B)

Vascular Endothelial Growth Factor-B (VEGF-B) is yet another angiogenic growth factor with the cysteine-knot motif that defines all members of the PDGF family. It was first discovered by accident when searching for new retinoic acid binding proteins in a murine library. Later, it was mapped on to chromosome 11q13 of the human genome (Paavonen *et al.*, 1996). The gene encoding this macromolecule is made up of seven exons (*Fig 2.8*). Alternative splicing of exon 6 due to use of different splice-acceptor sites results in a frameshift and hence in two overlapping reading frames. These encode the two splice variants, VEGF-$B_{167}$ and VEGF-$B_{186}$. These isoforms have distinct, non-homologous C-terminal domains with the C-terminus of the longer variant more hydrophobic. Of the two isoforms, VEGF-$B_{167}$ retains the ability to bind heparin (by virtue of exon 6 which is homologous to the exon 6 of the PlGF gene) whereas VEGF-$B_{186}$ is unable to bind heparin because of the frameshift.

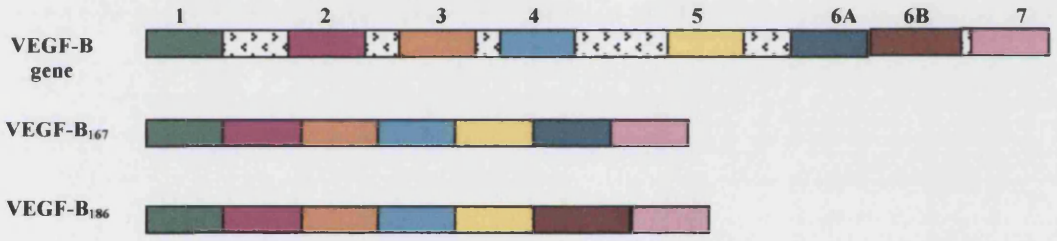


***Fig 2.8*** *A schematic representation of the two spliced variants of the VEGF-B gene. The gene has 7 exons (each coloured differently) separated by introns (shaded). A frameshift in the exon 6 gives rise to the two isoforms*

VEGF-$B_{167}$ and VEGF-$B_{186}$ travel as 21 and 32 kDa proteins under reducing conditions during gel electrophoresis. Amino acid sequence alignment of VEGF-B with other cysteine-knot proteins reveals that it shares highest homology of about 43% with VEGF-$A_{165}$. The next closest relative is $PlGF_{152}$. Like other cysteine-knot proteins VEGF-B also exists as a disulphide-linked homodimer. Both the isoforms can form heterodimers with VEGF-A when co-expressed. VEGF-$B_{167}$ remains associated with the cell surface unless released by heparin treatment whereas VEGF-$B_{186}$ is secreted

in a soluble form because of the lack of the basic, heparin-binding region. One unique feature of VEGF-$B_{186}$ is that unlike other PDGF family members it is the only member that is *O*-glycosylated.

VEGF-B is expressed abundantly in skeletal and cardiac muscles with the two isoforms seemingly being expressed in equal amounts in the various tissues. VEGF-B is known to bind both Flt-1 (VEGFR-1) and neuropilin-1 receptors with high affinity. VEGF-B can bind to VEGFR-2 and VEGFR-3 but with very low affinity and hence its inability to induce a strong mitogenic signal for endothelial cells. However, it was later found that the ability of VEGF-B to stimulate cell proliferation is enhanced when it interacts with tenascin-X in the extracellular matrix (Ikuta *et al.*, 2000). It is believed that perhaps VEGF-B acts in a paracrine mode, as the receptor to which it selectively binds is exclusively present in the endothelial cells (Aase *et al.*, 1999). The binding of VEGF-B to Flt-1 regulates the expression and activity of urokinase-type Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor 1 (PAI-1; Olofsson *et al.*, 1998).

It is becoming increasingly evident that VEGF-B has potential clinical use in therapeutic angiogenesis. It was found that mice lacking in *Vegfb* gene have smaller hearts and are unable to recover completely from cardiac ischemia suggesting that the gene was essential for the development of a fully functional coronary vasculature (Bellomo *et al.*, 2000). The partial overlapping of expression patterns of VEGF-B with that of VEGF-A suggests that the VEGF-B/VEGF-A heterodimers may be biologically important to regulate the bio-availability and signalling properties of VEGF-A.

### 2.4.2 MATRIX METALLOPROTEINASES (MMPS) AND TISSUE INHIBITORS OF MATRIX METALLOPROTEINASES (TIMPS)

Matrix metalloproteinases are a family of structurally related zinc-containing endopeptidases that mediate the breakdown of connective tissue macromolecules and are responsible for much of the turnover of the extracellular matrix.

***Table 2.1** The different members of the MMP family*

| Sub-type | MMP | Pseudonym | Mol wt | | Substrates | |
|---|---|---|---|---|---|---|
| | | | Latent | Active | Collagen substrates | Other substrates |
| **Collagenases** | MMP-1 | Fibroblast collagenase | 55 | 45 | I, II, III, VII, VIII and X | Casein, gelatin, MMP-2 & MMP-9 |
| | MMP-8 | Neutrophil collagenase | 75 | 48 | I, II, III, V, VII, VIII and X | Gelatin, aggrecan, fibronectin |
| | MMP-13 | Collagenase-3 | 60 | 48 | I, II, III and IV | Gelatin, aggrecan, tenascin, PAI-2 |
| | MMP-18 | Xenopus collagenase | 55 | 42 | Not known | |
| **Gelatinases** | MMP-2 | 72 kDa type IV collagenase | 72 | 66 | I, II, III, IV, V, VII, X and XI | Gelatin, aggrecan, elastin, versican, laminin, fibronectin |
| | MMP-9 | 92 kDa type IV collagenase | 92 | 86 | IV, V, VII, X and XIV | Gelatin, aggrecan, elastin, versican, α-PI |
| **Stromelysins** | MMP-3 | Stromelysin-1 | 57 | 45 | II, III, IV, IX, X and X | Gelatin, aggrecan, elastin, versican, casein, laminin, activates MMP-1, 8 and 13 |
| | MMP-10 | Stromelysin-2 | 57 | 44 | III, IV and V | Gelatin, aggrecan, elastin, casein, activates MMP-1, 7, 8 and 9 |
| | MMP-11 | Stromelysin-3 | 51 | 44 | | α-PI, laminin |
| **Membrane type- MMPs** | MMP-14 | MT1-MMP | 66 | 56 | I, II and III | Aggrecan, elastin, MMP-2, 13, gelatin |
| | MMP-15 | MT2-MMP | 72 | 60 | | Fibronectin, |
| | MMP-16 | MT3-MMP | 64 | 52 | | MMP-2 |
| | MMP-17 | MT4-MMP | 57 | 53 | | Fibrin and gelatin |
| | MMP-24 | MT5-MMP | 63 | 45 | | |
| | MMP-25 | MT6-MMP | not | known | IV | Gelatin, laminin-1 fibronectin, |

*This table has been taken from a review by Elliot and Cawston (2001).*

These proteases are normally synthesised as latent forms known as zymogens. This conversion may occur via other enzymes or auto-catalytically (Okada 2001). It is hypothesised that the proteases interact with each other resulting in the activation of other proteases that then collectively degrade all

the extra-cellular matrix (ECM) components. Over-expression and activation of one protease may provide a proteolytically active environment for the onset of local proteolysis of the basement membrane during angiogenesis. Degradation of the extracellular matrix is also important for tumour cell invasion. Experiments have revealed that most proteases can degrade the ECM components both extracellularly at neutral pH under normal physiological conditions and at acidic pH under pathological conditions (Griffiths, 1991; Montcourrier *et al.*, 1997; Baron, 1989). Coopman *et al.* (1996) have shown a correlation between the invasive ability of different cancer cell lines and their ability to phagocytose the ECM.

The MMPs form a large family of enzymes consisting of at least 26 MMPs although the exact role of each member, in both normal physiology and pathological conditions is still not very clear (Elliott and Cawston, 2001). All MMPs share several common features. They all exhibit a common domain structure, where each domain contributes towards a particular function of the enzyme. The MMPs have an N-terminal catalytic domain, and a C-terminal domain, which is involved in substrate binding and in proper orientation and presentation of the N-terminal domain at the catalytic site (Becker *et al.*, 1995; Lovejoy *et al.*, 1999). Apart from these common features, some MMPs contain additional domains that confer particular function to the enzyme. For example, MT-MMPs (with the exception of MMP-17 and MMP-25) contain a trans-membrane domain as well as a cytoplasmic tail (Fernandez-Catalan *et al.*, 1998). MMPs are all characterised by the presence of calcium and zinc ions, which have a role to play in both the activity and structural integrity of the enzyme. The active site of the enzyme remains blocked in the latent form of these molecules. Upon activation the pro-peptide gets cleaved off and the enzyme gets activated. The catalytic zinc is coordinated to the conserved amino acid residues that form the active site in the metalloproteinases, indicating a structural basis for the ion-dependent activity of all MMPs (Bode *et al.*, 1999).

MMP activity is regulated by tissue-inhibitor of metalloproteinases (TIMPs). The phylogeny of the TIMP indicates that TIMP from Drosophila

forms the root of the evolutionary tree. Cluster analysis (*Fig 2.9*; Brew *et al.*, 2000) and topological analysis reveal that TIMPs in humans have evolved by the process of gene duplication at various time intervals. Humans have four isoforms of TIMP identified so far: TIMP-1, -2, -3 and -4. Human TIMPs are homologous to TIMPs from insects, *C. elegans*, chicken, amphibians and fishes, both at the cDNA and the protein level. Of the four known human TIMPs, TIMP-1 is the closest to the Drosophila TIMP. Mammalian TIMPs have two domains, namely the N-terminal and the C-terminal domain. The N-terminal domain, which exemplifies a characteristic OB-fold (oligonucleotide-binding fold) topology, can form an active MMP inhibitor on its own.

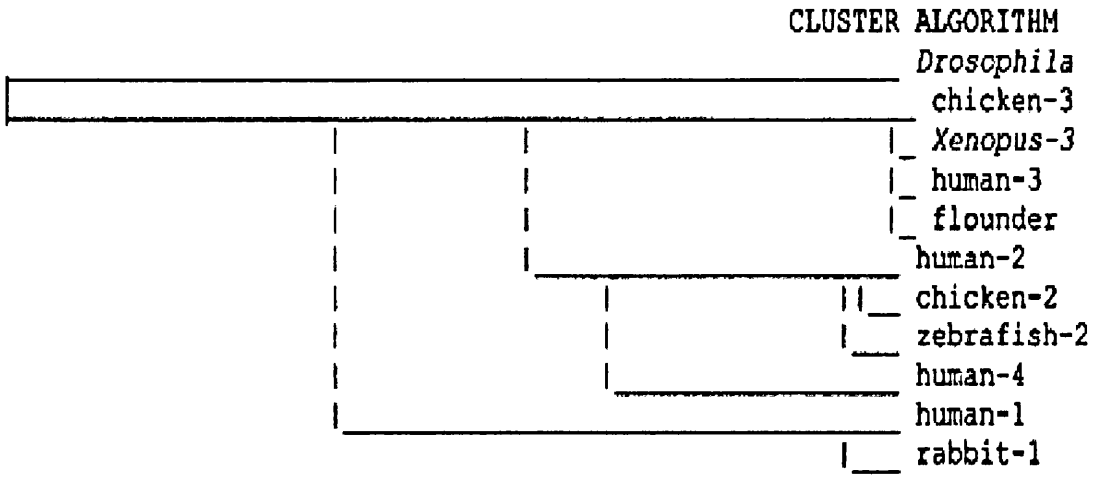


***Fig 2.9*** *Phylogenetic trees representation of the sequence divergence among the TIMPs (Brew et al., 2000). Figure reproduced, from the reference mentioned. human-1, 2, 3, and 4 refer to the four isoforms of TIMP.*

The TIMP genes show a conserved nature with respect to gene organisation within the synapsin locus, right across the animal kingdom, from Drosophila to humans (Pohar *et al.*, 1999). The sequence comparison of TIMPs (*Fig 2.10*) suggests that they may be concurrent with the origins of metazoan connective tissue. The four human TIMPs are differentially expressed in tissues and are temporally regulated by the influx of MMPs. These four TIMPs inhibit different MMPs with different inhibition efficiencies. The TIMPs bind to the MMPs in a slow, non-covalent and

irreversible ratio of 1:1 (Henriet *et al.*, 1999) with low nano molar inhibition constants.

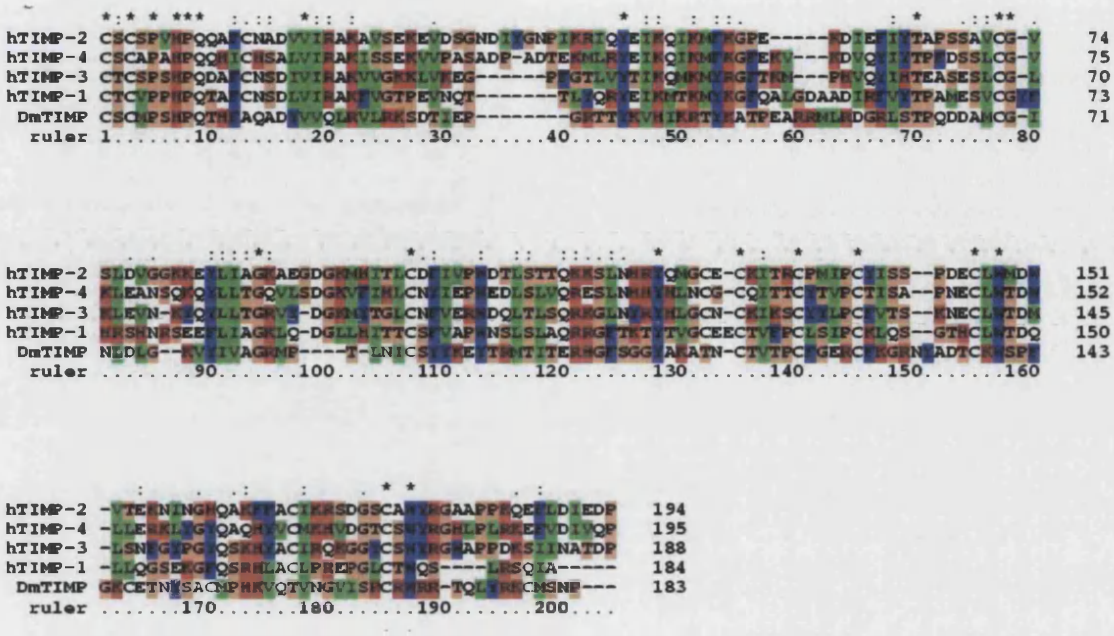


***Fig 2.10*** *Sequence alignment of the four isoforms of human TIMPs: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 with the Drosophila TIMP (DmTIMP)*

Although the four TIMPs show a high degree of divergence in their sequence, different TIMPs can bind to most MMPs. But there are differences in their inhibitory properties. The three-dimensional structures of TIMPs with MMPs resolved so far have enhanced our understanding of the interactions between TIMPs and MMPs (Gomis-Ruth *et al.*, 1997; Fernandez-Catalan *et al.*, 1998). Structural studies on other MMP/TIMP complexes will help us understand the complexities of interactions between these multi-domain molecules and further our knowledge about the biological relevance of having such a variety of MMPs and TIMPs.

An imbalance in the activity of MMPs and the TIMPs is believed to be the underlying reason for several major pathological conditions such as inflammatory and degenerative diseases like rheumatoid arthritis (Cawston, 1998), cancer and tumour progression. The central role played by the metalloproteinases and their inhibitors in a wide range of proteolytic events,

both in normal and pathological circumstances, has projected the MMPs and TIMPs as potential therapeutic targets.

## 2.5. ROLE OF HEPARAN SULPHATE PROTEOGLYCANS IN ANGIOGENESIS

Proteoglycans are negatively charged polysaccharide chains composed of repeating disaccharide units, attached to a core protein (Lindahl *et al.*, 1994). Proteoglycans are found in almost all cell types. The wide spectrum of their biological functions ranges from being a purely mechanical support molecule to the one with complex cellular functions such as adhesion, proliferation and differentiation. These biological effects are due to the ability of these molecules to act as receptors for a wide range of molecules. One group of proteoglycans known as the Heparan Sulphate Proteoglycans or HSPGs is involved in both physiological as well as pathological angiogenesis.

The biological functions of HSPGs are due to their ability to bind to different kinds of angiogenic molecules including growth factors, extracellular matrix proteins, and protease inhibitors (Lindahl *et al.*, 1994). Some of the angiogenesis related HSPG binding molecules are:

- Fibroblast Growth Factors
- Vascular Endothelial Growth Factor
- Placenta Growth Factor
- Heparin-binding Epidermal Growth Factor
- Hepatocyte Growth Factor
- Transforming Growth Factor-β
- Interferon-γ
- Platelet Factor-4
- Interleukin-8
- Macrophage Inflammatory Protein
- Interferon-γ inducible protein-1
- Platelet-Derived Growth Factor
- Plieotropin

The interaction of HSPGs with the molecules depends on distinct structural features. These interactions depend mainly on the size of the polysaccharide chain and on the degree and distribution of the sulphate groups (Maccarana, 1993). HSPG binding does not depend solely on the sulphate groups (*Table 2.2*). The interaction also depends on the biochemical properties of the protein. The HSPG binding regions on most proteins have been identified as a cluster of basic amino acids (Zhang *et al.*, 1991; Zhu *et al.*, 1990; Eriksson *et al.*, 1991).

***Table 2.2*** *The different sulphate groups required on HSPGs for potentiation of binding of these ligands to their receptors.*

| **Angiogenic molecules** | **The important sulphate groups** |
|---|---|
| FGF-2 | 2-O- and N-sulphate groups (Maccarana, 1993) |
| FGF-1 and FGF-4 | 2-O- and 6-O-sulphate groups (Guimond *et al.*, 1993) |
| HGF | 6-O-sulphate groups of $GlcNSO_3$ (Lyon and Gallagher, 1994) |
| HIV-Tat Protein | 2-O- and 6-O- and N-sulphate groups (Rusnati, *et al.*, 1994) |
| Interleukin-8 | 6-O-desulphated heparin, N-desulphated/N-acetylated heparin (Rusnati *et al.*, 1994) |
| PlGF-2 | 2-O- and 6-O-sulphate groups (Midgal *et al.*, 1998) |

HSPGs mediate the binding of these growth factors to their respective tyrosine kinase receptors (Mustonen and Alitalo, 1995). For example recent solution experiments have shown that heparin although is not absolutely necessary for the formation of the receptor-ligand complex, it does enhance the binding of FGFR to FGF-2 (Rusnati *et al.*, 1994). What is common about the ternary interaction among the HSPGs, growth factors and the receptors is

the ligand-induced receptor dimerisation for activating the tyrosine kinase receptors that is required to induce a mitogenic response (Ruoslahti and Yamaguchi, 1991; Vlodavsky *et al.*, 1991). The storage of the HSPGs in the ECM increases the local concentration of the growth factors and a decrease in the radii of diffusion. This favours ligand oligomerisation, receptor interaction and thus signalling (*Fig 2.11*).

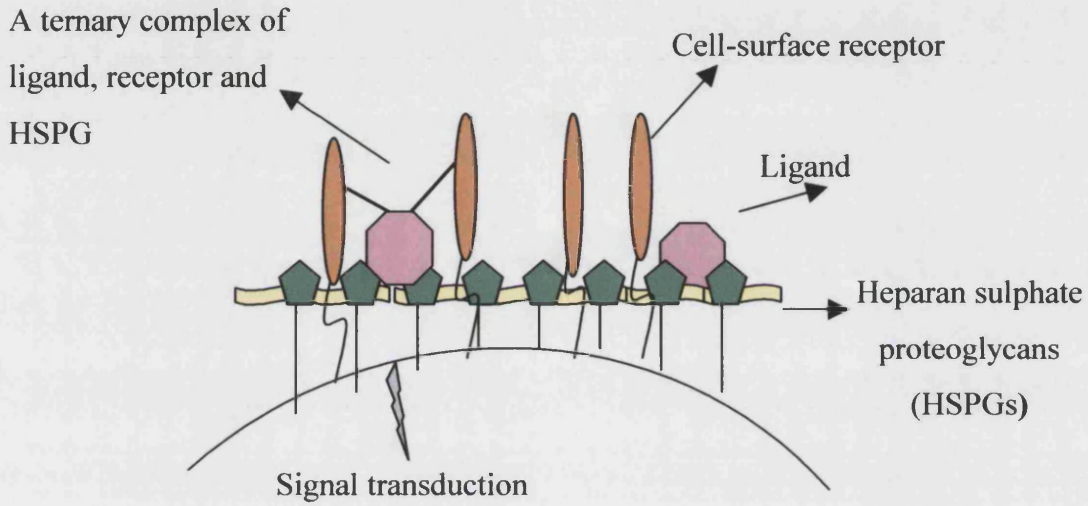


***Fig 2.11*** *Role of HSPGs in ligand-induced receptor dimerisation and thus cell signalling*

Molecules that bind to an angiogenic growth factor that will interfere with the latter interacting with ECM associated proteoglycans are potential compounds with angiostatic activity (*Table 2.3*). These molecules fall into two categories:

- Molecules that compete with the heparin-binding growth factors for HSPG interaction (eg. poly-cationic compounds).
- Molecules that compete with the HSPGs for growth factor binding interaction (eg. poly-anionic compounds).

*Table 2.3 Some known inhibitors of HSPGs*

| Inhibitors | Mechanism of action |
|---|---|
| Protamine | Compete with the heparin-binding growth factors for HSPG interaction |
| Chemokines | Compete with the heparin-binding growth factors for HSPG interaction |
| Tecogalan sodium | Compete with the heparin-binding growth factors for HSPG interaction |
| Alpha-2 Macroglobulin | Compete with the HSPGs for growth factor binding interaction |
| Suramin and its derivatives | Compete with the HSPGs for growth factor binding interaction |
| Pentosan polysulphate | Compete with the HSPGs for growth factor and receptor binding interaction |

# CHAPTER-3

# THE CRYSTAL STRUCTURE OF PLACENTA GROWTH FACTOR-1 (PLGF-1)

## 3.1 INTRODUCTION

Two decades earlier there were not many angiogenic molecules that had even been purified. Ten years down the line they are not only being purified by several laboratories, but one can now access the complete amino acid sequence of most of the angiogenic molecules known. In the past decade or so, the field of angiogenesis can claim three-dimensional structures of several molecules. In 1997, Muller *et al.* (1997b) determined the three-dimensional crystal structure of Vascular Endothelial Growth Factor-A (VEGF-A), the molecule central to the understanding of the process of blood vessel formation. While important physiological relevance of this polypeptide was and is still being discovered, a gene encoding another growth factor was located on Chromosome 14 of the human genome. This growth factor was named as the Placenta Growth Factor-1. It became apparent from its amino acid sequence that it was closely related to VEGF-A. It was found that despite ~40% similarity, there were significant differences in the physiological behaviour of the two moleules. Although both of them share the ability to bind one of the tyrosine kinase receptors, Flt-1 (VEGFR1), they do not elicit the same kind of angiogenic response. These interesting differences between VEGF-A and PlGF-1 prompted us to undertake structural studies on PlGF-1. What is described in this chapter is how we went about determining the crystal structure of the protein in question. Initially, the structure was solved at 2.0Å resolution (Iyer *et al.*, 2001). More recently a higher resolution dataset was collected and the structure and the resolution of the structure extended to 1.55Å (Iyer and Acharya, unpublished results).

## 3.2 MATERIALS AND METHODS

### 3.2.1 EXPRESSION AND PURIFICATION OF PlGF-1

Placenta Growth Factor-1 protein was provided to us by our collaborators (Drs. D. Maglione and M.G. Persico, CNRS, Napoli, Italy). The material used for crystallographic studies was recombinant human PlGF-1 (rhPlGF-1). Production of pure rhPlGF-1 was based on recombinant DNA technology. The gene encoding the mature protein was cloned into a prokaryotic expression vector by polymerase chain reaction (PCR). This vector was then used to transform DE3 *Escherichia coli* strain, and 1mM

isopropyl-2-D-thiogalactopyranoside was used to induce the synthesis of PlGF-1. Inclusion bodies were purified from the induced bacteria and refolded in denaturing buffer and oxidising/reducing buffer. The refolded PlGF-1 protein was then purified first by anion exchange chromatography followed by reverse phase chromatography. Final recovery of the protein was about 140mg per litre of initial bacterial culture. The angiogenic nature of the protein was tested using a CAM assay; the purified recombinant PlGF-1 was able to induce a strong and dose-dependent angiogenic response (Iyer *et al.*, 2001; Maglione *et al.*, 1991).

PlGF-1 is biologically active as a dimer. The dimeric nature of the material used for crystallisation was confirmed by the SDS-PAGE (*Fig 3.1*), under reducing conditions, done on samples from the peak of reverse-phase containing bulk of the purified material (dimer) as well as the rejected peak of reverse-phase containing monomers.

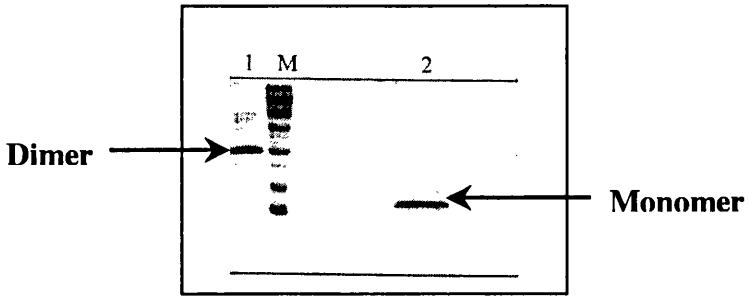


***Fig 3.1*** *An SDS-PAGE analysis of the protein, rhPlGF-1, under non-reducing (lane 1) and reducing (lane 2) conditions. The lane marked 'M' shows the Molecular weight markers used for the experiment. The gel was stained with coomassie.*

### 3.2.2 CRYSTALLISATION STRATEGY FOR PlGF-1

Crystals of recombinant human PlGF-1 were available at the time I started my PhD study (grown by Dr. D.D. Leonidas, a former member of our laboratory). I could reproduce these crystals for high-resolution structure determination. They were grown using the hanging drop vapour diffusion method. The drops contained 8mg/ml protein at pH 6.0 in 0.05M MES buffer, 10mM $CaCl_2$ and 7.5% (v/v) 2-methyl-2, 4-pentanediol (MPD) equilibrated

against a reservoir solution containing 0.1M MES buffer (pH 6.0), 20mM $CaCl_2$ and 15% (v/v) MPD. Single crystals appeared after 5-6 days at 16°C. The crystals show highly ordered pyramidal morphology (*Fig 3.2*). These crystals could be flash-frozen at 100K using a cryoprotectant solution containing 0.1M MES buffer (pH 6.0) and 30% (v/v) MPD.

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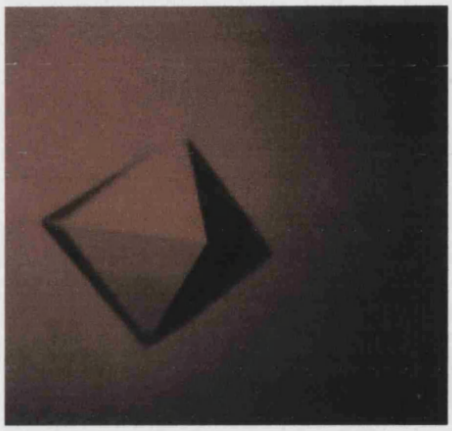

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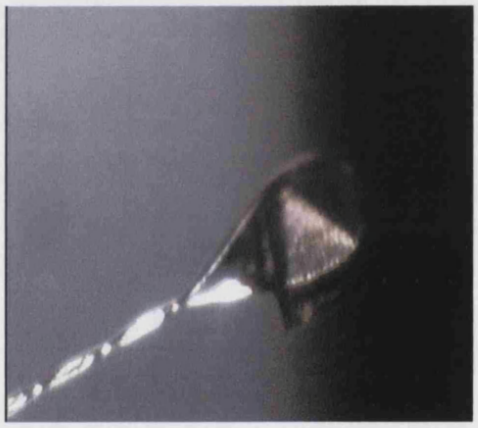


***Fig 3.2*** *(A) Crystal of Placenta Growth Factor-1. (B) PlGF-1 crystal held in the loop during X-ray data collection*

### 3.2.3 DATA COLLECTION, PROCESSING, AND SCALING

Initially a complete dataset to 2.0Å was collected at 100K from a single crystal using the Synchrotron Radiation Source (station PX 9.5, Daresbury, UK). I used this dataset structure determination (Iyer *et al.*, 2001). The data was recently extended to 1.55Å resolution (collected at 100K on station PX 9.6, Daresbury, UK). A total of 515 images were collected ($\lambda$=0.87Å) using a CCD-ADSC detector system. Both high and low-resolution dataset were merged to get a master dataset at 1.55Å.

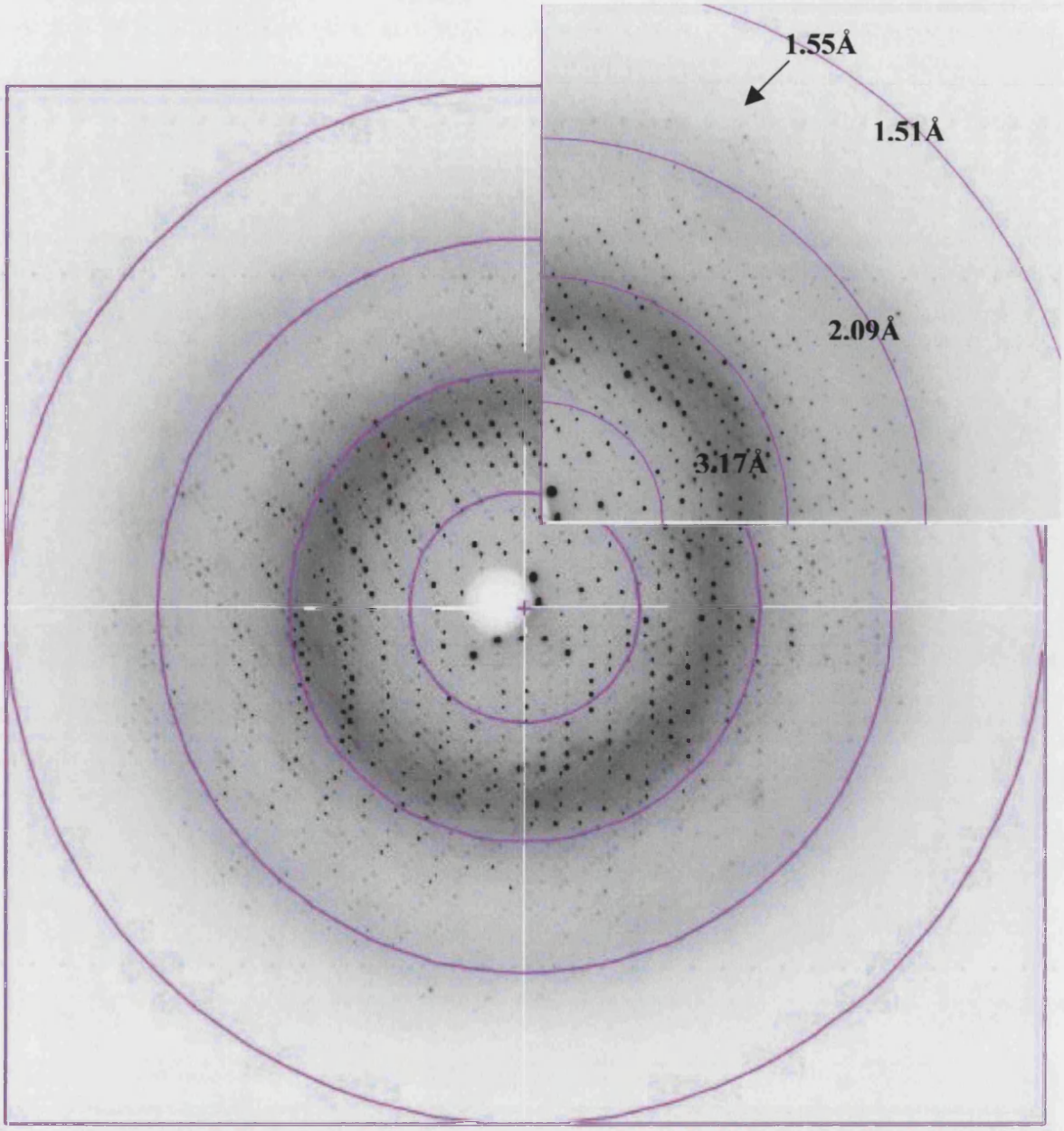



***Fig 3.3*** *A typical diffraction image from a PlGF-1 crystal diffracting to 1.55Å resolution (1.5Å at the edge). The inset represents a portion of the image zoomed in to show the high-resolution spots. The circles have been drawn to mark the resolution range. The data collection parameters used were:* $\lambda$ = *0.87Å;* $\Delta\varphi = 0.8°$ *and exposure = 10 seconds.*

Data processing was performed with the HKL package (Otwinowski and Minor, 1997) in the tetragonal space group, P4. Data reduction was carried out using the program, TRUNCATE, of the CCP4 suite (Collaborative Computational Project, 1994). Even though the crystal diffracted to 1.5Å resolution, reflections in the last shell were very weak giving a very high $R_{sym}$ of 71%. Hence the data was scaled to only 1.55Å. The following table (*Table 3.1*) details the differences between the 2.0Å and the 1.55Å datasets.

***Table 3.1*** *Comparison of data processing statistics of both the 2.0Å and the 1.55 Å resolution datasets*

| Data parameters | PlGF-1 (2.0Å) | PlGF-1 (1.55Å) |
|---|---|---|
| Unit cell dimensions (Å) | *a*=*b*= 62.6, c= 84.1 | *a*=*b*= 62.2, c= 84.1 |
| Reflections measured | 161,044 | 1,078,548 |
| Unique reflections | 21,067 | 48,576 |
| $R_{sym}$ (%) | 6.3 | 7.8 |
| I/σI (outermost shell) | 19.6 (3.6) | 10.3 (3.4) |
| Completeness (outermost shell) (%) | 99.4 (98.8) | 99.4 (98.9) |

*The outermost shell corresponds to the resolution bin covering 2.07 – 2.00 Å*

The solvent content for the crystal was calculated using the CCP4 supported program, matthews_coeff. This helped to determine the number of molecules in the asymmetric unit. Calculations showed that for one PlGF-1 dimer (molecular weight of ~29,000 Da) in the asymmetric unit, the Matthew's coefficient was 2.6$Å^3$/D with the solvent content of around 51%.

Although the crystal lattice was determined as tetragonal (P4), it still remained to be determined which of the subgroups the crystal belonged to. This was confirmed by looking at the systematic absences in each of the sub-space groups. Two criteria were chosen for a reflection to be deemed as present. Reflections that hold true to the general as well as special conditions

specified for a particular subgroup should be present and the I/σI value for each reflection that conforms to the former criterion should be greater than 2. To assign the correct space group, data were scaled in all the subgroups ($P4_1$, $P4_2$, and $P4_3$) and comparisons were made. Those belonging to the higher subgroup (P422) were ignored on the basis of more number of asymmetric units in each of the higher sub-space groups, as this did not correspond to a sensible value for Matthew's coefficient. Examination of the systematic absences showed that the actual space group was one of the two enantiomers, $P4_1$ or $P4_3$ and not $P4_2$.

### 3.2.4 STRUCTURE SOLUTION

The structure of PlGF-1 was determined by molecular replacement using the program AMoRe (Navaza, 1994) in the CCP4i module. The SCALEPACK reflection file was converted into an MTZ format suitable for input to AMoRe (SCALEPACK2MTZ). The first routine in AMoRe (SORTING) sorted the reflection file according to the space group symmetry. A poly-alanine (homodimer) model based on the structure of VEGF-A elucidated at 1.93Å resolution [PDB code-2VPF; Muller *et al.*, 1997b] was used as the search model (TABLING) to aid structure solution (one homodimer per asymmetric unit). Data in the range 15.0-3.0Å were used for both the rotation and the translation function searches. A sphere radius of 20Å was used for the cross rotation search (ROTING). Both $P4_1$ and $P4_3$ gave clear top solution with a peak height of 19.0 and an R-factor of 61%. However, space group ambiguity between the two enantiomers was resolved during the translation search (TRAING). The best solution was found in the $P4_3$ space group with a peak height of 26.3 and an R-factor of 58.1%. The peak height of the corresponding solution in the $P4_1$ space group decreased to 17.5 and had an R-factor of 60.0%. The optimised solution had a correlation coefficient of 56% and an R-factor of 51% after rigid body refinement (FITING) against data in the above range (*Table 3.2*). The initial coordinates for crystallographic refinement were obtained by applying the appropriate rotation and translation parameters to the search model (MR2IC and PDBSET). The use of a well-refined search model and the high degree of

sequence homology between PlGF-1 and VEGF-A helped in finding a clear solution in AMoRe without having to vary any of the search parameters like the resolution range, sphere radius or the model cell dimensions.

*Table 3.2 Molecular Replacement results for PlGF-1*

| *Rotation* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | Peak ht | CC_I |
|---|---|---|---|---|---|---|---|---|---|
| SOLUTIONRC | 45.1 | 90.27 | 232.92 | 0.00 | 0.00 | 0.00 | 61 | 19 | 12.9 |
| SOLUTIONRC | 44.9 | 89.8 | 52.91 | 0.00 | 0.00 | 0.00 | 61 | 19 | 12.8 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| SOLUTIONRC | 49.2 | 97.2 | 347.45 | 0.00 | 0.00 | 0.00 | 62.1 | 14.8 | 7.3 |
| *Translation* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | Peak | CC_I |
| SOLUTIONTF1 | 45.1 | 90.27 | 232.92 | 0.233 | 0.733 | 0.00 | 58.1 | 26.3 | 37.3 |
| SOLUTIONTF2 | 44.9 | 89.80 | 52.91 | 0.233 | 0.733 | 0.00 | 58.1 | 26.3 | 37.3 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | .... | .... | ..... |
| SOLUTIONTF19 | 76 | 146.34 | 191.95 | 0.008 | 0.925 | 0.00 | 61.5 | 13.5 | 21.8 |
| *Fiting* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | CC_I | |
| **SOLUTIONF_1** | **44.3** | **92.95** | **231.53** | **0.232** | **0.736** | **0.00** | **51.1** | **53** | |
| SOLUTIONF_2 | 45.7 | 87.13 | 51.53 | 0.236 | 0.732 | 0.00 | 51.2 | 53 | |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | |
| SOLUTIONF_19 | 75.5 | 146.66 | 190.99 | 0.009 | 0.927 | 0.00 | 56.9 | 32.8 | |
| SOLUTIONF_20 | 14.5 | 33.5 | 11.05 | 0.427 | 0.509 | 0.00 | 56.9 | 32.7 | |

*The optimised solution from FITING is highlighted in yellow. The rotational parameters are described by α, β, and γ. The translation search parameters are described by $T_x$, $T_y$ and $T_z$. $R_f$ refers to the R-factor corresponding to each solution and CC_I gives the correlation coefficient based on observed intensities.*

### 3.2.5 CRYSTALLOGRAPHIC REFINEMENT

#### 3.2.5.1 PlGF-1 AT 2.0Å RESOLUTION

All crystallographic refinement and model building was carried out using the programs CNS (Brünger *et al.*, 1998) and O (Jones *et al.*, 1991), respectively. Procedures carried out with CNS included the slowcool protocol of simulated annealing at 2500K using a maximum likelihood target function, restrained individual B-factor refinement, conjugate gradient minimisation, and bulk solvent correction. The behaviour of the $R_{free}$ value [using a test set comprising of 811 (3.7%) reflections] was monitored throughout refinement. Iterative cycles of refinement alternated with model building in 'O' improved the quality of the structure and brought down both the conventional ($R_{cryst}$) as well as the $R_{free}$ values. During the final stages of refinement, water molecules

were inserted into the model based on the 2Fo-Fc and Fo-Fc density using the program ARP-WARP (Lamzin and Wilson, 1993). One bound MPD molecule per monomer from the crystallization medium was identified and was included in the final stages of the refinement. The program PROCHECK (Laskowski *et al.*, 1993) was used to assess the quality of the final structure. Analysis of the Ramachandran plot showed all the residues to be within allowed regions of the plot. The final coordinates of the structure were deposited with the Protein Data Bank (PDB code 1FZV, Iyer *et al.*, 2001).

### *3.2.5.2 PlGF-1 at 1.55Å resolution*

Refinement of the PlGF-1 structure at high resolution was performed using the program SHELXL (Sheldrick and Schneider, 1997). The initial model used was the refined structure at 2.0Å resolution. The refinement was carried against $F^2$-values rather than the F-values using the conjugate gradient least square algorithm during the early stages.

***Table 3.3*** *Refinement statistics for PlGF-1, both at 2.0Å and 1.55Å*

| | **2.0Å** | **1.55Å** |
|---|---|---|
| [a]$R_{cryst}$ (%) | 21.6 | 18.1 |
| [b]$R_{free}$ (%) | 24.7 | 21.4 |
| Number of protein atoms (homodimer) | 1,546 | 1,566 |
| Number of solvent molecules (homodimer) | 132 | 155 |
| Number of MPD molecules (homodimer) | 2 | 2 |
| R.m.s. deviation in bond lengths (Å) | 0.010 | 0.016 |
| R.m.s. deviation in bond angles (°) | 1.5 | 1.5 |
| Average B-factor for protein atoms (Å$^2$) | 32.6 | 36.6 |
| Average B-factor for main-chain atoms (Å$^2$) | 32.6 | 34.8 |
| Average B-factor for side-chain atoms (Å$^2$) | 32.7 | 38.5 |
| Average B-factor for solvent molecules (Å$^2$) | 44.8 | 42.6 |
| Average B-factor for MPD atoms (Å$^2$) | 31.9 | 31.9 |
| B-factor (from Wilson plot) (Å$^2$) | 33.9 | 26.9 |

[a]$R_{cryst} = \Sigma \; ||Fobs| - |Fcalc|| / \Sigma \; |Fobs|$, *where* $F_o$ *and* $F_c$ *are observed and calculated structure factors, respectively.*

[b]$R_{free}$ *is equal to* $R_{cryst}$ *for a random subset of reflections (3.7%) not used in refinement*

In the later stages, restrained anisotropic refinement was carried out for the protein molecule to increase the radius of convergence and for handling disordered regions in the structure. The solvent molecules as well as the 2 MPD molecules (one per monomer) were not subjected to anisotropic refinement. Positions of difference electron density map peaks were modelled as waters and included in the next round of refinement. This was repeated in a iterative process till the $R_{free}$ value reached its minimum. The final round of refinement was then carried against all the data using the *Full-matrix* algorithm.

## 3.3 RESULTS

### 3.3.1 OVERALL STRUCTURE

The crystal structure of PlGF-1 was initially determined at 2.0Å resolution with a crystallographic R-factor of 21.6% and an $R_{free}$ value of 24.7% (Iyer *et al.*, 2001). Later on a higher resolution dataset was collected and the resolution was extended to 1.55Å. The following is a description of the structure at 1.55Å resolution, which refined to an $R_{cryst}$ value of 18.1% and an $R_{free}$ value of 21.4%. Examination of the Ramachandran plot (*Fig 3.4*) shows 93.2% (as compared to 91.5% in the 2.0Å structure) of the residues in allowed regions and no residues in the disallowed regions. PlGF-1 consists of a homodimer, organised in an anti-parallel arrangement with the two-fold axis perpendicular to the plane of the β-sheet (*Fig 3.5*). Two inter-chain disulphide bonds covalently link the homodimer. The most prominent feature of the structure is the presence of a 'cysteine-knot' motif, positioned symmetrically opposite at one end of each monomer. The 'knot' consists of an eight-residue ring formed by one inter-chain and three intra-chain disulphide bonds. The ring structure formed by two disulphide bonds on two, adjacent β-strands, β3 and β7, has the third intra-chain disulphide bond penetrating the covalent linkage and connecting strands β1 and β4. Cys60 and Cys69 (from each monomer) represent the cysteines involved in the formation of the two inter-chain disulphide bridges, which determine the anti-parallel dimerisation of PlGF-1. The cysteine ring contains a conserved glycine residue at position 68, which seems to be important in optimising the conformation of the side-chains

in the 'knot'. The 'cysteine-knot' motif appears to be important for the stabilisation of the dimer, as there are only a few contacts between the β-strands (β1-β1′) at the dimer interface.

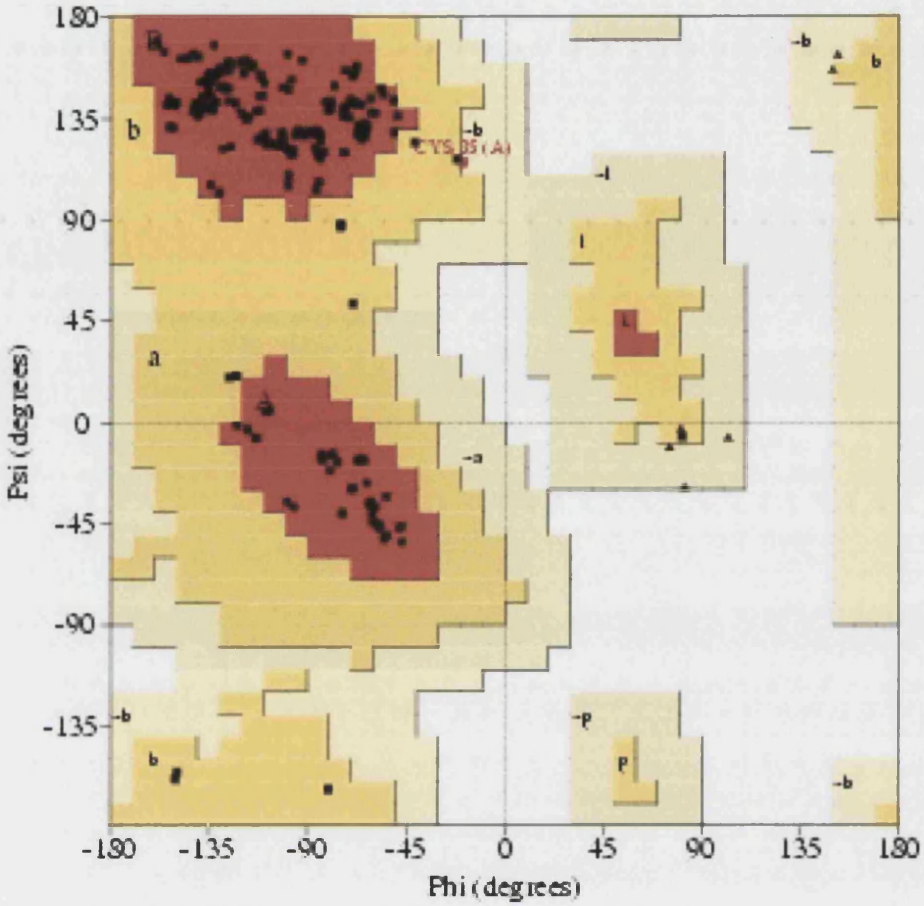



***Fig 3.4*** *Ramachandran plot for the refined structure of PlGF-1 at 1.55Å resolution. The red coloured region of the plot (A,B,L) represents residues in the most favoured regions; yellow region (a,b,l,p) represents residues in additional allowed regions; grey/buff coloured region (~a,~b,~l,~p) represents residues in generously allowed region and white area of the plot represents the sterically disallowed regions. The glycine residues are represented by triangles; A/a/~a represent alpha helices; B/b/~b represent beta strands, L,l,~l represent left-handed helices; p/~p represent epsilon.*

The structural core of the PlGF-1 monomer consists of a four-stranded, highly irregular, solvent accessible β-sheet. The hydrophobic core on the opposite end of the 'cysteine-knot' provides additional stability to the central portion of the structure. The hydrophobic core is formed by residues from both the monomers of the PlGF-1 homodimer.

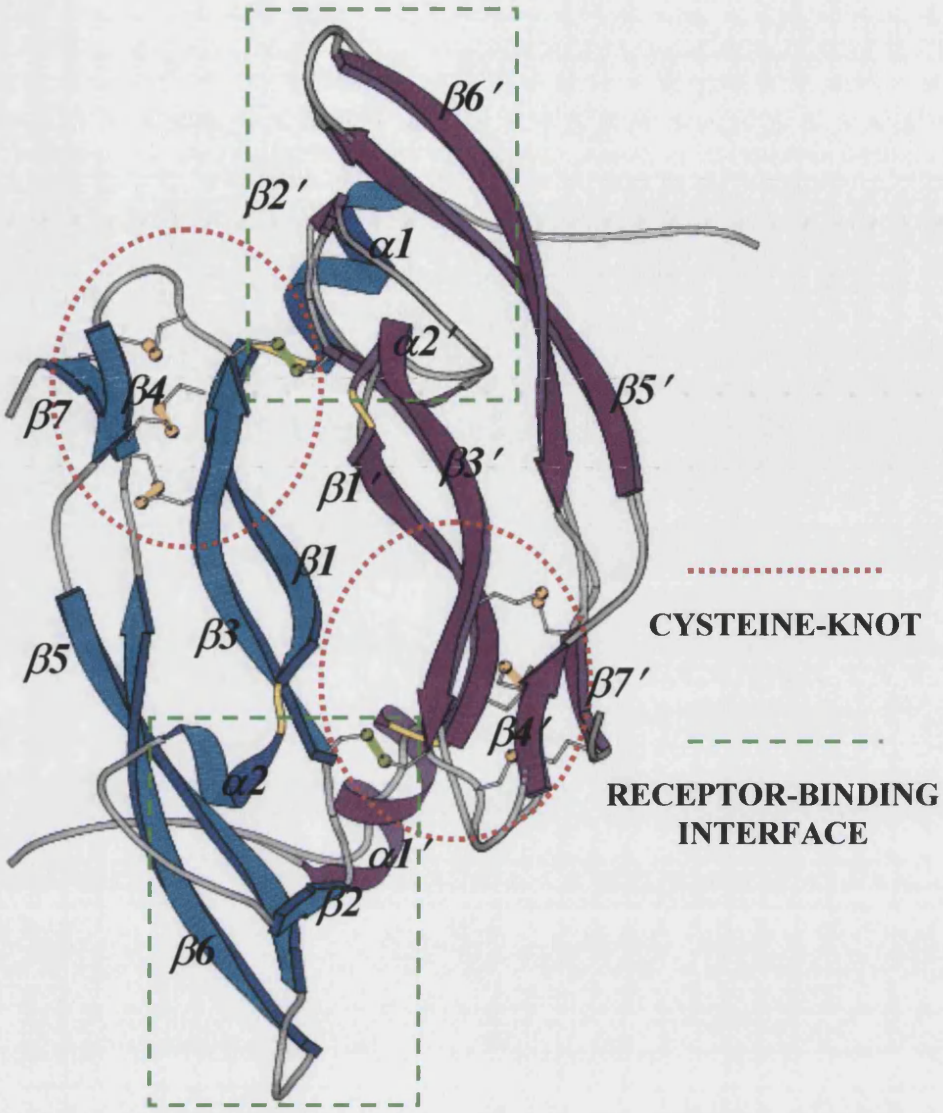



***Fig 3.5*** *The three-dimensional crystal structure of Placenta Growth Factor-1 (PlGF-1; Iyer et al., 2001). The structure has been colour coded to differentiate between the two subunits. Monomer A has been coloured purple and monomer B is in cyan. The cysteine-knot motif (consisting of three intra-chain and one inter-chain disulphide bonds) and the receptor-binding surface have been highlighted at each pole of the homodimer. The secondary structure elements have been labelled as α for helices and β for strands from chain A and as α′ and β′ from chain B. The numbering of the elements follows the chain from N-terminal to the C-terminal end. The figure was drawn using the program MOLSCRIPT (Kraulis, 1991). (Please refer to Page 178 for details on the cysteine-knot).*

Fourteen potential hydrogen bond interactions (*Table 3.4*) are observed between the two monomers. Two water-mediated hydrogen bonds (*Fig 3.6*)

between Glu-39 from each monomer form a bridge between two strands (β1 and β1′) across the centre of the dimer interface.

***Table 3.4*** *Hydrogen bonding interactions between the two subunits of the PlGF-1 dimer*

| SOURCE (subunit A) | TARGET (subunit B) | DISTANCE (Å) |
|---|---|---|
| Val24 N | Thr86 O | 2.8 |
| Val24 O | Gln88 N | 2.9 |
| Phe26 N | Gln88 Oε1 | 2.9 |
| Arg32 Nε | Glu39 Oε1 | 2.7 |
| Arg36 Nη2 | Glu39 Oε1 | 2.8 |
| Glu39 Oε1 | Arg32 Nε | 2.8 |
| Glu39 Oε1 | Arg36 Nη1 | 3.0 |
| Cys60 O | Ser33 Oγ | 2.8 |
| Thr86 O | Val24 N | 2.8 |
| Gln88 N | Val24 O | 2.9 |
| Gln88 Oε1 | Phe26 N | 2.9 |
| Ser33 Oγ | Cys60 O | 2.8 |

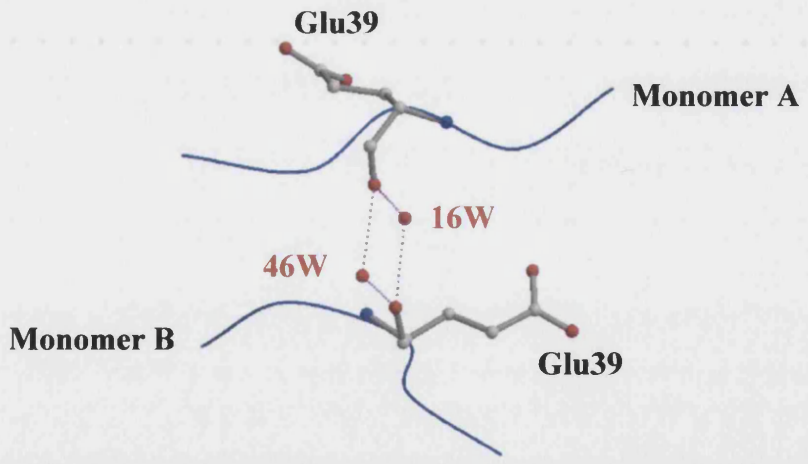



***Fig 3.6*** *The two water-mediated hydrogen bonds between chain A and B, across the centre of the dimer interface.*

The 1.55Å structure consists of a total of 155 water molecules (23 more than that found in the 2.0Å structure) and 2 MPD molecules (one ligand per monomer). Out of these 155 solvent molecules, 65 waters are assigned to

chain A and 58 to chain B. Six water molecules are common to both the monomers and around 50 water molecules are conserved between PlGF-1 and VEGF-A. The total surface area of the homodimer is 11617.4Å$^2$ (calculated using a CNS script). Dimerisation leads to a loss of 2614.05Å$^2$ surface area, buried at the dimer interface (similar values were calculated for the 2.0Å structure). The structure at both these resolutions are very similar and do not show many significant differences apart from the higher resolution structure having more water molecules and the some of the residues being better defined (*Fig 3.7*).

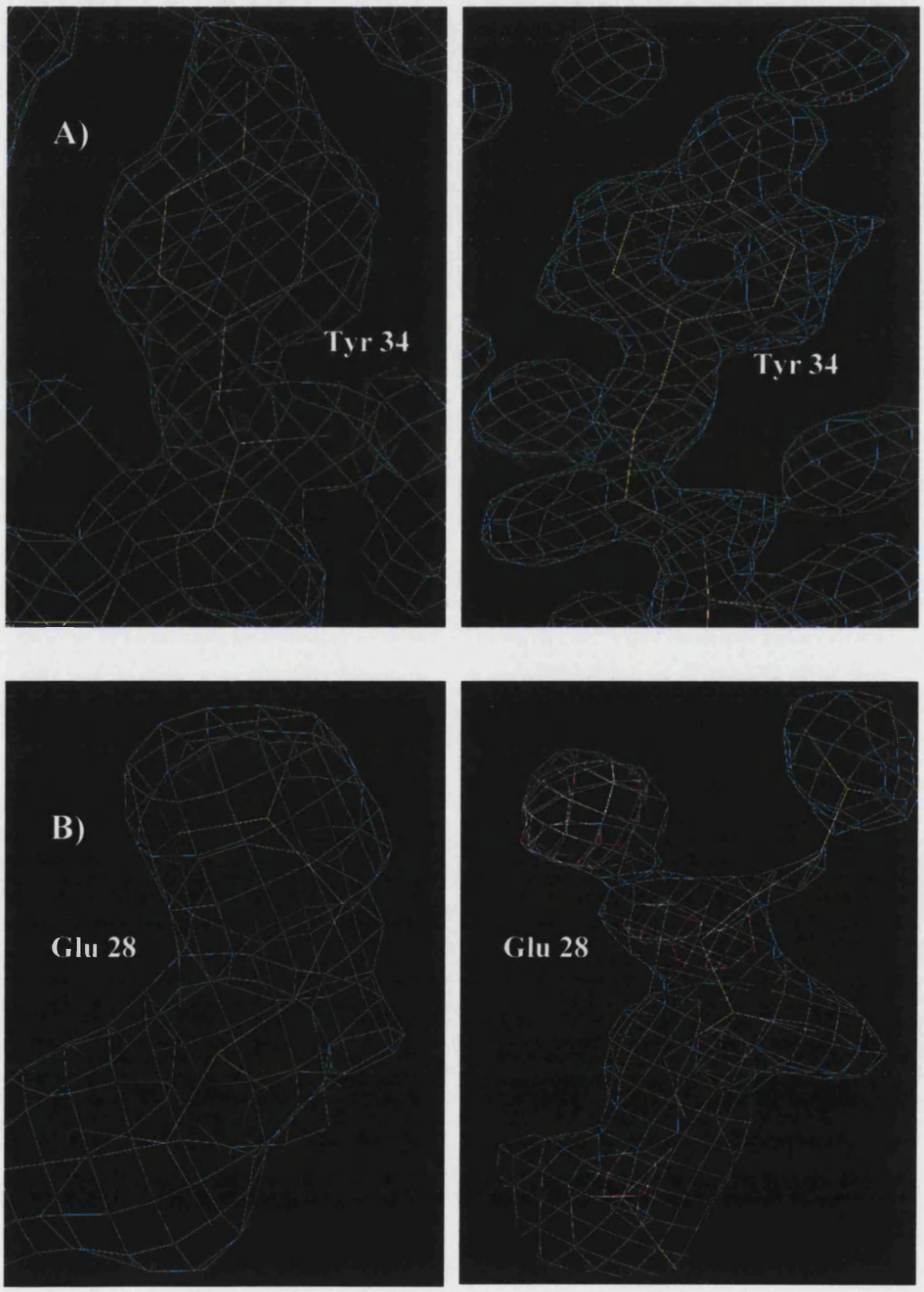



***Fig 3.7*** *Pictures depicting the quality of electron density map at high resolution (1.55Å) as opposed to the same regions in the 2.0Å map. A) a well defined Tyrosine, B) alternate conformation for Glutamate and C) complete side-chain density for Lysine with added interactions at high resolution*

### 3.3.2 PlGF-1: SIMILARITIES AND DIFFERENCES WITH VEGF-$A_{121}$ AND THE OTHER CYSTEINE-KNOT PROTEINS

The cysteine-knot family of growth factors comprises of polypeptides like Platelet-Derived Growth Factor-BB, Vascular Endothelial Growth Factor-A, VEGF-B, VEGF-C, VEGF-D and PlGF. Alignment of the amino acid sequences (*Fig 2.3*) of all these molecules show that the cysteines involved in the formation of the disulphide ring structure have been conserved through evolution, maintaining the structural integrity (*Fig 3.8*) of the members of this family.

R.m.s. deviations were calculated (*Table 3.5*) after superpositioning the $C^{\alpha}$ traces of different members of the cysteine-knot superfamily using the program O. Overall, the structure of PlGF-1 exhibits remarkable topological similarity with that of VEGF-$A_{121}$ (Muller *et al.*, 1997a; 1997b) despite significant functional diversity. The $C^{\alpha}$ traces of PlGF-1 and VEGF-$A_{121}$

homodimer superimpose onto each other with an r.m.s deviation of 1.37Å using 177 $C^{\alpha}$ atoms.

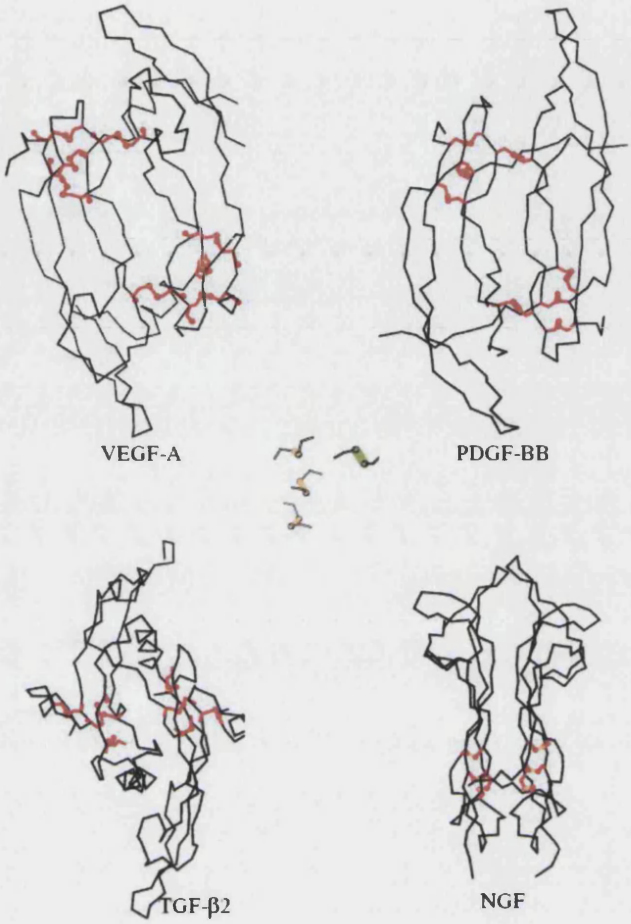



***Fig 3.8*** *$C^{\alpha}$ traces of VEGF-A (2VPF; Müller et al., 1997b), PDGF-BB (1PDG; Oefner et al., 1992) and TGF-β2 (1TFG; Schlunegger and Grutter, 1992) and NGF (1BTG; Holland et al., 1994) highlighting the conserved cysteine-knot motif in all the members, similar to that of PlGF-1 (1FZV; Iyer et al., 2001).*

***Table 3.5*** *Comparison of PlGF-1 with other members of the cysteine-knot family of growth factors with the rmsd values in Å.*

| | PlGF-1 | VEGF-A | PDGF-BB | NGF | TGF-β2 |
|---|---|---|---|---|---|
| **PlGF-1** | - | 1.4(177) | 1.7 (116) | 25.3 (200) | 24.4 (190) |
| **VEGF-A** | | - | 1.8 (113) | 25.2 (192) | 24.4 (190) |
| **PDGF-BB** | | | - | 25.4 (172) | 25.3 (172) |
| **NGF** | | | | - | 19.9 (206) |
| **TGF-β2** | | | | | - |
| *The number in the bracket represents structurally equivalent residues.* | | | | | |

### 3.3.3 RECEPTOR RECOGNITION

VEGF-A recognises both Flt-1 and KDR, which have overlapping binding sites on the polypeptide (Muller *et al.*, 1997a; 1997b). It has also been shown that domains 2 and 3 of Flt-1 are necessary and sufficient for binding VEGF-A with near-native affinity. The crystal structure of VEGF-A in complex with Flt-$1_{D2}$ at 1.7Å resolution has revealed that domain 2 is predominantly involved in hydrophobic interactions with the 'poles' of the VEGF-A dimer (Wiesmann *et al.*, 1997). PlGF-1 is known to bind and bring about autophosphorylation of Flt-1 receptor only. Of the two growth factor molecules, Flt-1 has a higher affinity for PlGF-1. PlGF-1 can displace VEGF from both truncated and full-length Flt-1 receptors (Clauss *et al.*, 1996; Barleon *et al.*, 1996).

#### 3.3.3.1 STRUCTURAL DIFFERENCES BETWEEN PlGF-1 AND VEGF-A AT THE RECEPTOR-BINDING INTERFACE

The receptor-binding interface is made of residues from both the subunits of the homodimer. In PlGF-1, the interface comprises of residues 25-33 (16-24)* from the α1 helix, residues 70-75 (61-66) from the loop between β3-β4 and residues 112-114 (103-105) from β7 strand of one monomer and residues 55-57 (46-48) of strand β2′ and residues 88-98 (79-89) from β′5, loop between β5′-β6′ and β6′ *(the numbers in brackets represent corresponding residues in VEGF-A).

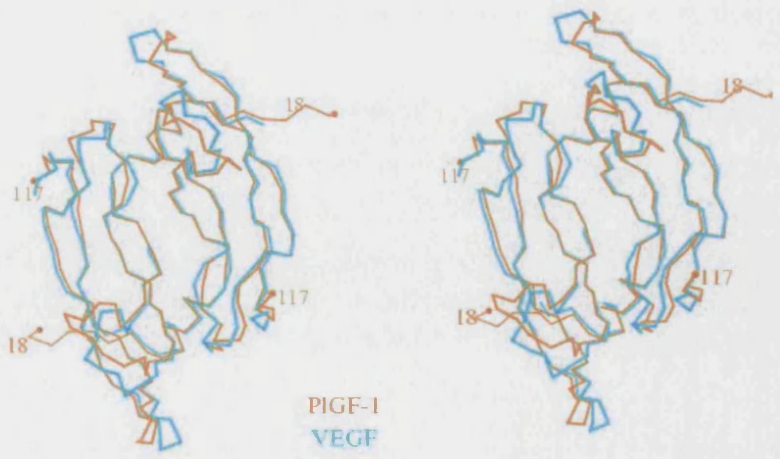



***Fig 3.9*** *Stereo view of the superpositioned $C^{\alpha}$ traces of PlGF-1 and VEGF-A. The PlGF-1 trace is in cyan and that for VEGF-A is in brown.*

Superposition of the $C^{\alpha}$ traces of VEGF-A and PlGF-1 (*Fig 3.9*) reveal conformational differences between PlGF-1 and VEGF-A at the N-terminal region, some loop regions and the C-terminal region. Interestingly, these loop regions form a part of the receptor-binding interface in both molecules.

### 3.3.3.2 LACK OF KDR RECOGNITION

A detailed mutagenesis study of VEGF-A by Muller *et al* (1997a) identified eight amino acid residues forming part of the KDR binding site. These were grouped as major and minor *'hot-spots'* for receptor recognition. Amino acid sequence alignment (*Fig 3.10*) shows that of the six most important KDR binding determinants (the major *hot-spot*) of VEGF-A, only two residues from VEGF-A (Glu-64 and Ile-83) are conserved in PlGF-1 (Glu-73 and Ile-92) and both residues from the minor *hot-spot* are conserved in the two structures.

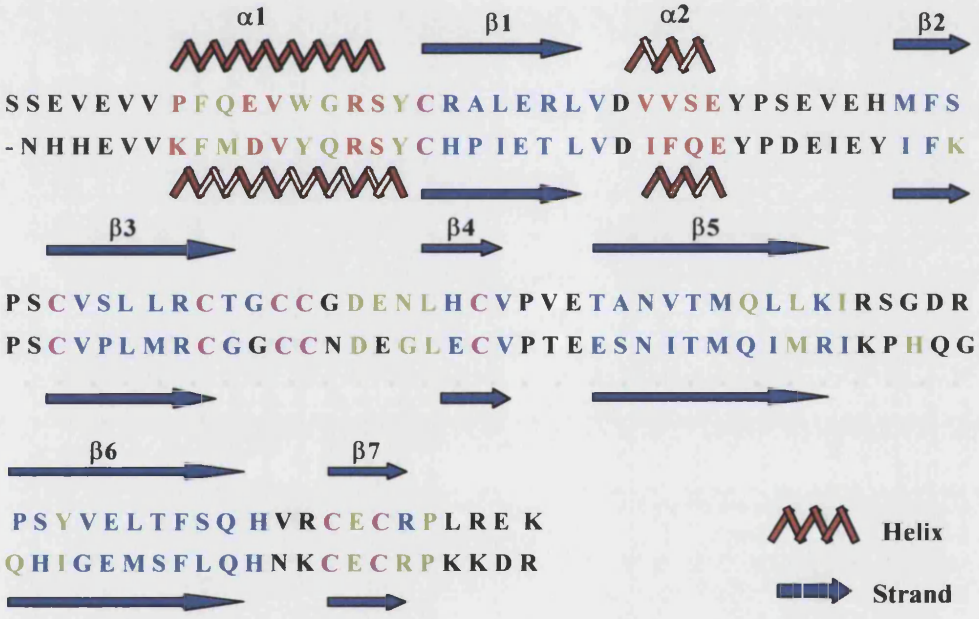



***Fig 3.10*** *The sequence alignment of PlGF-1 (top sequence) and VEGF-A (bottom sequence). PlGF-1 residue numbering starts from 18 and that for VEGF-A starts from 10. Residues coloured red represent helix and blue coloured residues make up the strands. The alignment highlights the residues that are receptor-binding determinants. The residues coloured green recognise Flt-1 and KDR in VEGF-A. The corresponding residues in PlGF-1 (for Flt-1) have also been shaded similarly for comparison. The conserved cysteines are shown in pink.*

In PlGF-1, residues Asp-72, Glu-73, Val-52, Met-55, Val-45, Asp-43 and Ser-59 form the walls of the groove. The corresponding groove in VEGF-A is formed by residues Asp-63, Glu-64, Ile-43, Ile-46, Phe-36, Asp-34 and Ser-50, which are implicated for recognition of domain 3 of Flt-1 (*Fig 3.11*).

This comparison shows that there is a high degree of conservation of residues in this region between the two molecules. However, at the structural level one can visualise significant changes in conformation that could be one of the contributing factor for the distinct receptor specificity for PlGF-1.

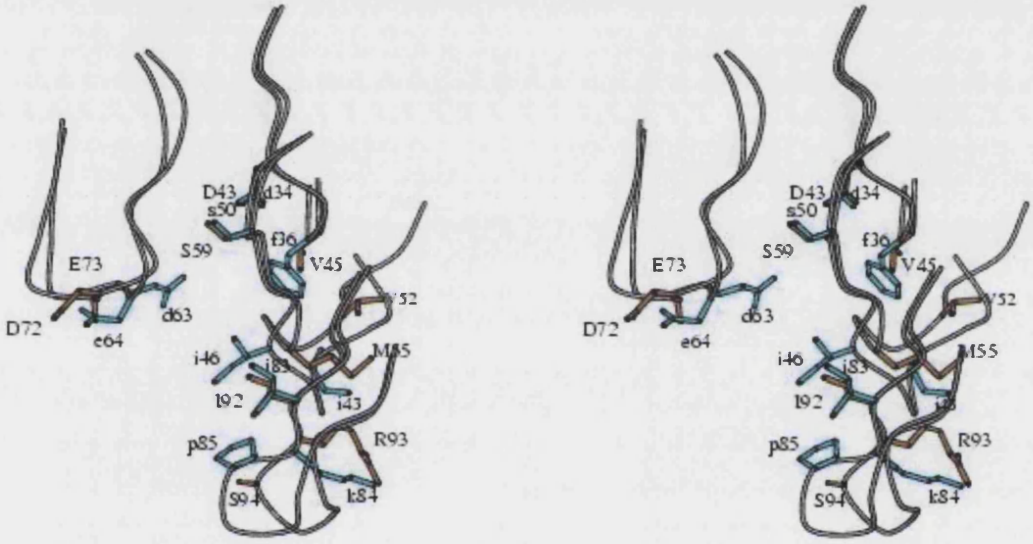



***Fig 3.11*** *Superpositioned $C^{\alpha}$ trace representations of the groove between the two subunits of PlGF-1 (residues coloured in brown) and VEGF-A (residues coloured in cyan). The amino acids of PlGF-1 are labelled with small lettering whereas the corresponding amino acids in VEGF-A are with capital lettering.*

### 3.3.4. PlGF-1•FLT-$1_{D2}$: A MODELLED COMPLEX

The crystal structure of VEGF-A in complex with Flt-$1_{D2}$ was determined at 1.7Å resolution (Wiesmann *et al.*, 1997). If we visualise a similar picture for a hypothetical complex, PlGF-1•Flt-1, with domain-1 of the receptor pointing away from PlGF-1, the domain 2-3 linker region will occupy the groove (6.8Å wide) between the two monomers and domain 3 will make contact with its bottom face. This would bring domain 4 of the receptor

at each pole of the dimer into direct contact with each other, making inter-receptor contacts and hence leading to receptor dimerisation.

Using the structural data from the VEGF•Flt-$1_{D2}$ complex, we constructed a model to visualise the binding mode between PlGF-1 and Flt-1. The PlGF-1•Flt-$1_{D2}$ complex (*Fig 3.12*) was modelled by superimposing the atomic co-ordinates of the VEGF•Flt-$1_{D2}$ complex [PDB code-1FLT; Wiesmann *et al.*, 1997] on to the PlGF-1 model followed by energy minimisation using the program X-PLOR (Brünger, 1992b).

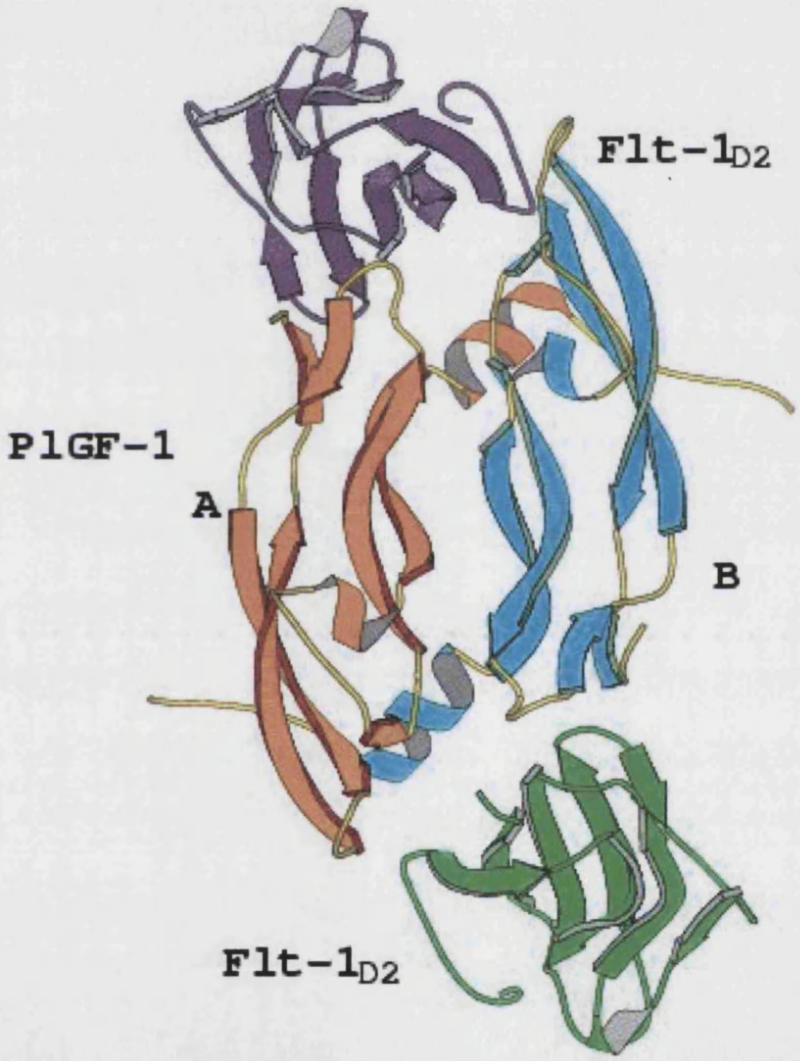
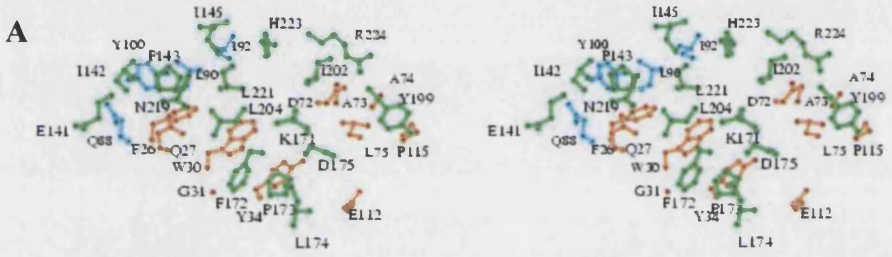


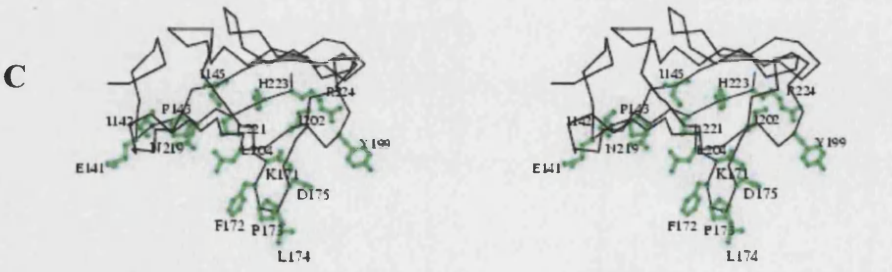

***Fig 3.12*** *The modelled PlGF-1•Flt-1 complex based on the crystal structure of VEGF•Flt-$1_{D2}$ complex [PDB code-1FLT; Wiesmann et al., 1997]. The two monomer of PlGF-1 have been coloured differently with chain A in orange and chain B in cyan. The Flt-$1_{D2}$ domains at the two poles of the homodimer are also in different colours (green and purple).*

In order to quantify the shape complementarity (Sc) of the receptor-ligand interfaces and get an estimate of the "goodness of fit" between the two protein surfaces, the coordinates output after minimisation in XPLOR were put through SC (CCP4, 1994) to compute values of Sc for the interface region between two molecules. Shape complementarity statistic for interface between PlGF-1 and Flt-1 was calculated to be around 0.43 for a probe radius of 1.7Å. When compared with the Sc value (0.63) for the receptor-ligand interface in the crystal structure of VEGF-A•Flt-1 complex, the Sc statistics for the modelled complex is on the lower side. But since this value is not computed from actual experimental data, it appears to be reasonable. Also the buried surface area (2614.05Å$^2$) at the interface (computed using a CNS script) is similar to what is expected from two interacting protein surfaces.




*Residues at the interface of the PlGF-1•Flt-1 complex model.*


*Residues at the receptor-binding interface of PlGF-1.*




*Residues at the ligand-binding interface of Flt-1.*

***Fig 3.13*** *Stereo representations of the residues from the modelled complex*

The residues at the interface of PlGF-1•Flt-$1_{D2}$ complex seem to comprise of about 25 amino acids from the PlGF-1 molecule, residues being contributed by both the monomers and around 17 residues from each of the 2 Flt-$1_{D2}$ molecules, one at each pole of the PlGF-1 homodimer. The binding appears to be mainly mediated through a large number of hydrophobic interactions. The general mechanism of Flt-1 recognition by PlGF-1 seems to be very similar to that reported for VEGF-Flt-1 interaction. The stereo pictures in *Fig 3.13* show the residues from both the components of the receptor-ligand complex (model), which interact at the binding interface.

***Table 3.6*** *Putative intermolecular polar interactions (3.4Å) between PlGF-1 and Flt-$1_{D2}$ at the receptor-ligand interface in PlGF-1•Flt-1 complex.*

| PlGF-1 | Flt-$1_{D2}$ | Distance (Å) |
|---|---|---|
| Molecule A | | |
| Gln27 Oε1 | Asn219 Nδ2 | 2.9 (3.2) |
| Asp72 Oδ1 | Arg224 Nε | 3.2 (3.3) |
| Asp72 Oδ1 | Arg224 Nη2 | 3.2 (3.2) |
| Ala74 N | Arg224 Nη2 | 3.0 (3.1) |
| Molecule B | | |
| Gln27 Oε1 | Glu141 O | 3.2 (3.5) |
| Gln27 Oε1 | Pro143 N | 3.2 (3.5) |
| Tyr34 OH | Lyz171 Nζ | 3.1 (3.3) |
| Glu112 Oε2 | Asp175 Oδ1 | 3.2 (3.0) |
| **Asp72 Oδ1** | **Arg224 Nε** | **3.0** |
| **Tyr100 OH** | **Pro143 O** | **3.2** |

*The values in the brackets are from the modelling studies using the 2.0Å structure. The interactions given in bold font are exclusive to the complex where the 2.0Å structure was used.*

The intermolecular hydrogen-bonding interactions between the Flt-1 receptor and PlGF-1 at 2.0Å or at 1.55Å resolution were almost identical except for the absence of the two contacts highlighted in *Table 3.6*. A similar

case was observed with the van der Waals contacts between the receptor and the ligand at both resolutions. Also, it was observed that the interactions in the

*Table 3.7 Putative intermolecular van der Waals contacts between PlGF-1 and Flt-$1_{D2}$ at the receptor-ligand interface in the modelled*

| PlGF-1 | Flt-$1_{D2}$ | Contacts |
|---|---|---|
| | **Molecule A of PlGF-1** | |
| Phe26 | Pro143, Leu221$^{(2)}$ | 3 |
| Gln27 | Pro143$^{(5)}$, Leu204$^{(3)}$, Asn219$^{(4)}$ | 12 |
| Trp30 | Leu221 | 1 |
| Tyr34 | Lys171, Phe172, Pro173 $^{(2)}$ | 4 |
| Asp72 | Tyr199, Ile202$^{(12)}$, Arg224 | 14 |
| Ala73 | Arg224 | 3 |
| Ala74 | Arg224 | 5 |
| Leu75 | Tyr199$^{(4)}$, Ile202 | 5 |
| Gln88 | Ile142 | 1 |
| Tyr100 | Ile142$^{(3)}$, Pro143 | 4 |
| Glu112 | Leu174 | 1 |
| Pro115 | Tyr199 | 11 |
| | **Molecule B of PlGF-1** | |
| Phe26 | Pro143$^{(4)}$, Leu221$^{(2)}$ | 6 |
| Gln27 | Glu141$^{(3)}$, Ile142$^{(2)}$, Pro143$^{(10)}$, Leu204$^{(2)}$, Asn219$^{(3)}$ | 20 |
| Gly31 | Phe172 | 2 |
| Tyr34 | Phe172 | 3 |
| Asp72 | Ile202$^{(6)}$, Arg224$^{(7)}$ | 13 |
| Asn74 | Arg224 | 6 |
| Leu75 | Tyr199 | 2 |
| Gln88 | Ile142 | 3 |
| Leu90 | Ile145 | 2 |
| Ile92 | His223 | 1 |
| Tyr100 | Ile142$^{(4)}$, Pro143 | 5 |
| Glu112 | Asp175 | 4 |
| Pro115 | Tyr199 | 10 |

VEGF-A•Flt-1 complex were largely similar to those in the modelled complex as far as van der Waals contacts were concerned (*Table 3.7*).

### 3.3.5. MUTAGENESIS STUDIES ON PlGF-1

Based on the native structure of PlGF-1 and the interactions between PlGF-1 and domain 2 of the receptor, Flt-$1_{D2}$, five residues from PlGF-1 seemed to stand out more than the others, those which were involved in either polar or van der Waals interactions with the Flt-1 residues. These residues were Gln27, Asp72, Glu73, Pro98 and Tyr100 (*Table 3.7 and 3.8*). So, in order to assess the contribution of each of these residues towards binding to Flt-1, it was decided to perform mutagenesis studies on PlGF-1. All these 5 residues were mutated to alanine. The mutations involved 4 single mutations: Q27A, D72A, P98A and Y100A and one double mutant: D72AE73A. The double mutant was decided upon because they were the only two residues making direct hydrogen bonds with the Flt-1 residue, Arg224. The variants were then tested for their ability to bind to soluble Flt-1 (sFlt-1). These experiments were performed by our collaborators and the results of this mutagenesis study is represented graphically in *Fig 3.14 and Fig 3.15*.

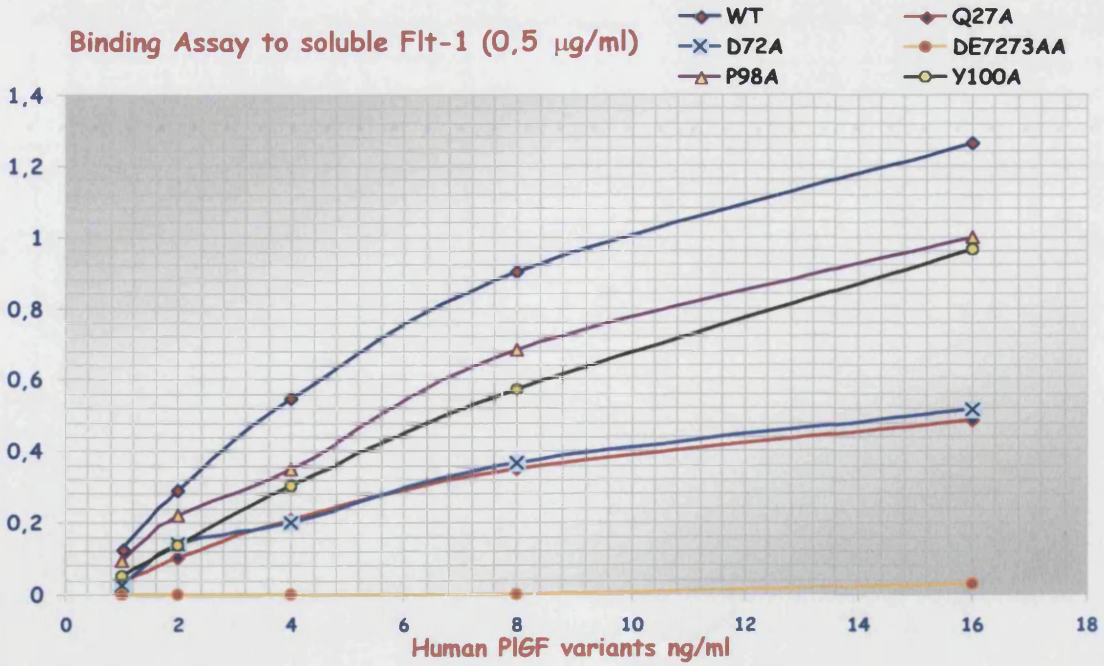



***Fig 3.14*** *Affinities of the wild-type PlGF-1 and the various mutants as determined by using a binding assay that measures binding affinities using varying concentrations of the PlGF-1 variants in the presence of 0.5µg/ml of Flt-1.*

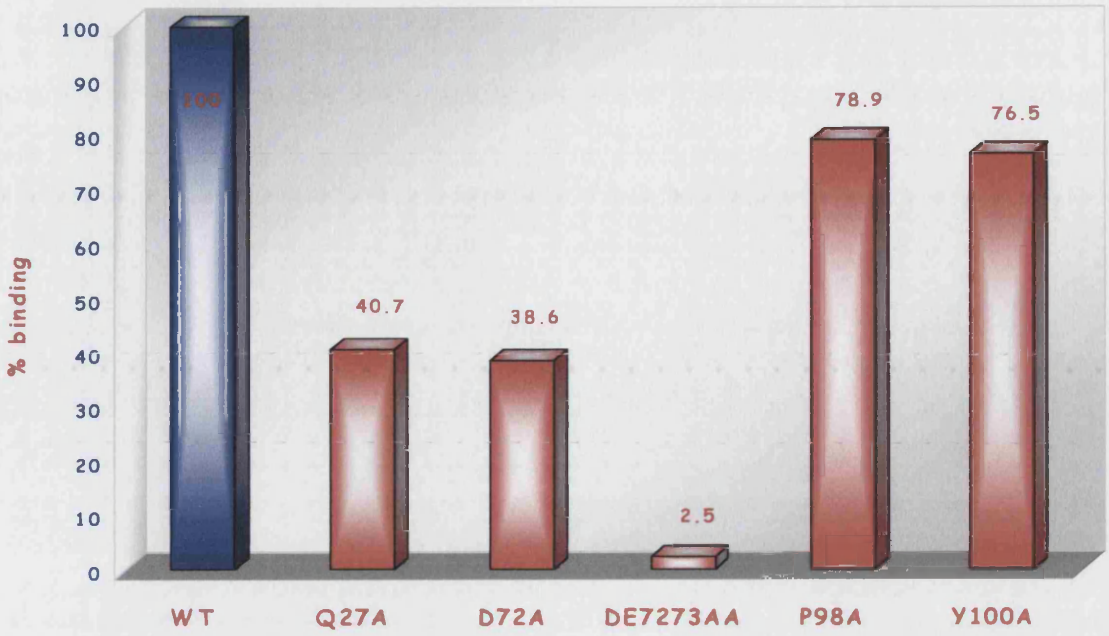



***Fig 3.15*** *Percentage binding of human PlGF-1 variants to soluble sFlt-1. The bar on the extreme left represents 100% binding by the Wild-type (WT) human PlGF-1. The bars for the various mutants have been coloured red.*

It can be seen from the graphs that as expected the double mutant D72AE73A loses its ability to bind to sFlt-1 completely. The corresponding residues in VEGF-A produce similar effects. What is unexpected though is the effect of Q27A mutation. This mutation seems to be affecting PlGF more than it did VEGF-A. However, the mutagenesis studies with VEGF-A were done to determine the binding affinities for KDR and not Flt-1 (Muller *et al.*, 1997a).

## 3.4. DISCUSSION

Placenta Growth Factor-1 is yet another example of a functionally diverse protein arisen through gene duplication. Divergent evolution has given rise to proteins that have very little sequence identity but exhibit remarkable topological similarities. The members of the cysteine-knot superfamily are all dimeric forms although each one is stabilised in different ways. The disulphide bonds bring together the β hairpins, which display a topologically equivalent hydrogen-bonding pattern. The identical alignment of the

equivalent half cysteines in the disulphide cluster is a result of a remarkably similar hydrogen-bonding pattern around the disulphide cluster.

Structure-based drug design of small molecule antagonists or agonists requires a detailed three-dimensional structural and functional characterisation of the target molecule. The structure of PlGF-1 shows that although it has a structural fold very similar to that of VEGF-A, it displays important conformational differences, especially in the flexible loops that form part of the receptor-binding region of PlGF-1. It has been known that the solvent-exposed loops undergo concerted movements, which decide the receptor-specificity for VEGF-A. In PlGF-1, the flexibility of the loop connecting strands β5 and β6 is somewhat restricted compared to the corresponding loops in VEGF-A. This is because of amino acid changes involving two prolines that affect the movement of the loop *vis-à-vis* their physical properties. There are some other changes in the sequence of PlGF-1 at the receptor-binding interface that probably correspond to the inability of PlGF-1 to induce proliferation of endothelial cells despite its ability to bind Flt-1 like VEGF-A. These changes include R93K, G95H and Y100I. These changes might also explain why PlGF-1 has a lower affinity for Flt-1 than VEGF-A and its inability to displace VEGF from Flt-1. A single change from Ile46 in VEGF to Met55 in PlGF-1 significantly affects its binding affinity for KDR. Structurally, it was seen that unlike Ile46 in VEGF-A, the side chain of Met55 is facing away (*Fig 3.11*) from the groove where Flt-1/KDR is proposed to bind the ligand (Muller *et al.*, 1997a; Keyt *et al.*, 1996).

The structure of the PlGF-1•Flt-1 complex is yet to be elucidated. However, based on the structure of the VEGF-A•Flt-1 complex (Wiesmann *et al.*, 1997) it can be postulated that Flt-1 binds at the monomer-monomer interface of PlGF-1 and not exclusively to each of the subunits in the dimer. Domain deletion experiments had revealed that domain 2 and 3 of Flt-1 contain the key determinants required for signalling. These experiments have mapped the ligand-binding residues to the second domain and those important for receptor dimerisation have been characterised as domain 3 residues (Davis-

Smyth *et al.*, 1996). Mutagenesis studies and the crystal structure of the complex reveal the binding determinants on Flt-1 for VEGF-A. Davis-Smyth *et al.* (1996) have identified Glu137, Arg159, Lys171, Glu208, His223 and Arg224 as critical determinants of PlGF-1 binding. Modelling studies showed that Gln27, Tyr34, Asp72, Tyr100 and Glu112 could make both hydrogen bonding and van der Waals contacts with the residues implicated in PlGF-1 binding. However, these modelling data need to be substantiated with the complex structure of PlGF-1 with Flt-1 that will unravel the exact orientation of PlGF-1 when it binds Flt-1. This would be very useful, as the modelling study is based on the assumption that both PlGF-1 and VEGF-A bind to Flt-1 in an analogous fashion.

The native structure of PlGF-1 contains 155 solvent molecules out of which 30 are buried structural waters that bring together the different secondary structure elements within the two monomers. The nature of interaction of protein residues with solvent molecules, especially at the receptor-ligand interface is of great interest as it has direct implications for drug design. The energetics of complex formation involves considerable tradeoffs between loss of protein-water interactions and gain of protein-protein interactions. A careful analysis of the solvent structure in the native PlGF-1 reveals that there is an apparent correlation between the number of water molecules bridging the receptor and the protein in the modelled PlGF-1•Flt-1 complex and the number of water molecules found at the protein surface in the unliganded molecule. Although most of the receptor-ligand interactions are mediated via hydrophobic residues in both Flt-1 and PlGF-1, there are about 11 waters at the receptor-binding interface of PlGF-1 that make hydrogen-bonding interactions with the same residues (Gln27, Trp30, Gly31, Tyr34, Asp72, Ala73, Tyr100 and Pro115) that contact Flt-1 (*Table 3.6*) in the modelled complex. These solvent molecules can perhaps be assigned as displaceable water molecules. These will most probably be sacrificed when PlGF-1 forms a complex with the Flt-1 receptor and the interactions they make in the unbound protein will be compensated by protein-protein interactions.

The unliganded structure of PlGF-1 is a starting point for designing drug molecules that can either activate Flt-1 and therefore potentiate VEGF-A stimulation or inhibit Flt-1 stimulation by binding either to Flt-1 or PlGF-1 itself. The drugs can either be small molecule activators/inhibitors or peptides that target the protein-protein interaction sites. Such peptides have a great pharmacological value and are possible candidates that can be developed further as potential therapeutics.

Only certain types of tumours, namely thyroid tumours and germ cell tumours, produce PlGF, unlike VEGF-A, that is produced by all tumour types. PlGF is expressed in these tumours and in inflamed tissues (Takahashi *et al.*, 1994; Kodama *et al.*, 1997), and this expression is probably a consequence of angiogenic factors being released from the extracellular matrix. Most of the earlier experiments were done with recombinant human PlGF-1, which was angiogenic only under certain conditions. These raised several doubts and questions regarding the role of endogenous PlGF-1 in angiogenesis. The recent results reported by Carmeliet *et al.* (2001) based on gene inactivation experiments in mice, answer some of these queries. Their experiments showed that loss of PlGF correlated with impaired angiogenesis. Although PlGF deficiency hardly affects vascular development, the absence of the protein affected VEGF-dependent endothelial cell survival, migration and proliferation. Their results indicate that even though PlGF on its own is not an effective inducer of angiogenesis, it does potentiate the activity of VEGF-A in physiological conditions. Also, the expression of PlGF in pathological conditions indicates that it might induce the angiogenic switch by increasing the bio-availability of VEGF.

Another important aspect that links PlGF and VEGF is the ability of the two cytokines to form heterodimers. It has been now proven that PlGF-1 binds to VEGF-A and reduces the bio-availability of VEGF-A (Cao *et al.*, 1996) or, at the other extreme, augments the activity of low concentrations of VEGF-A (Park *et al.*, 1994). PlGF/VEGF heterodimers are somewhere between PlGF and VEGF-A homodimers *vis-à-vis* their mitogenic ability. Chick chorioallantoic membrane (CAM) assays show that the heterodimers,

unlike the PlGF homodimers, can bind to KDR through which they exert their stimulatory effects on endothelial cell proliferation (Kurz *et al.*, 1998).

VEGF-A is the angiogenic cytokine that has been experimentally shown to be responsible for initiation of angiogenesis. It is believed that VEGF is a promising candidate in therapeutic angiogenesis because of its ability to induce vasodilation and enhance vascular permeability. Therapeutic angiogenesis, as opposed to anti-angiogenesis therapy, involves stimulation of blood vessel formation in diseases like ischemia, atherosclerosis, thrombosis, restenosis etc. Studies based on VEGF-mediated angiogenesis therapy show mixed results (Isner, 1999), and as a consequence it is not possible to draw any substantial conclusion from them. Also, such a therapy is shrouded by risks of unwanted neo-vascularisation (Zachary *et al.*, 2000; Maglione *et al.*, 2000).

Recently, however, PlGF seems to be making waves not only because of its similarity to VEGF but also because of its ability to augment the activity of VEGF. A fundamental reason that makes PlGF such a promising factor is that on its own PlGF cannot initiate endothelial cell stimulation. Relatively speaking, PlGF-1 has no permeability activity compared to VEGF (Maglione *et al.*, 2000). So, in effect, this growth factor should not have any adverse effects on the blood vessels, both near and far off from the site of administration. PlGF would only enhance the effects of the available low concentrations of endogenous VEGF (Park *et al.*, 1994). Another important factor that goes in favour of PlGF is that it can stimulate the recruitment and activation of monocytes via the Flt-1 receptor (Clauss *et al.*, 1996). Monocyte recruitment is yet another essential step in the process of formation of mature vascular networks, especially collateral growth of blood vessels. It is not known as yet whether or not VEGF alone is sufficient for collateral vascular growth.

It was shown in animal models that recombinant PlGF-1 explicates protection from isoprenaline-induced ischemic lesions in the heart without influencing the heart beat rate (Maglione *et al.*, 2000). Although these findings need to be confirmed with human-based experiments, it is safe to suggest that

PlGF-1 could be considered as a potential therapeutic agent for ischemic myocardial infarction. Also, evidence and reasoning seem to suggest that since PlGF acts synergistically with VEGF (Carmeliet *et al.*, 2001), a combination therapy of VEGF and PlGF would probably be more effective in the treatment of cardiovascular diseases.

## 3.5 FURTHER CRYSTALLOGRAPHIC STUDIES WITH PlGF-1

### 3.5.1 INTRODUCTION

Concomitant with the identification of inducers of blood vessel formation, came the discovery of molecules that inhibit the process. Thrombospondin-1 (TSP-1), endostatin, angiostatin, interferon-γ (IF-γ), prolactin etc are some of the endogenous inhibitors of angiogenesis. VEGF-A is a primary regulator of the process and stimulates mitogenesis of vascular endothelial cells by interactions with KDR (VEGFR-2) and/or is involved in organisation of the vasculature by interactions with Flt-1 (VEGFR-1). Because of association of VEGF-A with various pathological conditions like tumours, arthritis, atherosclerosis, cancer etc, antagonists targeting VEGF-A are clinically very important. A variety of antagonists have been described e.g. small-molecule inhibitors, endogenous inhibitors like angiostatin and endostatin, synthetic inhibitors and peptide inhibitors. Natriuretic peptides were the first peptide inhibitors shown to inhibit endothelial cell proliferation and invasion by inhibition of VEGF synthesis (Pedram *et al.*, 1997). Inhibition of EC proliferation can also be brought about by peptides that are derived from exon 7 of the VEGF-A gene (Soker *et al.*, 1997). This exon represents the structural difference between VEGF-$A_{121}$ and VEGF-$A_{165}$ (Leung *et al.*, 1989; Keck *et al.*, 1989; Houck *et al.*, 1991; Poltorak *et al.* 1997) and facilitates the binding of VEGF-$A_{165}$ to neuropilin-1 (NP-1).

Since KDR plays an important role in VEGF-A mediated proliferation and invasiveness of ECs during vascularisation and tumour-induced angiogenesis, most of the peptide inhibitors identified target VEGF-A-KDR interaction. Of the isolated peptides that bind KDR specifically, the peptide with the sequence **ATWLPPR** completely inhibited VEGF-A mediated

angiogenesis by blocking VEGF-A binding to KDR (Binetruy-Tournaire *et al.*, 2000). Screening of phage display library led to the construction of two different types bi-specific antibodies. One diabody was directed against two different epitopes on KDR (VEGFR-1) and blocked VEGF-A induced activation of the receptor as well as mitogenesis of human ECs. More recently, however, a bi-functional diabody was constructed against both the VEGF-A receptors, Flt-1 (VEGFR-1) and (KDR) VEGFR-2. This antibody completely inhibits all of VEGF-A activities (Lu *et al.*, 2001).

In 1998, Fairbrother *et al.* (1998) used a technique involving a multicopy display of random peptides on f1 filamentous phage particles to identify small disulphide-constrained peptides targeting the receptor-binding site of VEGF-A. These peptides were able to block interaction of VEGF-A with both Flt-1 (VEGFR-1) and KDR (VEGFR-2) by binding to a region that contained the binding determinants for both the tyrosine kinase receptors. Since receptor-dimerisation is an important consequence of receptor-ligand interaction, prevention of this step would inhibit signal transduction and eventually prevent blood-vessel formation.

With the increasing evidence linking PlGF-1 with VEGF-A, especially on account of their ability to form heterodimers (Cao *et al.*, 1996) and the synergistic relationship between the two (Carmeliet *et al.*, 2001), it is becoming evident that PlGF-1 inhibitors are just as important. Now that the crystal structure of the native protein has been solved the next step is structure-based design of inhibitors. The VEGF-A binding peptide (Fairbrother *et al.*, 1998) provided a starting point for this experiment, the aim of which was to elucidate the structure of PlGF-1 in complex with the receptor-blocking peptide.

### 3.5.2 CRYSTALLISATION STRATEGIES

Recombinant human PlGF-1 was provided to us by our collaborators (Drs. D. Maglione and M.G. Persico, CNRs, Naples, Italy). The pure protein was used to grow native crystals for soaking experiments as well as to grow co-crystals of PlGF-1 with the inhibitor peptide. Two different peptides were

synthesised, a 20mer and a hexamer, at Department of Biochemistry, School of Medical Sciences, Bristol. Peptide I (the 20mer) was the same as the receptor-blocking peptide (**v108**) used to determine the structure of the complex of the peptide and VEGF-A (Wiesmann *et al.*, 1998). The sequence of the peptide was '**RGWVEICAADDYGRCLTEAQ**'. Peptide II (the hexamer) was derived from the 20mer but was based on the peptide sequence designed to bind PDGF and prevent the ligand-induced receptor-autophosphorylation. These novel growth factor binding molecules have potent anticancer and anti-angiogenic activity (Blaskovich *et al.*, 2000). The sequence of the hexamer used for crystallisation purposes was '**AADDYG**'.

The concentrations of the peptides used for soaking experiments were 0.5mM, 1.0mM, 2,0mM, 3.0mM and 5mM. For co-crystallisation experiments the concentrations initially used were according to that used for growing crystals of VEGF-A with v108 [10% molar excess of 20mg/ml of VEGF-A w.r.t. 1.5mM peptide (v108); Wiesmann *et al.*, 1998]. Since, most of the initial co-crystallisation trials produced very tiny crystals, the concentration of PlGF-1 was dropped from 27mg/ml to 16mg/ml (the same as that used to grow native PlGF-1 crystals).

First attempts at crystallisation were done with the 20mer peptide (v108). Soaking experiments were carried out with various concentrations (as mentioned above). Since soaking experiments did not work favourably, two possibilities were tried. One was to truncate the 20mer to a hexamer (based on Blaskovich *et al.*, 2000) to facilitate the entry of the peptide into the crystal lattice and to set up co-crystallisation experiments. Initially, the native PlGF-1 conditions (Iyer *et al.*, 2001) were used to crystallise the complex. Later on a variety of crystallisation conditions were tried. The crystals were grown at 16°C by the hanging drop vapour diffusion method. Drops containing 2µl of the PlGF-1•peptide solution and 2µl of the reservoir solution were equilibrated against 700µl of the reservoir. Crystals appeared after 3-4 days when grown using native PlGF-1 condition. But the other crystallisation conditions produced crystals after about 10-15 days. Some of the conditions

that produced crystals have been summarised in the form of a table (*Table 3.8*).

*Table 3.8* *Some of the crystallisation trials for PlGF-1 with the peptide*

| | Crystallisation Condition | Observations |
|---|---|---|
| a | 20mM $CaCl_2$, 1.0M MES (pH 6.0), 15.0% (v/v) MPD | Very small, pyramidal crystals |
| b | 0.2M ammonium acetate, 0.1M Na citrate (pH 5.6), 30% MPD | Big pyramidal crystals |
| c | 30% MPD, 5% PEG 4000, 01M Na HEPES (pH 7.5) | Big pyramidal crystals |
| d | 30% MPD, 10% PEG 4000, 01M Imidazole HCl (pH 8.0) | Big pyramidal crystals |
| e | 47% MPD, 2% tert-butanol | Big pyramidal crystals |
| f | 50% MPD | Big pyramidal crystals |

### 3.5.3 DATA COLLECTION, PROCESSING AND SCALING

X-ray diffraction data sets were collected on different stations at SRS, Daresbury, UK. Data collection from these crystals was performed by transferring the crystal into their respective cryoprotectant solution, mounting them in a loop and then exposing them to the X-rays under a stream of liquid nitrogen at 100K. Data processing statistics are given in *Table 3.9*.

### 3.5.4 STRUCTURE REFINEMENT AND RESULTS

Since all the crystals were characterised to belong to the same space group as the native PlGF-1 crystal, it was not necessary to determine the initial protein phases. The structure of PlGF-1 solved to 2.0Å resolution (Iyer *et al.*, 2001) was used as a starting model for refinement purposes. A single round of refinement including simulated annealing lowered the R-factors considerably. Both $2|F_{obs}|-|F_{calc}|$ and $|F_{obs}|-|F_{calc}|$ maps were calculated for each dataset. Examination of the electron density maps did not reveal any region of the map that was not accounted for.

***Table 3.9*** *Data collection statistics from crystals grown during co-crystallisations*

| Datasets | Resolution (Å) | $N_m$ | $N_u$ | completeness (%) | I/σI | $R_{sym}$(%) |
|---|---|---|---|---|---|---|
| **CRYSTALLISATION TRIALS WITH THE 20MER (v108)** | | | | | | |
| 5.0mM soak | 1.55 | 1,078,548 | 48,576 | 99.4 (98.9) | 10.3 (3.4) | 7.8 (50.1) |
| 3.0mM soak | 2.7 | 107,223 | 9169 | 89.5 (89.8) | 8.6 (2.2`) | 10.4 (41.8) |
| Native condition | 1.7 | 541,655 | 37,720 | 95.6 (79.7) | 19 (3.7) | 5.0 (31.3) |
| **CRYSTALLISATION TRIALS WITH THE HEXAMER** | | | | | | |
| 2.0mM soak | 2.0 | 380,550 | 21,388 | 99.9 (99.9) | 23.5 (4.1) | 8.0 (54.3) |
| 1.0mM soak | 2.59 | 111,530 | 10,116 | 98.9 (92.1) | 11.2 (2.3) | 12.0 (55.6) |
| 0.5mM soak | 2.6 | 144,574 | 10,021 | 99.9 (99.8) | 22.2 (7.7) | 10.4 |
| crystal b | 2.2 | 149,240 | 15,533 | 96.0 (96.8) | 13.2 (3.3) | 7.6 (41.9) |
| crystal c | 2.27 | 161,420 | 15,070 | 99.7 (100) | 177.5 (9.9) | 7.5 (15.4) |
| crystal d | 2.13 | 382,862 | 17,846 | 98.9 (95.5) | 24.2 (10.5) | 7.7 (15.4) |
| crystal e | 2.13 | 177,288 | 18,331 | 99.8 (99.6) | 21.0 (9.6) | 6.7 (13.0) |
| crystal f | 2.13 | 268,211 | 17,372 | 97.3 (92.7) | 19.7 (9.4) | 7.8 (14.5) |

*The crystals b, c d, e and f represent crystals grown from conditions tabulated in Table 3.8. All the crystals grown in these conditions belonged to space group $P4_3$ and their unit cell dimensions were isomorphous with the native PlGF-1 crystals.*

### 3.5.5 DISCUSSION

The use of the inhibitor peptide (v108) in the present study was based on the crystal structure of this peptide in complex with VEGF-A (Wiesmann *et al.*, 1998). This peptide was shown to bind to the receptor-binding surface of VEGF-A. The crystal structure of this showed that the inhibitor peptide contacts VEGF-A residues via the α2-β2 loop region and the β strands β5 and β6 of one monomer and residues from the helix α1 of the other monomer (*Fig 3.10*). The residues identified at the interface form the binding site for both Flt-1 and KDR (Keyt *et al.*, 1996; Muller *et al.*, 1997a; Wiesmann *et al.*, 1997). The significant degree of overlap between the receptor-binding and the peptide-binding sites on VEGF-A and the fact that the general mechanism of Flt-1 recognition by PlGF-1 seems to be very similar to that of VEGF-A justified the use of the same peptide for PlGF-1 as well.

In order to make sure that the interactions between the peptide and PlGF-1 were similar to that between VEGF-A and the inhibitor, we decided to dock the peptide against the native structure of PlGF-1. This was accomplished by superimposing the complex of VEGF-A with the receptor-blocking peptide on to the native PlGF-1 structure (Iyer *et al.*, 2001) followed by energy minimization using the program X-PLOR (Brünger, 1992).

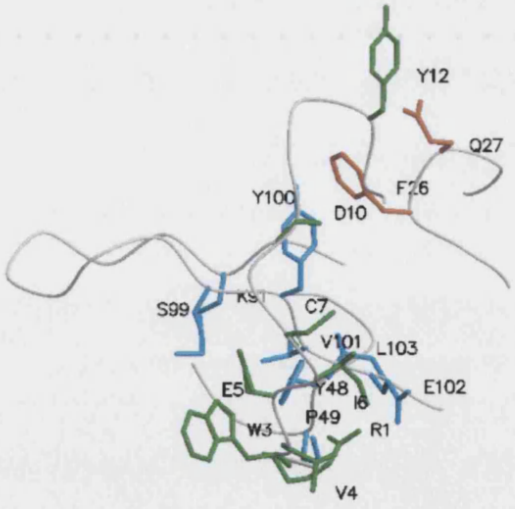



***Fig 3.16*** *Interface residues in the modelled PlGF-1•Peptide complex (based on the crystal structure of VEGF-A in complex with the inhibitor peptide (v108); Wiesmann et al., 1998). The residues from the peptide have been coloured in green and the PlGF-1 residues have been coloured in cyan.*

Interactions between PlGF-1 and the peptide residues at the binding interface (*Fig 3.16*) seem to be similar to that between VEGF-A and the inhibitor. Residues Gln27, Ile92, Tyr100 and Pro115 of PlGF-1 make hydrogen-bonding interactions (*Table 3.10*) with the residues from the peptide. Similar to VEGF-A residues, these residues form a part of the receptor-binding surface in PlGF-1.

***Table 3.10*** *The Hydrogen bonding interactions between the peptide residues and the PlGF-1 residues at the interface in the modelled PlGF-1•Peptide complex.*

| H-Bonding interactions PlGF-1•Peptide | |
|---|---|
| Gln27 | Tyr12 |
| Tyr48 | Arg1 |
| Glu102 | Gly2 |
| Glu102 | Val4 |
| Tyr100 | Glu5 |
| Tyr100 | Gly13 |

Our initial attempts at obtaining the complex through soaking experiments failed although several concentrations of the peptide as well as different soaking times were tried. Inspection of the packing of PlGF-1 in the crystal lattice with its symmetry related molecules (*Fig 3.17*) showed it was a very tight arrangement. There wasn't enough space between the molecules for a 20 amino acid long peptide to be able to enter the crystal lattice through simple soaking. This prompted the truncation of the 20mer to a hexamer (*Fig 3.18*). The six amino acids retained were based on the results of a study undertaken by Blaskovich *et al.* (2000) wherein they designed a molecule GFB-111 that bound PDGF (Platelet-derived Growth Factor) at its receptor-binding site and had a highly potent anticancer and anti-angiogenic activity. Experiments with the hexamer, both soaking and co-crystallisations trials did not come through. Interestingly, the crystallisation conditions (*Table 3.8*) indicated MPD to be a common and perhaps an important factor for crystal

***Fig 3.17*** *Packing arrangement of PlGF-1 and its symmetry related molecules in the crystal lattice.*

GFBP: GDDY

20-mer: RGWVEICAADDYGRCLTEAQ

Hexamer: AADDYG

***Fig 3.18*** *The amino acid sequences of the GFB (growth factor binding peptide), the 20mer (v108) and the hexamer.*

growth as none of the conditions without MPD ever produced any crystals. MPD seems to be very important for crystal contacts as a result of which all the crystallisation conditions, despite varying buffer and pH, produce tetragonal crystals in identical space group and with almost similar cell dimensions. A careful inspection of the region around MPD in the PlGF-1 crystal lattice shows that MPD and the peptide have overlapping binding sites (*Fig 3.19*). Although they do not interact with the same PlGF-1 residues, the presence of MPD molecule appears to hinder the incorporation of the peptide into the crystal lattice during crystal growth. Since conventional attempts at producing co-crystals have been unsuccessful, perhaps mutation of residue Thr104 might help arriving at a condition that does not require MPD for crystal growth. It is evident from these observations that it is imperative to displace MPD from its position in order to facilitate binding of the peptide to

PlGF-1. De-convolution of common structural features (those involved in interactions with PlGF-1) between MPD and the peptide might help in the design of a small molecule inhibitor targeting the receptor-binding site on PlGF-1.

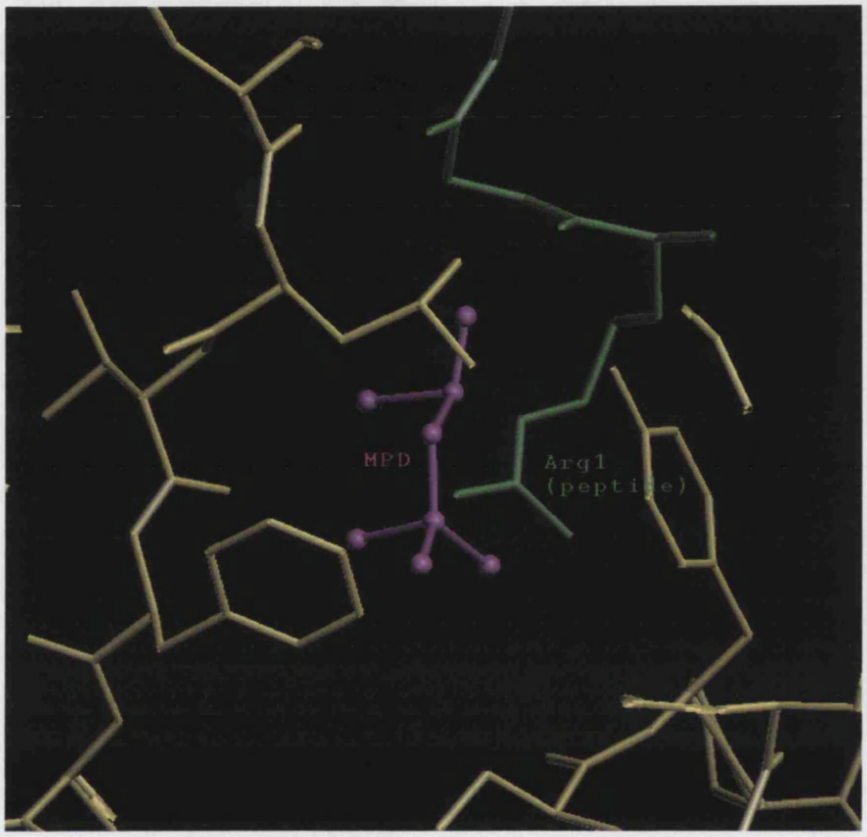



***Fig 3.19*** *Overlay of the native structure of PlGF-1 (yellow) and the modelled PlGF-1•PEP complex. Both the MPD (magenta) in the native structure and the peptide (green) in the modelled structure occupy an overlapping position.*

## 3.6 FUTURE PROSPECTS

Conformational differences at the receptor-binding interface between the structures of PlGF-1 and VEGF-$A_{121}$, despite a high degree of sequence homology, have uncovered crucial structural insights that are valuable in the rational design of novel angiogenesis inhibitors. Further structural studies are required on PlGF-1 that would be largely based on receptor-ligand complexes. The structural basis behind Flt-1 receptor specificity of PlGF-1 and the other isoforms may lead to the development of receptor/ligand specific therapeutics.

This needs to be complemented with the characterisation of the entire sequence of events that occur during PlGF-1 induced signal transduction. The next step would then be to correlate the signalling events with the topological details of the ligand and the receptor at the binding interface. Also, the elucidation of the three-dimensional structure of PlGF-2, the heparin binding isoform, will take our understanding of these proteins a step further. Although PlGF-2 has a highly basic insert of 21 amino acids at the C-terminal end of the molecule, the cysteines involved in the formation of the ring structure are all conserved and hence the structural core of PlGF-2 should be very similar to PlGF-1. These two isoforms do not have any other differences in their sequence apart from the insert. It would be interesting to see how different PlGF-2 is from its isoform, and mapping of the binding determinants for heparin as well as NP-1 should enhance our understanding of molecular details involved in PlGF recognition.

# CHAPTER-4

# STRUCTURAL STUDIES ON PLACENTA GROWTH FACTOR-2 (PLGF-2)

## 4. INTRODUCTION

An alternative splice variant of PlGF was isolated from choriocarcinoma cell lines and characterised as a PlGF isoform (Maglione *et al.*, 1993). The mRNA, expressed in these cell lines, contained an additional stretch of 63 nucleotides as compared to the placental cDNA (Maglione *et al.*, 1991). This mRNA encoded for a 170 amino acid form of PlGF that was identical to the previously characterised PlGF-1 except for an additional stretch of 21 basic amino acids at the C-terminal end (*Fig 4.1*). This protein was referred to as Placenta Growth Factor-2 or PlGF-2 (Maglione *et al.*, 1993).

```
PlGF-1   LPAVPPQQWALSAGNGSSEVEVVPFQEVWGRS
PlGF-2   LPAVPPQQWALSAGNGSSEVEVVPFQEVWGRS
PlGF-1   YCRALERLVDVVSEYPSEVEHMFSPSCVSLLRC
PlGF-2   YCRALERLVDVVSEYPSEVEHMFSPSCVSLLRC
PlGF-1   TGCCGDEDLHCVPVETANVTMQLLKIRSGDRP
PlGF-2   TGCCGDEDLHCVPVETANVTMQLLKIRSGDRP
PlGF-1   SYVELTFSQHVRCECRPLREKMKPER********
PlGF-2   SYVELTFSQHVRCECRPLREKMKPERRRPKGR
PlGF-1   **********************CGDAVPRR
PlGF-2   GKRRRENQRPTDCHLCGDAVPRR
```

***Fig 4.1.*** *Sequence comparison of PlGF-1 and PlGF-2 highlighting the stretch of 21 basic amino acids at the C-terminus end in PlGF-2 that is absent in PlGF-1. Around 50% of this insert comprises of arginines and lysines (Maglione et al., 1993).*

## 4.1. PLACENTA GROWTH FACTOR-2: RECEPTORS AND HEPARIN

Placenta Growth Factor-2 (PlGF-2), like PlGF-1, can bind Flt-1 (VEGFR-1) receptor and not KDR (VEGFR-2). It has been reported that PlGF-2 is more active than PlGF-1 in inducing endothelial cell proliferation, angiogenesis and permeabilisation of blood vessels (Ziche *et al.*, 1997). PlGF-2 is also known to bind Neuropilin-1 (NP-1; Midgal *et al.*, 1998) and Neuropilin-2 (NP-2); (Gluzman-Poltorak *et al.*, 2000). The neuropilins are

class 3 semaphorins and serve as axon guidance factors during embryogenesis (Giger *et al.*, 1998). NP1 is made of a large extracellular domain and a short cytoplasmic domain of 40 amino acids. The extracellular domain is further subdivided into 3 sub-domains: a, b and c. NP-1 plays an important role in embryonic vasculogenesis and maturation of blood vessels (Kawasaki *et al.*, 1999). This was proved by gene knockout experiments in mice (Kitsukawa *et al.*, 1997) that died in about 12.5 embryonal days and showed severe abnormalities in the cardiovascular system and defective angiogenesis in the CNS and the pericardium (Yamada *et al.*, 2001). The biological role of NP-2 in the vascular system is not as well characterised as NP-1. Fuh *et al.* (2000) showed that Flt-1 forms a complex with the extracellular domain of NP-1. This binding (Kd = 1.8nm) is mediated by the tandem repeat, b1b2, in the b-domain of NP-1 (Mamluk *et al.*, 2002).

The 21 basic amino acids encoded by exon 6 of the PlGF gene confers, on PlGF-2, a heparin binding ability that is lacking in other PlGF forms (Hauser and Weich, 1993). Binding of PlGF-2 to NP-1 is dependent on heparan sulphate (Midgal *et al.*, 1998). The binding of PlGF-2 to NP-1 is potentiated by the presence of sulphate moieties on the glucosamine-O-6 and on the iduronic acid-O-2 groups of heparin. Heparin competes with Flt-1 to bind to NP-1 suggesting that both heparin and Flt-1 bind to the same site (b1b2 domain) on NP-1 (Mamluk *et al.*, 2002). The overall picture that emerges from this is that heparin physically interacts with both PlGF-2 and Flt-1 to regulate interactions of PlGF-2 with NP-1. While heparin potentiates binding of PlGF-2 to NP-1 by binding to NP-1 itself, Flt-1 negatively regulates angiogenesis by competitively binding NP-1.

The two PlGF isoforms, PlGF-1 and PlGF-2, do not have any other differences in their sequence apart from the highly basic insert of amino acids. Despite this addition at the C-terminal end of the PlGF-2 sequence, the cysteines involved in the formation of the ring structure are all conserved and hence the structural core of PlGF-2 should be very similar to PlGF-1. The binding determinants on Flt-1 are already known (Davis-Smyth *et al.*, 1998). Given the interesting complexity of the role played by PlGF-2 with ability to

interact with multiple structurally unrelated ligands (Flt-1, NP-1 and heparin), the next aim of the present study was to determine the three-dimensional crystal structure of PlGF-2 (native or in complex with heparin). This would help in identification of binding determinants of heparin on PlGF-2. The characterisation of the specific interactions of heparin with PlGF-2 could be of interest in understanding structure-function relationships, especially since different kinds of angiogenic molecules including growth factors, extracellular matrix proteins, and protease inhibitors have the ability to bind heparin or heparan sulphate proteoglycans.

## 4.2. EXPERIMENTAL PROCEDURES

### 4.2.1. PURIFICATION OF PLACENTA GROWTH FACTOR-2

Recombinant, lyophilised human-Placenta Growth Factor-2 protein (rhPlGF-1) was provided to us by our collaborators (Drs. D. Maglione and M.G. Persico, CNRS, Napoli, Italy). The gene encoding the mature protein was cloned into a prokaryotic expression vector by polymerase chain reaction (PCR). After preparation and induction of the inclusion bodies, the protein was refolded by solubilising the inclusion bodies in a denaturing buffer (8M urea + 50mM Na-Phosphate buffer (pH 7.5) + 20mM ethylenediamine). The solution was diluted using a dilution buffer to get an OD of 0.5 at 280nm. Oxidised and reduced glutathione were added to the solution, which was then incubated for 20hrs at 20°C under agitation. The refolded PlGF-2 protein was purified in a two-step process. Phase-I of the chromatographic purification process involved a column containing S-sepharose Fast Flow resin followed by reverse phase chromatography in Phase II. The material from the reverse phase was ultra-filtered against water using a cut-off of 10,000 Da. The dimeric nature of PlGF-2 used for crystallisation was confirmed by the SDS-PAGE.

### 4.2.2. CRYSTALLISATION TRIALS

Finding the winning combination that supports crystal growth often begins with experiments designed on a coarse grid aiming to seek important

*Table 4.1* *Overview of the crystallisation conditions tried for PlGF-2*

| Experimental Design | Total no of conditions | Results |
|---|---|---|
| MDL Structure Screen 1 | 50 | range from clear drops to cloudy amorphous precipitates |
| MDL Structure Screen 2 | 50 | range from clear drops to cloudy amorphous precipitates |
| FGF condition (Pellegrini *et al.*, 2000) | 1 | amorphous precipitate |
| PlGF-1 condition (Iyer *et al.*, 2001) | 1 | clear drop |
| Sodium malonate @ pH 7.2 (McPherson *et al.*, 2001) | 4 | amorphous precipitate |
| MEMBFAC conditions @ pH 7.5 (Hampton Research) | 13 | range from clear drops to cloudy amorphous precipitates |
| Fibronectin fragment condition | 1 | crystalline precipitate |
| Conditions from the BMCD | 16 | protein denaturation in all the cases except one which produced $CaCl_2$ crystals |
| JBScreen 7 (MPD based) | 24 | range from clear drops to cloudy amorphous precipitates; one condition gave a crystalline precipitate |
| JBScreen 8 (MPD/Alcohol based) | 24 | range from clear drops to cloudy amorphous precipitates; about 5-6 conditions gave light crystalline precipitation. |
| JBScreen 9 (Alcohol and salt based) | 24 | range from clear drops to cloudy amorphous precipitates |
| Cross seeding of the clear drops from all the trials with PlGF-1 crystals | 32 | the PlGF-1 crystals did not dissolve when seeded into the drops |
| Optimisation of conditions based on JBScreen 8 | 18 | cloudy amorphous precipitates |
| Use of glycerol for conditions that had given crystalline precipitation | 2 | these conditions gave clear drops instead of crystalline precipitate. |

experimental variables that can then be navigated in greater detail. Crystallisation trials for PlGF-2 also started with the *sparse-matrix conditions* from Molecular Dimensions Limited (MDL Structure Screens 1 and 2). The following table (*Table 4.1*) gives an overview of all the conditions tried in an attempt to crystallise the protein. The vapour diffusion hanging drop method was the preferred crystallisation technique. A variety of conditions were tried at different temperatures (10°C, 16°C and 22°C), maintaining a drop size of 3µl, containing equal volumes of the protein solution and the reservoir solution, equilibrated against 700µl - 1000µl of the reservoir.

### 4.2.3. CIRCULAR DICHROISM ON PlGF-2

Circular dichroic spectra for PlGF-1 and PlGF-2 were recorded using an in-house Jasco 600 spectropolarimeter with computer control. Spectra were recorded at ambient temperature as an average of 8 to 15 scans. Protein samples (along with appropriate controls) at 580µg/ml concentration in 10mM sodium-potassium phosphate, pH7.0, were measured using a quartz cuvette with a 0.02cm path-length. The start wavelength for the far UV experiment was 210nm and the end wavelength was 188nm. Data points with 0.2nm step resolution were collected at a speed of 10nm/minute. The ellipticities were determined assuming a mean residue weight of 16725.4 for PlGF-1 (131 amino acids) and 19325.3 for PlGF-2 (152 amino acids).

## 4.3. RESULTS AND DISCUSSION

Nucleation and crystal growth are affected by chemical and physical parameters, as well as the crystallisation method used. A wide range of different conditions (~300) based on the various screening kits available from Hampton Research and Molecular Dimensions were used for attempting to crystallise PlGF-2. Almost all of the conditions tried resulted in producing a cloudy amorphous precipitate. Some produced clear drops although a couple of conditions gave some kind of crystalline precipitate. But despite varying different parameters in these conditions, the attempts to grow PlGF-2 crystals were so far not successful. Even cross-seeding all the drops of the previous experiments that were still clear, with PlGF-1 crystals, did not produce the

desired result. Heparin disaccharide was also used in one of the experimental designs in the hope that perhaps association of the macromolecule with one of its binding partners would help to stabilise the system and thus help initiate crystal growth. Despite the inability to arrive at the right combination of crystallisation conditions for PlGF-2, some basic observations can be made based on the results from all the trials so far. The protein seems to like MPD (as a precipitant preferably at a concentration lower than 50%) and Sodium acetate (either as a salt or a buffer). It was also seen that Tris-HCl at any pH caused turbidity of the protein solution in the drop as soon as it was added.

While crystallisation trials were being carried out, Circular Dichroism (CD) experiments were also performed to obtain come information on the secondary structure of PlGF-2. Since it is believed that the core structure of PlGF-1 and PlGF-2 are same, CD spectra for PlGF-1 were also recorded to allow comparisons between the two isoforms. The two spectra, surprisingly, were quite different from one another (*Fig 4.2*).

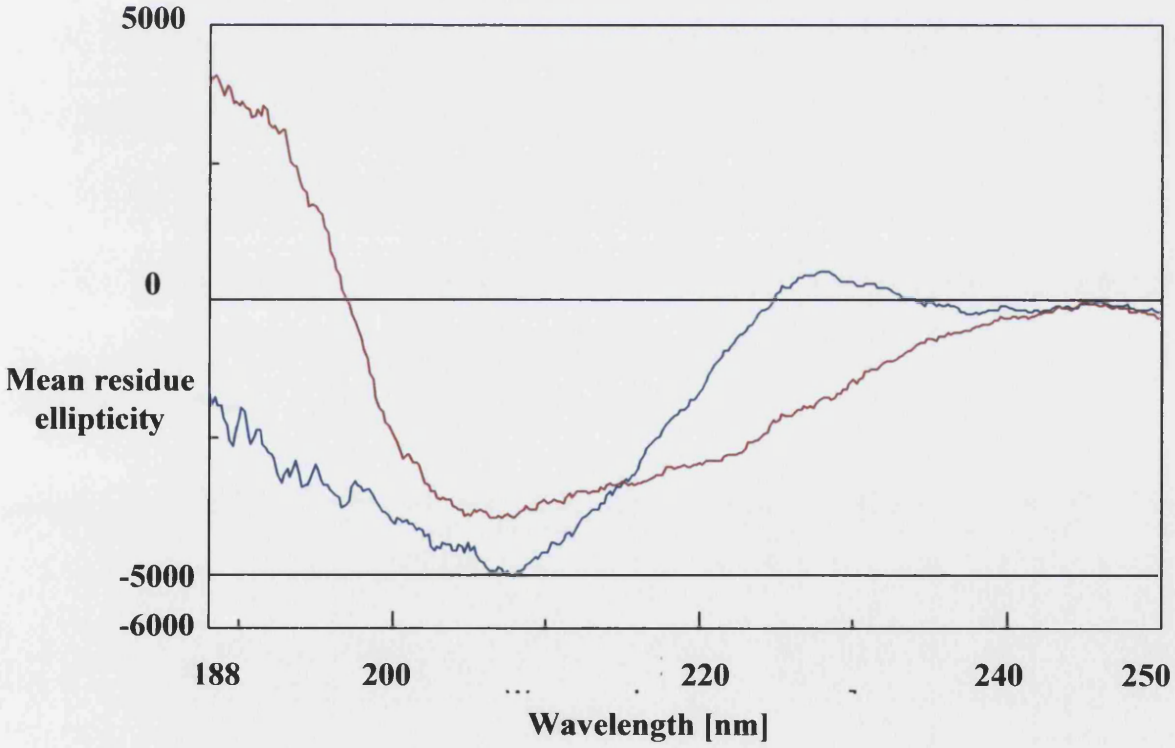



***Fig 4.2 Far UV** CD spectra of PlGF-1 (_____) and PlGF-2 (_____) in 10mM sodium/potassium phosphate buffer (pH 7.0).*

The PlGF-1 spectrum suggests that PlGF-1 primarily contains β-turns and random coil structure. It shows a positive maximum at about 230nm. This feature is quite similar to that seen for brain-derived neurotrophic factor (BDNF; Narhi *et al.*, 1993). The positive peak can be attributed to the presence of the cysteine-knot motif. It could be possible that the signal from the disulphides is so intense that it appears to mask the true structure that arises from the aromatics like tryptophan, tyrosine and phenylalanine. This peak interferes with the spectrum in the region of about 220nm and thus makes it difficult to fit the spectrum with the available model systems. PlGF-2 spectrum, on the other hand, is more in tradition with a usual CD spectrum and therefore easier to analyse (*Fig 4.2*). Analysis of the PlGF-2 CD spectrum using the programs, CONTINL and K2D found on the Dichroweb website (http://www.cryst.bbk.ac.uk/cdweb/html/home.html/), predicted a β-sheet protein, which is in agreement with what is expected. However, what is not explained is the absence of the positive peak in the PlGF-2 spectrum. The presence of a similar disulphide structure would have confirmed the homology between the two proteins as amino acid sequence alignment (*Fig 4.1*) clearly indicates that all the cysteines involved in formation of the disulphide ring structure are located in a similar environment in both PlGF-1 and PlGF-2. The only difference between the two isoforms is the stretch of positively charged residues inserted at the C-terminal end of PlGF-2. The most probable explanation for the absence of the positive peak at about 230nm in PlGF-2 could be that this insert somehow overrides the signal from the disulphide ring and thus allows recording of the true structure in terms of α-helices and β-sheets. Also it could be possible that the basic residues of the insert interact with the surrounding negatively charged residues or the phosphate ions from the buffer used for CD and fold into a helical structure. This could mean that the cysteine-knot conformation is disrupted in PlGF-2 because of the addition of the insert. However, these are only assumptions and each needs to be validated or otherwise by a three-dimensional structure of PlGF-2.

## 4.4. FUTURE WORK

Over the past few decades, much of the work in the field of angiogenesis has been devoted to VEGF-A, as this molecule seems to be of utmost importance in both physiological and pathological conditions. A deeper understanding of the biology of VEGF-A will come from studies that involve additional members of the VEGF family. These include PlGF, VEGF-B, VEGF-C and VEGF-D. Unlike previous findings, recent experiments by Carmeliet *et al.* (2001) have brought into focus the significance of PlGF isoforms in their roles as angiogenic molecules. PlGF-2, the longer isoform, is believed to more angiogenic than PlGF-1. Hence, elucidation of the three-dimensional structure of PlGF-2 would be a major advance in this field, especially since the structure of the heparin binding form of VEGF-A (VEGF-$A_{165}$) is still unknown.

Once the three-dimensional structure of PlGF-2 is known it can be used to identify regions of the protein responsible for its function by comparing it with proteins that are structurally different and yet functionally similar (e.g. Fibroblast Growth Factor). It will serve as a starting point for a rational program of experimentation such as site-directed mutagenesis, receptor and ligand-binding studies and so forth.

PlGF-2 can bind Flt-1 (Park *et al.*, 1994), heparan sulphate proteoglycans (HSPGs; Hauser and Weich, 1993; Mamluk *et al.*, 2002) and neuropilins 1 (Midgal *et al.*, 1998) and 2 (NP-1 and NP-2). Each of these ligands plays an important individual role in the development of the vascular system. However, it is also possible that, since PlGF-2 can bind receptors that associate with VEGF-A, functional redundancy could compensate for the loss of VEGF-A. All these views can be substantiated by structure determination of PlGF-2•Flt-1 complex, PlGF-2•heparin complex and PlGF-2•NP-1 complex. The PlGF-2•Flt-1 complex and PlGF-2•NP-1 complex will also help improve our understanding of the common mechanisms of angiogenesis and neurogenesis. Since heparin is very heterogeneous in nature, the PlGF-2•heparin complex will contribute to our limited knowledge of the structural

requirements for heparin binding to proteins. The structural and functional characterisation of the interaction between PlGF-2 and its binding partners might lead to additional therapeutic opportunities as well as to the refinement of the current strategies that are aimed at growing or blocking vessel formation.

# CHAPTER-5

# STRUCTURAL STUDIES ON VASCULAR ENDOTHELIAL GROWTH FACTOR-B (VEGF-B)

## 5. INTRODUCTION

Cancer research, over the past few decades, has focussed on tumour growth accompanied by increased vascularity. Over the past few years the quest for understanding the biology of VEGF-A has been in the forefront because VEGF-A is central to angiogenesis research. The discovery of the two tyrosine-kinase receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2), demonstrated the crucial role played by both these receptors as binding partners of VEGF-A in the development of the vascular system. A unique feature of Flt-1 is that although it has much weaker tyrosine-kinase activity than KDR, it has 10-fold higher affinity for VEGF-A (Ferrara and Davis-Smyth, 1997; Shibuya, 1999; Mustonen and Alitalo, 1995; Kendall and Thomas, 1993 and Sawano *et al.*, 1996). Because of its stronger ability to trap VEGF-A, Flt-1 negatively regulates physiological angiogenesis in the embryo (Fong *et al.*, 1995 and Hiratsuka *et al.*, 1998). However, when an Flt-1 specific ligand is over-expressed in certain pathological conditions, this receptor acts as a positive inducer of blood vessel formation. One such Flt-1 specific ligand is a VEGF-related protein, Vascular Endothelial Growth Factor-B (VEGF-B).

VEGF-B is a functional homologue of PlGF based on its binding specificity to Flt-1 (VEGFR-1). Like PlGF, VEGF-B also cannot bind KDR (VEGFR-2). However, amino acid sequence alignment shows that VEGF-B is more similar to VEGF-A than PlGF (*Fig 5.1*). These differences and similarities between VEGF-B and the other two members of the cysteine-knot family makes VEGF-B an attractive target for a structure-based initiative to further angiogenesis research.

This is a collaborative project with Sergio D. B. Scrofani from AMRAD Operations, Australia. The protein used for crystallisation was purified by our collaborators. The protocol used for expression and purification of refolded recombinant VEGF-B from *Escherichia coli* inclusion bodies is described in (Scrofani *et al.*, 2000). Both truncated (VEGF-$B_{10-108}$) and the full-length (VEGF-$B_{167}$) forms were used for crystallisation purposes.

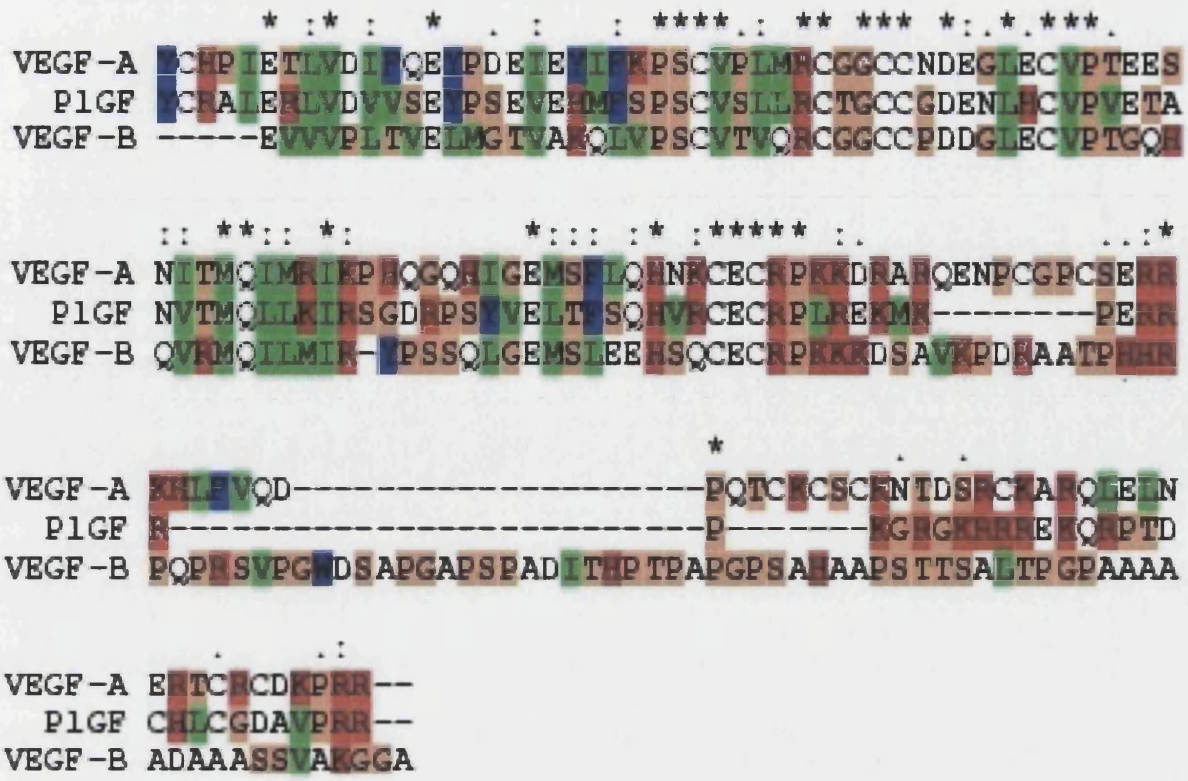



***Fig 5.1*** *Sequence alignment of VEGF-A (P15692), PlGF (P49763) and VEGF-B (49765). The residues indicated by a * represent identical residues in all the three sequences.*

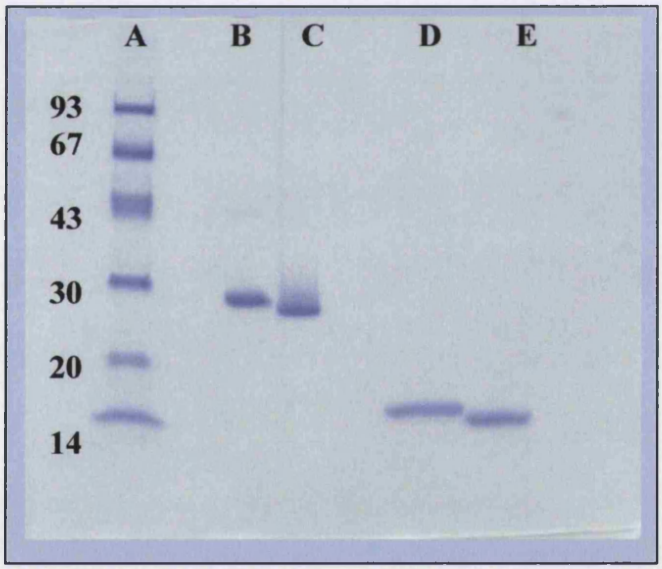



***Fig 5.2*** *Reducing and Non-reducing SDS-PAGE/Coomassie analysis of purified VEGF-$B_{10-108}$ before and after cleavage of the His-tag.*

*Lane A* *Molecular weight markers (from Pharmacia)*

*Lane B* *6-His tagged VEGF-$B_{10-108}$ (Non-reduced)*

*Lane C* *VEGF-$B_{10-108}$ (Non-reduced)*

*Lane D* *6-His tagged VEGF-$B_{10-108}$ (Reduced)*

*Lane E* *VEGF-$B_{10-108}$ (Reduced)*

## 5.1. CRYSTALLISATION OF VEGF-$B_{167}$ AND VEGF-$B_{10-108}$

Most of the times all the efforts and resources in order to crystallise a protein is invested into screening of optimal conditions. Since, VEGF-$B_{167}$ has an amino acid composition very similar to that of VEGF-$A_{167}$ and PlGF-2 and has a similar pI value (8.46), the first set of conditions that were tried were those that were reported for VEGF-A (Christinger *et al.*, 1996). However, none of these conditions were successful. The protein either precipitated out of solution or the drop remained clear. The next step was to try the sparse matrix conditions from Structure Screens 1 and 2 (Molecular Dimensions Ltd). Since protein material was limited, only conditions at pH 7.5 and 8.5 were tried, to begin with, from both the screens. Condition #8 of Structure Screen 2 produced crystalline spherulites whereas condition #11 of Structure Screen 2 produced a shower of fine and very tiny needles. Optimisation of both these conditions were carried out in parallel, however, the latter turned out to be more promising. The original condition comprised of 0.5M $(NH_4)_2SO_4$, 0.1M HEPES buffer (pH 7.5) and 30% MPD. Optimisation of this condition was carried out by varying MPD concentration (10-60%), substituting MPD with PEG400, varying ammonium sulphate concentration (0.2-2.4M) and varying the salts (e.g. $Na_2SO_4$, $Li_2SO_4$, $MgSO_4$, $ZnSO_4$, NaCl, HCOONa at different concentrations). These variations did not improve the crystal size and some of them did not produce any crystals e.g. substitution of MPD with PEG400 was not favourable as it lead to aggregation of the protein solution. Since some of these changes that gave positive results still produced very tiny needles, the protein concentration was dropped from 15mg/ml to 10mg/ml. As the protein seemed to prefer MPD for crystallisation, JBScreen 7 (MPD based) and JBScreen 8 (MPD and alcohol based) were also tried. Most of the conditions resulted in clear drops and only one condition produced fine needles once again. This condition was the same as condition #11 of Structure Screen 2. None of these trials resulted in any improvement in crystal morphology, so different methods of crystallisation such as microdialysis, batch crystallisation, crystallisation under oil were tried. One experimental design was based on a fine pH variation covering the whole range of HEPES buffer (pH 6.6–8.5). The crystal morphology seemed slightly better at pH 6.8 and therefore all further trials were carried out at this pH.

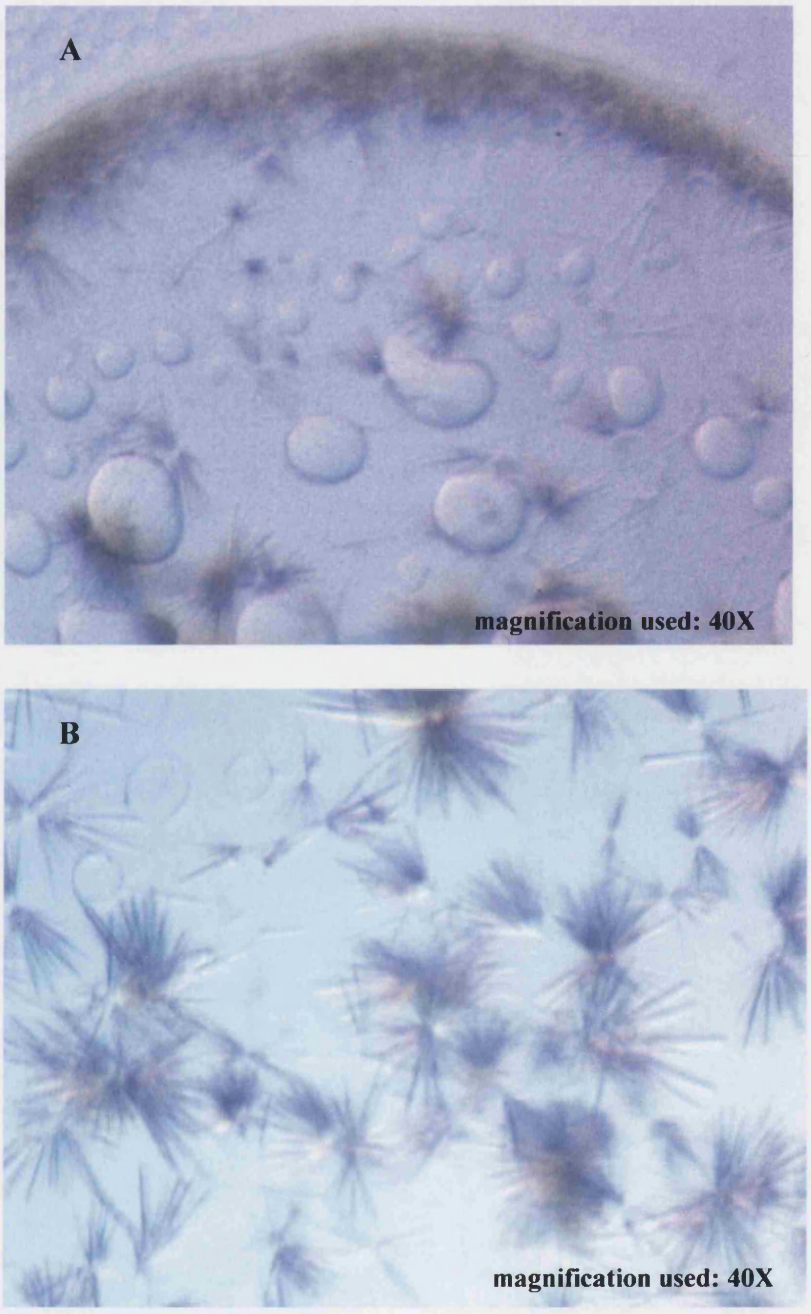



***Fig 5.3*** *Needle-like crystals of VEGF-$B_{10-108}$. Panel A shows the initial crystals that were obtained for VEGF-$B_{10-108}$. Panel B shows the slight improvement in crystal size and in the reduction of the oil-like droplets due to the addition of acetic acid.*

It seemed that the small size of the crystals was probably because the drops form oil-like coacervates (*Fig 5.3A*) in the presence of the organic solvent, MPD. This led to several nucleation sites within these droplets and hence very small and thin needle like crystals were being formed. At this point our

collaborators suggested that we reconstitute the protein in 1.0mM acetic acid. They used acetic acid to reconstitute their samples for NMR studies. It was believed that acetic acid would nullify the coacervate-like effect. Although the problem of droplets was reduced to some extent, the size of the crystals still did not improve (*Fig 5.3B*).

About this time we obtained a truncated form of this protein, VEGF-$B_{10\text{-}108}$. The pH-refined condition was tried for this form. The crystals were better looking than the very thin needles obtained previously for the full-length form. Then, like the full length VEGF-B (VEGF-$B_{167}$), VEGF-$B_{10\text{-}108}$ was also reconstituted in 1.0mM acetic acid to give a stock concentration of 10mg/ml. This, when used for crystallisations, improved the crystal size ever so slightly.

Since we had narrowed down the various parameters in the crystallisation condition, it seemed like a good idea to try crystallisations within a gel. Both hanging drop method and capillary diffusion methods were used. The hanging drop crystals gave positive results, however they were still not big enough to be used for data collection.

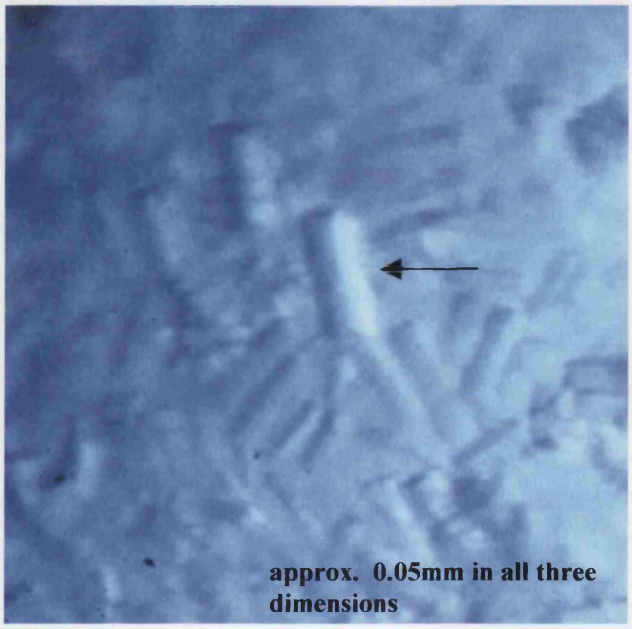



***Fig 5.4*** *Crystals of VEGF-$B_{10\text{-}108}$ (truncated form). The arrow is pointing towards the crystal from which the 3.5Å dataset was collected recently.*

The next option that was explored was that of varying the protein concentration. These trials showed that the minimum protein concentration required was around 2.5 mg/ml in the drop. This concentration was used to set up a streak seeding experiment. Although the nucleation sites introduced in each drop was still very high, one of the drops did produce rod shaped crystals, one of which was big enough (*Fig 5.4*) to be fished out for data collection, albeit with lot of difficulty especially because of skin formation in the drop. A dataset to 3.5Å was collected with this crystal at SRS, Daresbury (UK). At present further optimisation trials with gel crystallisations and seeding experiments are being carried out to improve the crystal quality and size by controlling number of nucleation sites as well as the vapour diffusion rate.

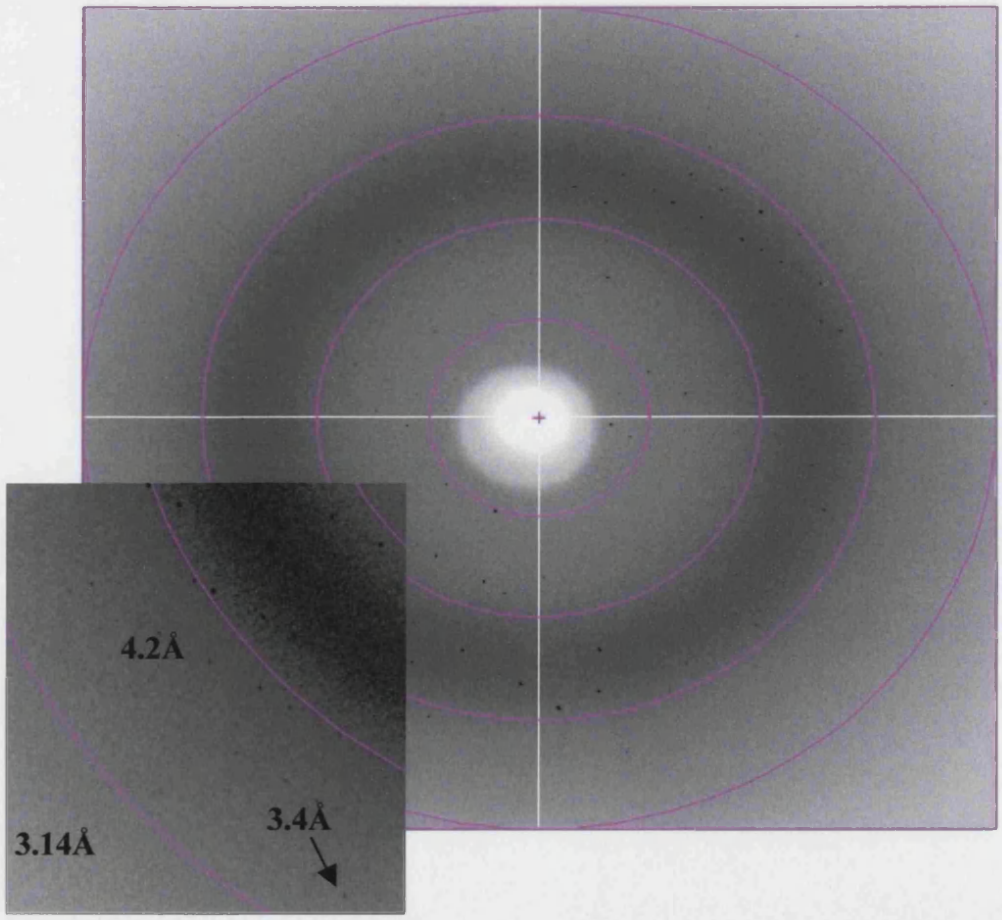



***Fig 5.5*** *A diffraction image from a VEGF-$B_{10-108}$ crystal. The inset represents a portion of the image zoomed in to show the spots. The circles have been drawn to mark the resolution range. The data collection parameters used were: $\lambda$ = 0.98Å; $\Delta\varphi$ = 1.5° and exposure = 30 seconds at SRS, Daresbury, UK.*

## 5.2 DATA COLLECTION, PROCESSING AND SCALING

A low-resolution dataset to 3.4Å was collected at 100K from a single crystal using the Synchrotron Radiation Source (station PX 14.2), Daresbury, UK. Figure 5.5 shows the first image that was collected from the crystal. Autoindexing of the first image using HKL2000 (Otwinowski and Minor, 1997) revealed that VEGF-$B_{10-108}$ had crystallised in the hexagonal space group, P6. A total of 71 images were collected ($\lambda$=0.87Å,) using a CCD-ADSC detector system. Due to poor quality of the crystals data were collected only to 3.4Å, which is reflected I the high $R_{sym}$ value (see *Table 5.1*).

***Table 5.1*** *Data collection and reduction statistics*

| | |
|---|---|
| Unit cell dimensions (Å) | $a$=$b$=121.62, c=40.04 |
| | $\alpha$=$\beta$=90°, $\gamma$=120° |
| Reflections measured | 43551 |
| Unique reflections | 4881 |
| $R_{sym}$ (%) | 17.9 |
| I/σI (outermost shell) | 6.9 (2.8) |
| Completeness (outermost shell) (%) | 98.8 (100) |

*The outermost shell corresponds to the resolution bin covering 3.52 – 3.39 Å*

The solvent content for the crystal was calculated using the CCP4 supported program, matthews_coeff, in order to determine the number of molecules in the asymmetric unit. Calculations showed that for one VEGF-$B_{10-108}$ dimer (molecular weight of 29,000 Da per dimer) in the asymmetric unit, the Matthew's coefficient was 3.0Å$^3$/D with the solvent content of around 57%. This indicated that the crystal belonged to one of the lower space groups ($P6_1$, $P6_2$, $P6_3$, $P6_4$ and $P6_5$). The higher subgroups (P622) were not considered as a possibility because the value for Matthew's coefficient did not lie in the accepted range (between 1.6-3.5Å$^3$/D), due to the higher number of asymmetric units for these space groups. To assign the correct space group, data were scaled in all the possible lower subgroups ($P6_1$, $P6_2$, $P6_3$, $P6_4$ and $P6_5$). Since the data was only up to 3.4Å, the scaling of the data in different

space groups did not produce a list of systematic absences and thus the space group could not be determined in this way.

## 5.3. STRUCTURE SOLUTION

The structure of VEGF-$B_{10-108}$ was determined by molecular replacement using the program AMoRe (Navaza, 1994) in the CCP4i module. The SCALEPACK reflection file was converted into an MTZ format suitable for input to AMoRe (SCALEPACK2MTZ). The first routine in AMoRe (SORTING) sorted the reflection file according to P6 space group symmetry. A tailored model based on the structure of VEGF-A elucidated at 1.93Å resolution [PDB code-2VPF; Muller *et al.*, 1997b] with non-identical residues between VEGF-A and VEGF-B mutated to alanine was used as the search model (TABLING). Data in the range 20.0-4.0Å were used for both the rotation and the translation function searches. A sphere radius of 20Å was used for the cross rotation search (ROTING). In order to resolve the space group ambiguity, translation searches (TRAING) were carried out for all the 5 sub-groups. Both $P6_1$ as well as $P6_4$ gave clear top solution. However, the best

***Table 5.2*** *Molecular Replacement Solutions for VEGF-$B_{10-108}$*

| *Rotation* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | Peak ht | CC_I |
|---|---|---|---|---|---|---|---|---|---|
| SOLUTIONRC | 29.5 | 87.8 | 277.06 | 0.000 | 0.000 | 0.000 | 61.2 | 9.7 | 7.3 |
| SOLUTIONRC | 30.2 | 92.2 | 97.01 | 0.000 | 0.000 | 0.000 | 61.1 | 9.7 | 7.2 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| SOLUTIONRC | 19.9 | 154.4 | 122.85 | 0.000 | 0.000 | 0.000 | 61.4 | 11.0 | 5.8 |
| *Translation* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | Peak | CC_I |
| SOLUTIONTF1 | 29.5 | 87.8 | 277.06 | 0.4762 | 0.2381 | 0.000 | 59.8 | 16.6 | 14.9 |
| SOLUTIONTF2 | 30.2 | 92.2 | 97.01 | 0.1429 | 0.5714 | 0.000 | 59.1 | 15.3 | 14.8 |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | .... | .... | ..... |
| SOLUTIONTF20 | 19.9 | 154.4 | 122.85 | 0.8889 | 0.8254 | 0.000 | 61.1 | 10.4 | 9.9 |
| *Fiting* | α | β | χ | $T_x$ | $T_y$ | $T_z$ | $R_f$ | CC_I | |
| **SOLUTIONF_1** | **27.6** | **87.4** | **279.61** | **0.4728** | **0.2344** | **0.000** | **52.6** | **44.5** | |
| SOLUTIONF_2 | 30.2 | 92.3 | 97.74 | 0.1383 | 0.5689 | 0.000 | 53.6 | 40.4 | |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | |
| SOLUTIONF_20 | 16.3 | 151.63 | 119.71 | 0.8849 | 0.8237 | 0.000 | 55.2 | 39.8 | |

*The optimised solution from FITING is highlighted in yellow. The rotational parameters are described by α, β, and γ. The translation search parameters are described by $T_x$, $T_y$ and $T_z$. $R_f$ refers to the R-factor corresponding to each solution and CC_I gives the correlation coefficient based on observed intensities.*

solution was found in the $P6_4$ space group after optimisation of the solutions in the FITING routine of AMoRe. The correct solution had a correlation coefficient of 44.5% and an R-factor of 52.6% after the FITING (*Table 5.2*). The initial coordinates for crystallographic refinement were obtained by applying the appropriate rotation and translation parameters to the search model (MR2IC and PDBSET). The use of a well refined search model and the high degree of sequence homology between VEGF-B and VEGF-A produced a top solution in AMoRe straightaway, without having to vary any of the search parameters like the sphere radius or the model cell dimensions.

## 5.4 CRYSTALLOGRAPHIC REFINEMENT

In the first round of refinement of the molecular replacement solution, the entire protein molecule was considered as a rigid body to find the best position for the molecule in the new crystal cell. This was followed by the annealing procedure at a constant temperature of 1200K, which brought down the R-factors to 43% ($R_{cryst}$) and 47.6% ($R_{free}$). Since the data was only up to 3.5Å, the number of observations at this resolution was very low. The parameters for refinement could not be defined accurately as the ratio of observations to variables (atomic coordinates, occupancy and B-factors) was not sufficient to justify refinement of even the overall B-factor for all the atoms. For this reason, electron density maps (**$2F_o$-$F_c$ and $F_o$-$F_c$** maps) were calculated after positional refinement of the structure. The maps were examined for very obvious regions of gross error (if any) and difference density for side-chains for the residues that had been modelled as alanines in the MR search model. Some regions of the map were quite reliably defined. However, there were some places where it was difficult to replace alanines with the original VEGF-$B_{10\text{-}108}$ residues and also some regions of the structure, especially the loop regions, were quite disordered. These regions were left as it is and the modified model was put back into the rigid-body refinement protocol of CNS (Brünger *et al.*, 1998). The R-factors dropped to 38% ($R_{cryst}$) and 43.7% ($R_{free}$) with an rmsd in bond length of 0.009Å. The Figure of Merit (FOM) of the refined structure at this stage was around 64% indicating that the refinement was proceeding in the right direction. However, because of the lack of sufficient number of independent reflections, the structure could not be

refined further. As a result a detailed structural analysis of the VEGF-B structure has to be left to a later date when a high-resolution dataset becomes available.

## 5.5 FUTURE DIRECTION

Analysis of the role of VEGF-B in blood vessel formation and function will be facilitated by the three-dimensional structure of VEGF-B. At present, we have only a partially refined structure of VEGF-$B_{10-108}$ at 3.5Å. In order to improve this resolution, attempts at present are directed towards improving crystal quality. Excessive nucleation is producing a large number of small protein crystals that are not suitable for X-ray diffraction analysis. The crystals grown at the moment are consistently tiny and very fragile. The formation of oil-like droplets and skin do not help towards the desired end. In order to obtain large single crystals it is imperative that the nucleation phase does not overlap with the growth phase as suggested by Saridakis and Chayen (2000). Also it might be worthwhile to drop the protein concentration further. At present some drops have been set up at 4°C at three different stock concentrations of protein (5mg/ml, 3.5mg/ml and 2.8 mg/ml). Recently another experimental design has been tried which is based on adding a series of additives (about 48 in all). Attempts to improve the quality of VEGF-$B_{167}$ (full-length form) crystals are also in progress.

Once a refined structure of VEGF-B becomes available, the next step would be to elucidate the structure-function relationship for VEGF-B, especially in heart development *vis-a-vis'* its ability to bind Flt-1 and heparin. Recently, Scotney *et al.* (2002) have characterised VEGF-B specific monoclonal antibodies that are being used in several animal-based assays to assess the role of VEGF-B in cardiovascular and other diseases as well as to identify potential therapeutic applications for these neutralising monoclonal antibodies (mAbs). Crystal structures of VEGF-B in complex with its various binding partners (Flt-1, heparin, neutralising mAbs etc) will help identify important VEGF-B residues that facilitate the angiogenic activity of the protein. Analysis based on mutagenesis studies and comparison of protein-protein interactions in the different complex structures (as mentioned above)

will facilitate the rational design of small molecule antagonists specific for VEGF-B. This will be crucial for underlining the precise role of the Flt-1 receptor in angiogenesis especially since its importance seems to be undermined by all the controversies surrounding it.

# CHAPTER-6

# STRUCTURAL STUDIES ON THE CATALYTIC DOMAIN OF MMP-1 IN COMPLEX WITH THE AMINO TERMINAL DOMAIN OF TIMP-1 (NTIMP-1:MMP-1: CD)

## 6. INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of enzymes that can degrade all the components of the extra-cellular matrix. It is a well-known fact that proteolytic degradation of the proteoglycans and collagens in the extra-cellular matrix (ECM) leads to tissue damage. Matrix can be regenerated if proteoglycan alone is removed. However, when collagen is lost or degraded, the extra-cellular matrix cannot be regenerated (Elliot and Cawston, 2001), as the interstitial triple helical collagens are the major structural proteins of the connective tissue. Members of the MMP family that cleave collagen are known as collagenases. MMP-1 (fibroblast collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase-3) are the only MMPs that can cleave collagens type I, II and III (Welgus *et al.*, 1981; Hasty *et al.*, 1987).

Crystal structures of the catalytic domain of several MMPs have been elucidated. The MMPs exhibit remarkable topological similarities in the overall structural fold of their catalytic domain. The catalytic-domain of the MMPs is spherical and encompasses a five-stranded β sheet and three α helices (*Fig 6.1)* and as the name suggests this domain is responsible for the enzymatic activity of the enzymes. The amino acid sequence of the catalytic domain is highly conserved among the MMP family (*Fig 6.2*).

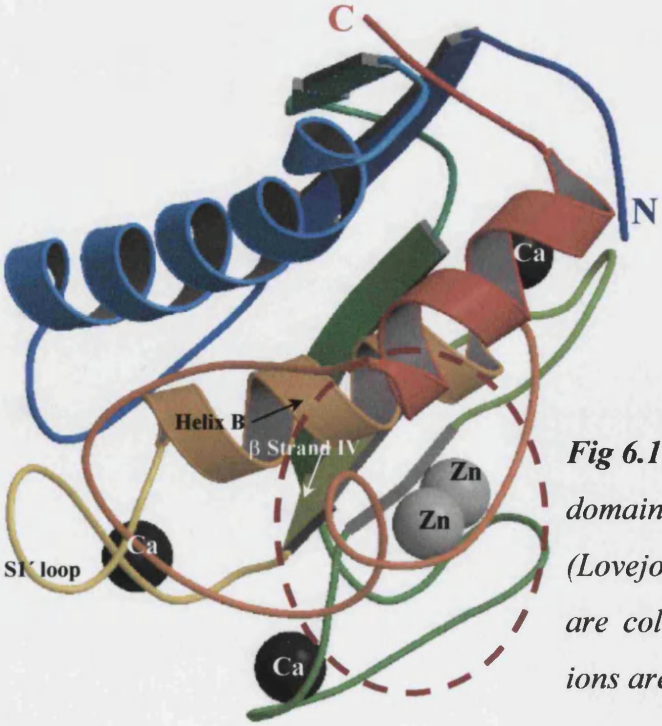



***Fig 6.1*** *Crystal structure of the catalytic domain of MMP-1 (PDB code: 1CGE) (Lovejoy, et al., 1994). The two zinc ions are coloured white and the three calcium ions are in black.*

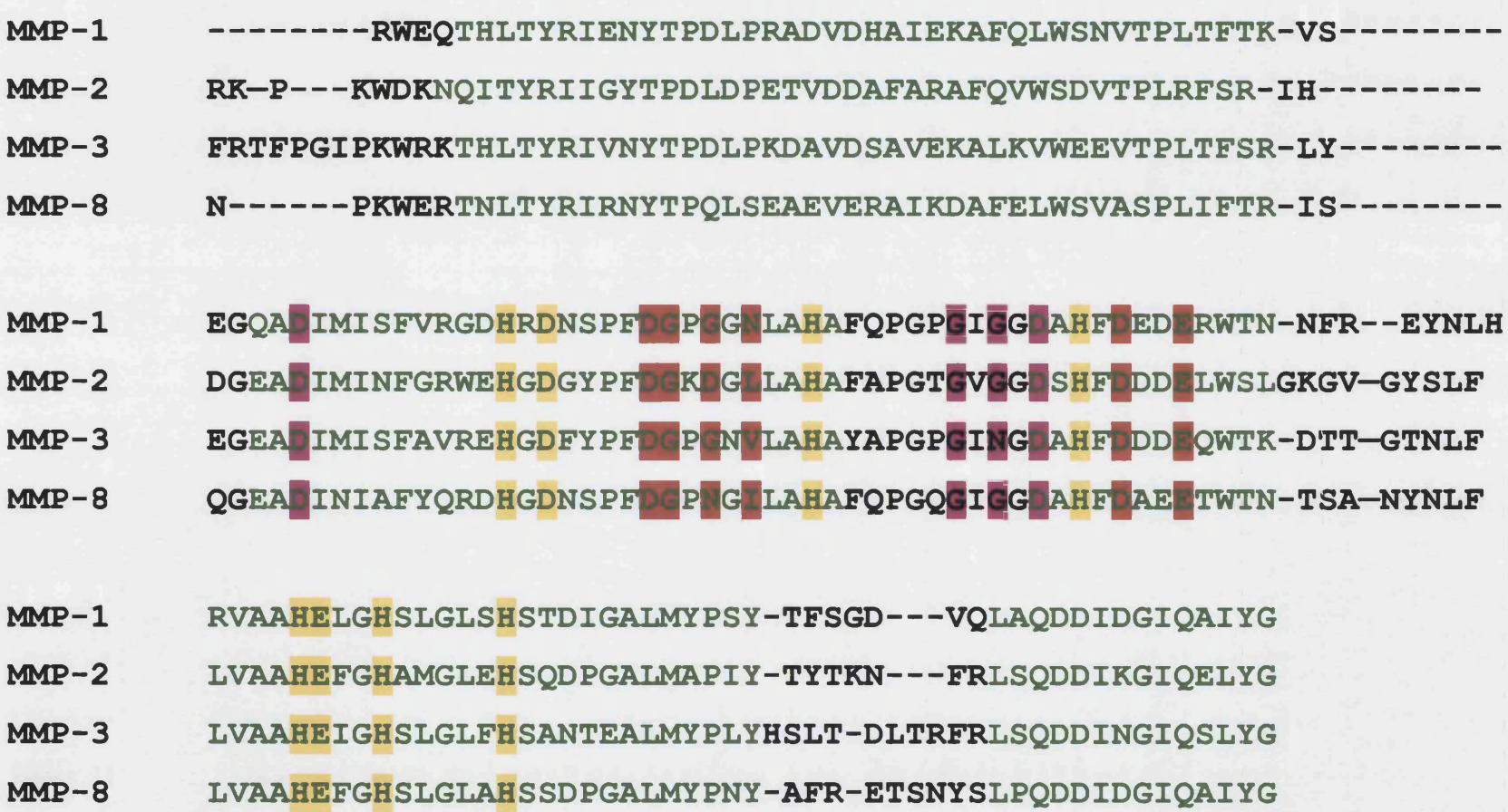



***Fig 6.1*** *Sequence alignment of the catalytic domains of MMP-1, MMP-2, MMP-3 and MMP-8. The regions sharing the same secondary structure are coloured green, the residues ligating with the structural and catalytic zinc have been shaded yellow, the residues shaded red and magenta represent residues that ligate the two calcium ions.*

The catalytic domain of MMP consists of a catalytic-zinc ion and a structural-zinc ion. The residues that coordinate with the two zinc ions are conserved among all the members of the MMP family (*The amino acid sequences in Fig 6.2 represent different types of members of the MMP family: MMP-1 and MMP-8 represent the collagenases, MMP-2 represents the gelatinases and MMP-3 represents the stromelysins*). The catalytic domain also comprises of three calcium ions, which seem to be important for the structural integrity of the enzyme. The catalytic or the N-terminal domain of all MMPs is separated from the C-terminal domain by an active site cleft. This cleft is made of the β-strand number IV, the 2$^{nd}$ α-helix (Helix B) and a random coil next to the second helix (*Fig 6.1*). The catalytic zinc lies at the bottom of this active site cleft. On the right hand side of this catalytic site, lies the selectivity/specificity pocket, S1′ subsite, framed by the S1′ loop. The major difference between the catalytic domains of different MMPs lies in the variable size of this well-defined hydrophobic pocket (Lovejoy, *et al.*, 1999).

The activity of different MMPs is regulated by endogenous inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs). There are four isoforms of the human TIMP known so far: TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The domain organisation is the same for all the TIMPs. It consists of an N-terminal domain and a C-terminal domain (*Fig 6.3*) (Williamson *et al.*, 1990; Pohar *et al.*, 1999). Despite the two separate domains the TIMP structures have a superficial appearance of being continuous because of the tight packing of the two domains. The N-terminal domain of the TIMPs is characterised by the widely distributed OB fold (oligonucleotide-binding fold). The three dimensional crystal structures of TIMP-1 (Gomis-Ruth *et al.*, 1997) and TIMP-2 (Tuuttila *et al.*, 1998) are available at present. The wedge-like structure consists of a six-stranded β-sheet in the N-terminal domain, a central helix separating the two domains and a C-terminal domain comprising of four β strands and a small α-helix. Although the OB fold is relatively rigid in the TIMP structure, the loop connecting the first two β strands in the N-terminal domain and the area of the molecule that attaches to the β-sheet via

the Cys-1-Cys-70 and the Cys-3-Cys99 disulphide bonds are very mobile (Williamson *et al.*, 1997; Fernandez-Catalan *et al.*, 1998).

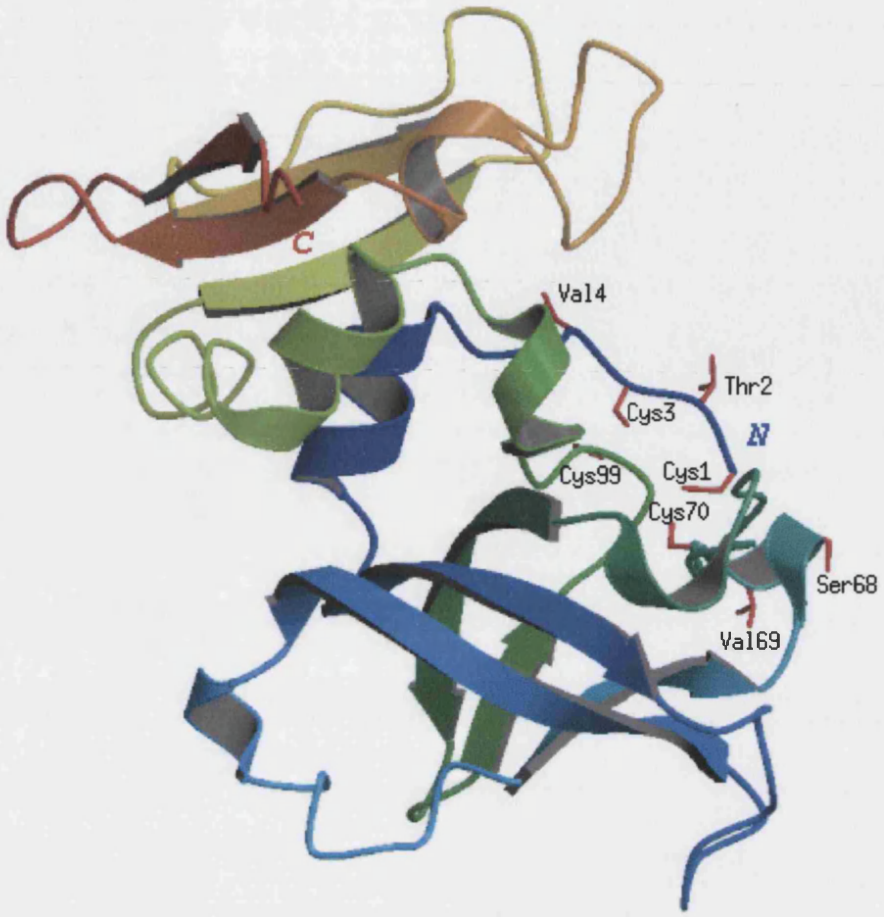



***Fig 6.3*** *Secondary structure of TIMP-1 (full-length form) based on the crystal structure of MMP-3•TIMP-1 complex (PDB code: 1UEA; Gomis-Ruth et al., 1997). The residues important for the activity of the protein have been highlighted.*

All TIMPs inhibit active MMPs except for TIMP-1, which cannot inhibit MT-MMPs (Willenbrock and Murphy, 1994). Mutagenesis studies on TIMP-1 have revealed that Thr2 and some other residues such as Val4, Ser68 and Val69 influence the affinity of different MMPs and hence are important for selectivity of the endogenous inhibitors towards these different proteases (Nagase *et al.*, 1999). These studies have provided a crucial platform for engineering of MMP-selective TIMPs.

Three-dimensional structures of the MMP•TIMP complexes help us address the structural basis of the MMP inhibitory action of TIMP. At present, the crystal structures that are available for the enzyme-inhibitor complexes are the catalytic domain of MMP-3 in complex with the full-length TIMP-1 (PDB code: 1UEA; *Fig 6.4*) Gomis-Ruth *et al.*, 1997), MT1-MMP•TIMP-2 (PDB code: 1BR9) (Tuuttila *et al.*, 1998) and the latent MMP-2 in complex with the full-length TIMP-2 (PDB code: 1GXD) (Morgunova *et al.*, 2002). The crystal structure of MMP-3 in complex with the full-length TIMP-1 revealed that Thr2 of TIMP-1 occupies the S1′ specificity pocket of the MMP-3 in order to mediate its inhibitory effect on the enzyme (Gomis-Ruth *et al.*, 1997). The characteristic feature of this enzyme-inhibitor interaction is the bi-dentate coordination of the amino and the carbonyl group of Cys-1 of TIMP-1 with the catalytic zinc of MMP-3. The carbonyl group of Thr2 interacts with the catalytically important residue, Glu202 of MMP-3 (Glu219 of MMP-1) and displaces a water molecule that is important for the proteolytic activity of the enzyme. The residues in the loop regions of the N-terminal domain of TIMP-1

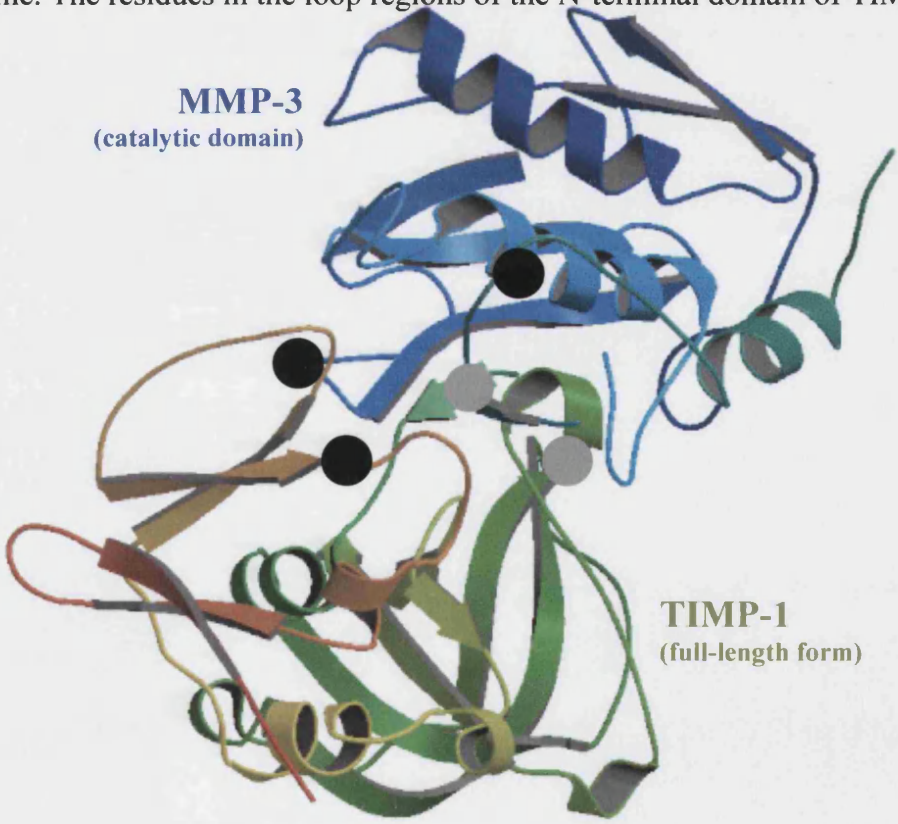



***Fig 6.4*** *Crystal structure of the catalytic domain of MMP-3 in complex with the full-length form of TIMP-1 (PDB code: 1UEA) (Gomis-Ruth et al., 1997). The two zinc ions are shown as grey spheres and the three calcium ions have been represented as black*

molecule, loopsbetween β-strands 1 & 2 and between 5 & 6, also facilitate binding of the inhibitor to the protease. The active site residues are shown in *Fig 6.5*.

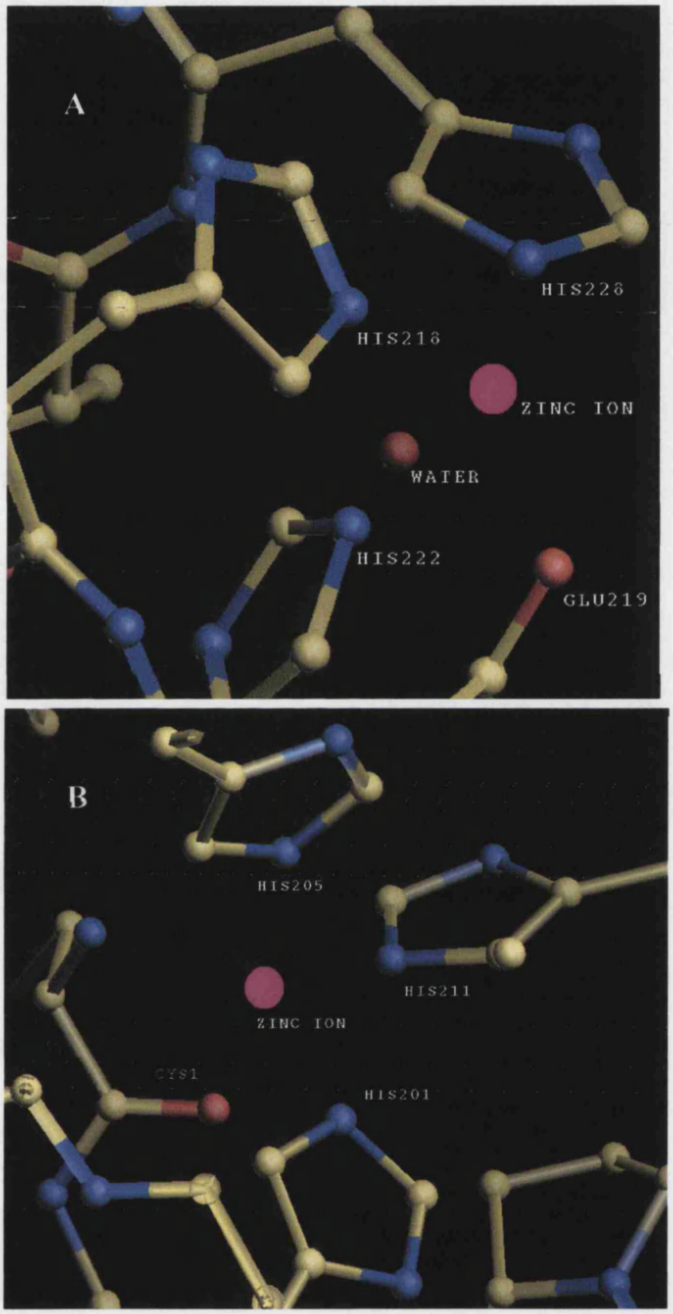



***Fig 6.5*** *The active site residues of the metalloproteinase. A) Native structure of MMP-1. B) MMP-3 in complex with TIMP-1. The interaction with the water molecule in the native structure is replaced by the carbonyl atom of Cys1 from TIMP-1.*

Association of the two molecules results in a structural change in the catalytic domain of the enzyme. Superposition of the structure of the bovine TIMP-2 bound to MT1-MMP with the free human TIMP-2 yielded an rmsd. value of 1.32Å. The N-terminal domain on its own corresponded to a difference of 1.11Å (Fernandez-Catalan *et al.*, 1998). Apart from the mobility of the loop regions in the N-terminal domain of TIMP-2, a large conformational change of nearly 15Å is also effected (Bode *et al.*, 1999). TIMPs and MMPs rapidly associate in the ratio of 1:1 to form a reversible intermediate followed by a slower, rate-limiting step during which the intermediate complex slowly isomerises to form a more stable and tight-binding irreversible complex. The second step is believed to be a consequence of the association of the hemopexin domain of the MMP with the TIMP molecule. Kinetic studies have also shown that the single isolated N-terminal domain of TIMPs still retain the MMP inhibitory activity although the full-length TIMP-1 has at least 8-10% higher free energy of interaction as compared to the association of the MMPs with the N-terminal domain of TIMP on its own (Huang *et al.*, 1997).

The matrix metalloproteinases play a very important role in the physiological turnover of the ECM leading to tissue remodelling during development and wound healing. MMPs have both cancer-promoting and cancer-inhibiting functions. MMPs promote angiogenesis by increasing the bioavailability of angiogenic inducers like VEGF-A (Hashimoto *et al.*, 2002), TGF-α and basic-FGF. On the other hand, they inhibit blood vessel formation by activating the latent TGF-β. But MMPs are known for their role in pathological degradation of the ECM during inflammation. Their unregulated activity leads to several chronic degenerative diseases like the rheumatoid arthritis (RA) and osteroarthritis (OA). These diseases lead to destruction of the cartilage and loss of joint function.

So far, despite the available crystal structures, the mechanism of MMP inhibition by TIMPs is not very clear, especially since there are 25 MMPs and only 4 TIMPs to regulate their activity. Studies have revealed that the serum

levels of both MMP-1 and TIMP-1 are imbalanced in both rheumatoid arthritis (inflammatory) as well as knee osteoarthritis (degenerative). The crystal structure of MMP-1 in complex with TIMP-1 is not yet known. It will facilitate a further insight into the molecular biology of the MMPs and TIMPs. The structure can be used to compare interactions that stabilise complex formation between different MMPs and TIMPs and also provide new direction for the design of drugs that have selective specificity towards individual MMPs. With this aim we embarked into the structural studies of the catalytic domain of MMP-1 in complex with the N-terminal domain of TIMP-1 (nTIMP-1•MMP-1:CD complex).

## 6.1 CRYSTALLISATION OF THE nTIMP-1•MMP-1:CD COMPLEX

The amino-terminal domain of TIMP-1 (nTIMP-1) was purified along with the catalytic domain of MMP-1 (MMP-1: CD) as a complex in the ratio of 1:1 by our collaborator, Professor Keith Brew (Department of Biomedical Sciences, Florida Atlantic University, Florida, USA). The crystallisation trials were initiated by setting up drops with conditions already published for various MMPs, both with and without small molecule inhibitors and TIMP-1. This strategy was not very fruitful and did not produce any crystals other than that of $CaCl_2$. So, conditions from both Structure Screen 1 and 2 were used. Structure Screen 1, condition #20 as well as Structure Screen 2, condition #19 produced diffraction quality crystals (*Fig 6.6*). These crystals grew overnight at 16°C to a reasonable size.

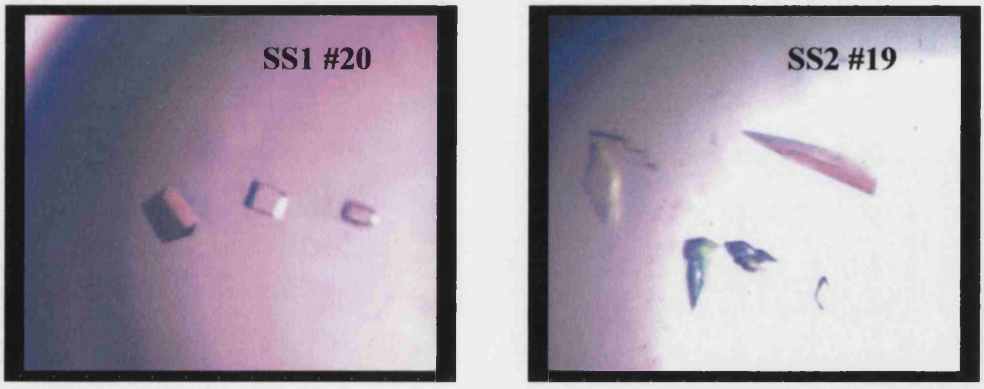



***Fig 6.6*** *Crystals of the nTIMP-1•MMP-1: CD complex from two different conditions (SS1 #20 and SS2 #19). The crystals used for data collection were grown from SS2 #19 (0.1M HEPES (pH 7.5), 10% PEG 8000 and 8% Ethylene Glycol).*

## 6.2 DATA COLLECTION, PROCESSING AND SCALING

A complete dataset to 2.54Å was collected at 100K from a single crystal, grown from Structure Screen 2 condition #19, using the Synchrotron Radiation Source (station PX 9.6, Daresbury, UK). The crystals from Structure Screen 1 condition #20 were also exposed to the X-rays but despite their beautiful morphology these crystals did not diffract beyond 3.0Å. A total of 150 images (*Fig 6.7 representing the first image recorded from the crystal*) were collected (λ=0.87Å,) using a CCD-ADSC detector system.

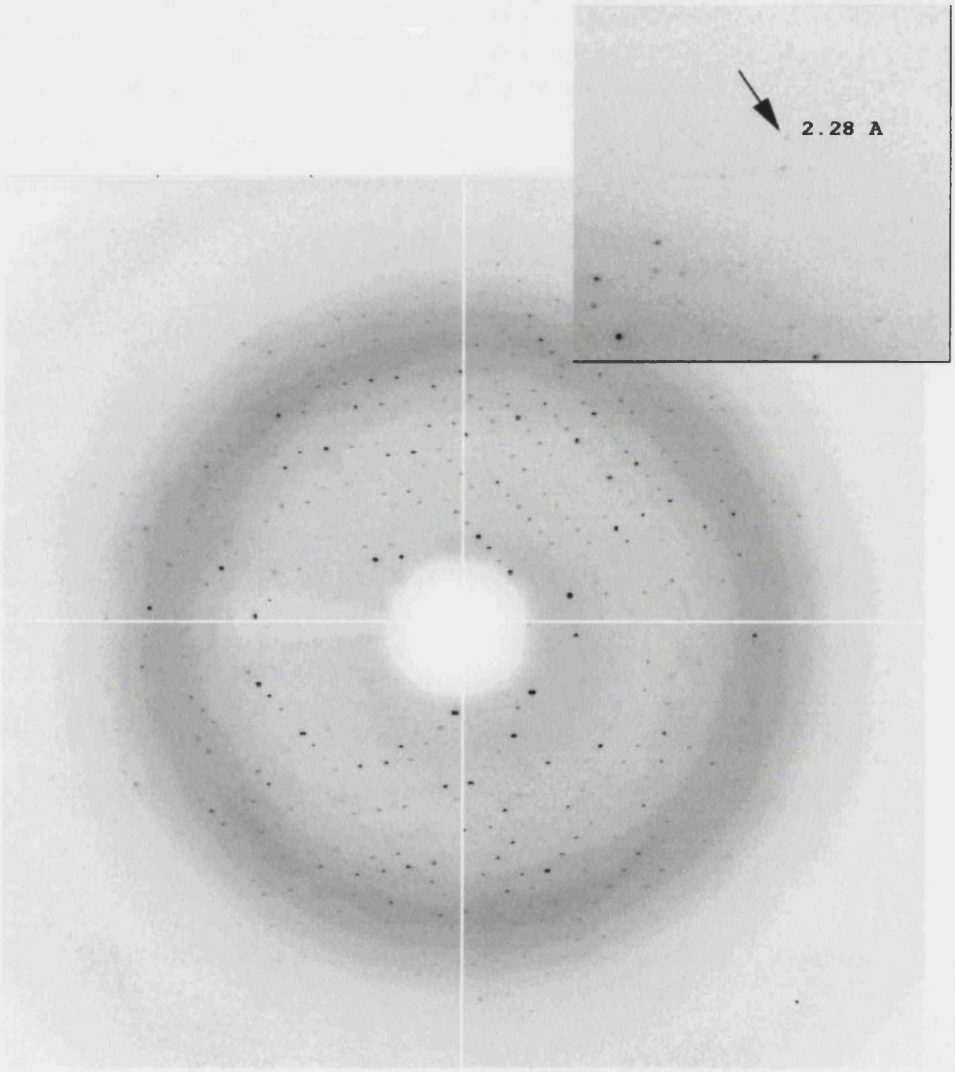



***Fig 6.7*** *A typical diffraction image from the nTIMP-1•MMP-1:CD complex crystal diffracting to 2.54Å resolution (2.28Å at the edge). The inset represents a portion of the image zoomed in to show the high-resolution spots. Data collection parameters used were: λ = 0.87Å; Δφ = 1.0°, Distance = 245mm and exposure = 15 seconds.*

Data processing was performed with the HKL package (Otwinowski and Minor, 1997) in the C-centred monoclinic space group, C2. Data reduction was carried out using the program, TRUNCATE, of the CCP4 suite. Even though the crystal diffracted to 2.28Å resolution, reflections in the last shell were very weak, hence the data was scaled to only 2.54Å. The following table (*Table 6.1*) details the data processing and scaling.

***Table 6.1*** *Data collection and reduction statistics*

| | |
|---|---|
| Unit cell dimensions (Å) | $a$=158.1 $b$=67.85, c=86.24 |
| | α=90°, β=100.29°, γ=90° |
| Reflections measured | 191,162 |
| Unique reflections | 30,014 |
| $R_{sym}$ (%) | 8.8 (45.1) |
| I/σI (outermost shell) | 8.3 (2.8) |
| Completeness (outermost shell) (%) | 98.4 (99.4) |

*The outermost shell corresponds to resolution bin covering 2.63-2.54Å.*

## 6.3 STRUCTURE SOLUTION ATTEMPTS

### 6.3.1 MOLECULAR REPLACEMENT TRIALS

The attempts to solve the structure of the nTIMP-1•MMP-1:CD complex were initiated with molecular replacement trials since the three-dimensional structures of both the catalytic domain of MMP-1 (Lovejoy *et al.*, 1999) and the N-terminal domain of TIMP-1 (Gomis-Ruth *et al.*, 1997) were available. The number of molecules in the asymmetric unit was deduced to be two nTIMP-1•MMP-1:CD complexes based on Matthews_coefficient. The resulting solvent content for the crystal was calculated to be ~64%. The first attempt at structure solution was made with AMoRe (Navaza, 1994). The crystal structure of the MMP-3 (catalytic domain) in complex with the full-length TIMP-1 (Gomis-Ruth *et al.*, 1997) was used as the search model. The TIMP-1 part of the structure was trimmed to retain just the N-terminal domain whereas for the MMP-3 part of the complex, the residues identical to MMP-1

were kept and the others were mutated to alanines. The two zinc ions and the three calcium ions from MMP-3 were excluded from the search model with the view that appearance of Fourier difference density for the zinc ions would confirm the solutions found. The number of molecules in the asymmetric unit was confirmed through calculation of the self-rotation function using POLARRFN (*Fig 6.8*) (CCP4, 1994).

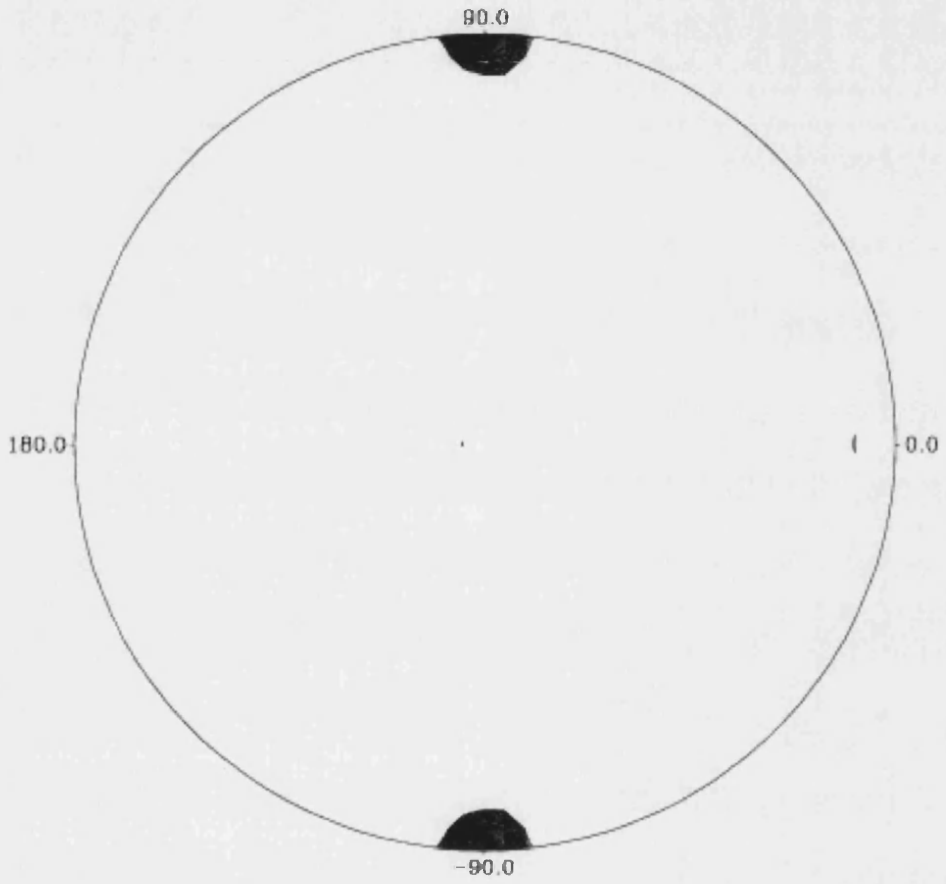



***Fig 6.8*** *Self rotation function as a stereographic projection of the section $\kappa = 180°$ for the nTimp-1•MMP-1:CD data. The top peak height was $100\sigma$*

Cross rotation function did not yield a unique solution. Hence, the search for the two complexes in the asymmetric unit was then carried out in two different runs, one for each domain comprising the complex, MMP-1: CD and N-TIMP-1. Molecular replacement calculations were computed in AMoRe (Navaza, 1994), XPLOR (Brünger, 1992b; Brunger and Rice, 1997),

Beast (Read, 2001), Molrep (Vagin and Teplyakov, 1997), EPMR (Kissinger *et al.*, 1999) and CNS (Brünger, 1992b; Brünger *et al.*, 1998). Several parameters like the radius of integration, resolution range, search model (N-TIMP-1, MMP-3 and MMP-1: both all-alanine models as well as the whole structure) were tried for molecular replacement calculations in AMoRe and Molrep. None of this yielded a unique solution although one of the search runs using Molrep for the MMP-1 domain of the complex did produce a solution that seemed promising. For this run of Molrep, the search model used was the 1.9Å structure of the catalytic domain of MMP-1 (PDB code: 1CGE; Lovejoy *et al.*, 1999). Rigid body and positional refinement of this solution in CNS (Brünger, 1992b; Brunger *et al.*, 1998) resulted in an $R_{cryst}$ of 45.5% and $R_{free}$ of 50.8% with a FOM of around 44.2%. Parameters similar to those used for the MMP-1 domain search were used for N-TIMP-1 as well, but this did not prove to be a fruitful exercise. XPLOR and CNS molecular replacement protocols were used to compute a Patterson search in different resolution ranges but this did not produce a distinct solution. Consequently, a direct rotation search was employed using a Patterson correlation (PC) target function in which the search model was rotated every 5° about all Euler angles in the asymmetric unit of the rotation function. At each orientation, PC target function was calculated using all data (again for different resolution ranges) and each orientation was subjected to PC refinement to further define the correct orientation of the molecule in the asymmetric unit. The top solution was 7σ higher than the average correlation coefficient for the search and increased to 8σ after PC refinement. Translation search was carried out against all data in the same resolution range. This search produced a solution that was about 6σ above the mean correlation coefficient for the search and 2σ above the second highest solution. To find the second molecule in the asymmetric unit, self-rotation function was calculated and used in the second translation search. The solution thus found did not show a significant increase in its correlation coefficient. A rigid body refinement of the two molecules proved that the solutions were not correct.

Unsuccessful attempts with AMoRe, Molrep, XPLOR and CNS, made us try other molecular replacement programs. First to be tried was Beast, which is based on using maximum-likelihood targets for calculating rotation and translation functions. This usually works best when the search model is derived from a well-refined crystal structure of at least medium resolution. Again for MMP-1 domain of the complex the 1.9Å structure of the catalytic domain of MMP-1 (PDB code: 1CGE; Lovejoy *et al.*, 1994) was used. As for the N-TIMP-1 domain, the TIMP-1 part from the MMP-3•TIMP-1 complex (PDB code: 1UEA; Gomis-Ruth *et al.*, 1997) and laminin-binding domain of agrin (PDB code: 1JB3; (Stetefeld *et al.*, 2001)) was used. The laminin-binding domain of agrin was used because it is structurally related to the N-terminal domain of TIMP-1 and the structure available is a well-refined model at 1.6Å. Beast also failed to produce any solution with significantly positive Log Likelihood Gain (LLG) scores despite varying (increase) the estimate of the effective RMS error.

The next program tried was EPMR. The random orientations it generated could not be translated into an optimised solution and thus EPMR also ended up being an unsuccessful attempt at structure solution. A careful read through the literature at this point of time revealed that molecular replacement calculations were not quite straightforward for almost all the structures solved previously for MMPs and TIMPs. This was attributed to high flexibility of the TIMP molecules in the structure. So, AMoRe (Navaza, 1994) was tried once again in order to find the position and orientation of the two complexes in the asymmetric unit. But this time, the loop regions in both MMP-1 and NTIMP-1 domains were excluded from the search model and only the secondary structure elements were used in both rotation and translation searches. Again molecular replacement calculations were computed for each component of the complex separately and as a complex. MMP solutions were found, which turned out to be the same as the ones found using Molrep but TIMP search was yet again unsuccessful. Since the same solutions were obtained for MMP-1 twice, tracing of the TIMP-1 molecules was attempted with FFFEAR (Cowtan, 1998) and with BONES in 'O' (Jones

*et al.*, 1991). However, the electron density for the TIMP-1 molecules was very patchy and not as well defined as for MMPs, which made the tracing not possible.

### 6.3.2 SINGLE ANOMALOUS DISPERSION (SAD) CLOSE TO THE ZINC EDGE

While structure solution trials using molecular replacement methods were being carried out, the presence of the two zinc ions in MMP-1 prompted us to collect a SAD dataset close to the zinc edge on PX 14.2, SRS, Daresbury (UK). The statistics of data processing and scaling are given in *Table 6.2*. A total of 366 images (a full sphere of data) were collected with an exposure time of 12 seconds for each image.

***Table 6.2*** *Data collection and reduction statistics for anomalous data*

| | |
|---|---|
| Unit cell dimensions (Å) | $a$=158.24 $b$=67.72, c=86.29 |
| | α=90°, β=100.29°, γ=90° |
| Reflections measured | 3,89,549 |
| Unique reflections | 27,532 |
| $R_{sym}$ (%) | 6.6 (45.1) |
| I/σI (outermost shell) | 26.7 (4.4) |
| Completeness (outermost shell) (%) | 99.9 (100) |

*The data was collected at λ = 1.042Å at a distance of 210mm and Δφ = 1.0°. The outermost shell corresponds to the resolution bin covering 2.70-2.61Å.*

The reflection file output by SCALEPACK was converted into an MTZ format using the SCALEPACK2MTZ routine. The SCALEIT program was then used to analyse the dataset for large anomalous differences (if any). The scaled dataset was then used to calculate difference anomalous Patterson map with FFT. The Patterson map thus produced was plotted along the Harker section using NPO and contoured at 1.5σ (*Fig 6.9*).

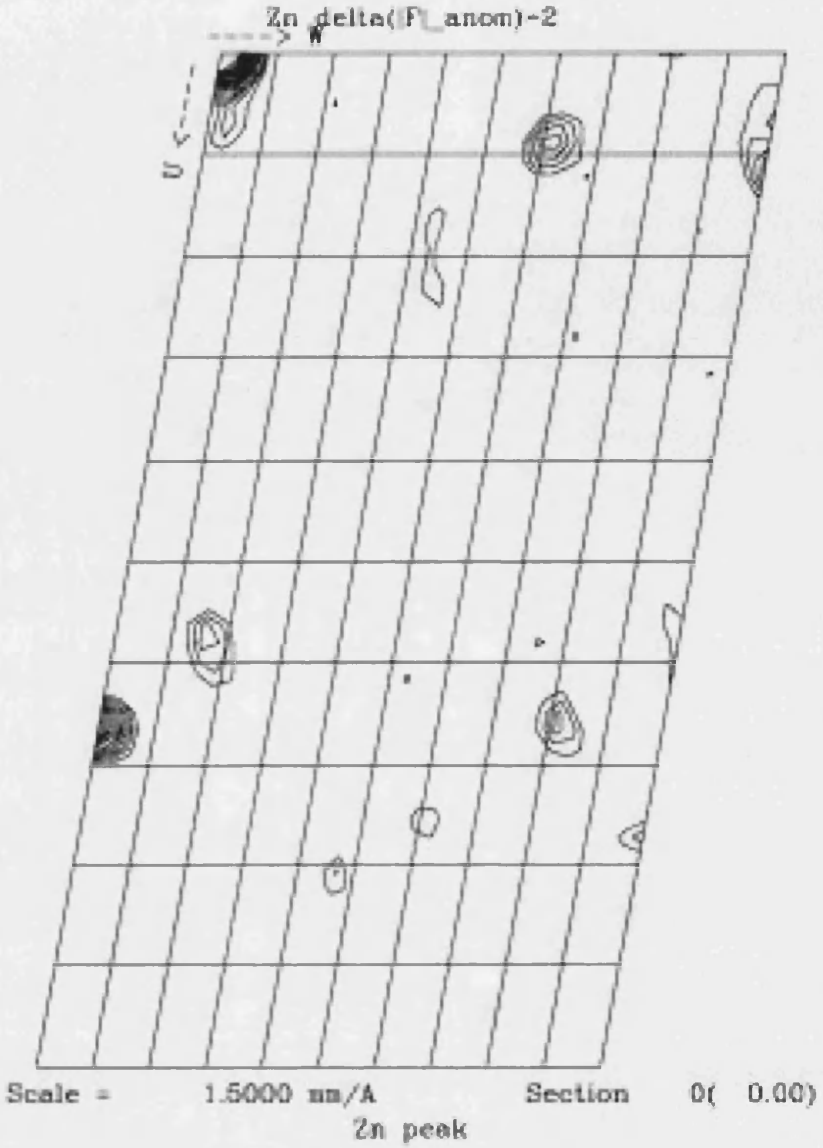



***Fig 6.9*** *Anomalous difference Patterson map for Zinc*

As mentioned before, the crystals belonged to monoclinic C2 space group (unique b axis) with two molecules in the asymmetric unit. Since each MMP-1 molecule has two zinc atoms bound to it (a catalytic zinc and a structural zinc), a total of 4 zinc sites had to be identified. The equivalent positions for this space group are x, y, z; -x, y, -z; 1/2+x, 1/2+y, z and 1/2-x, 1/2+y, -z. The CCP4 program, PEAKMAX, was used to compute a list of predicted peaks from the Patterson map. The top peak was compared with the anomalous zinc sites deduced manually from the Harker section plot. One of the peaks from the plot was the same as the top peak. This zinc site was

refined in MLPHARE. Difference Fourier map was calculated to find more sites. The top peak (2nd site) found in the second run of PEAKMAX was checked for consistency with the Patterson peaks like before. Although the peak did not correspond to any of the Patterson peaks, it was input as the 2nd site in another run of MLPHARE. This addition did not improve the phasing power or the FOM in any way. Further addition of sites resulted in worsening of the statistics. So, phases were calculated from the first two sites and subjected to Density Modification (DM; Cowtan and Zhang, 1999) to improve and extend phases. The sign of the sites were reversed in order to compute phases for the other hand. These phases were also subjected to DM. None of the two trials produced an interpretable map.

### 6.3.3 MULTIPLE ISOMORPHOUS REPLACEMENT (MIR) ATTEMPTS

MIR attempts for the crystals of the nTIMP-1•MMP-1:CD complex was carried out with different heavy atom compounds like Potassium tetrachloroplatinate (II) [$K_2PtCl_4$], [$K_2PdCl_4$] and Osmium chloride. The soak concentrations used were 10mM (with 5mM in the drop). The crystals were monitored for deterioration during the soak. The Platinum (Pt), Palladium (Pd) and the Osmium (Os) datasets were collected in X11, Hamburg, Germany. An overview of the data processing and scaling statistics is given in *Table 6.3*. All the datasets were collected at a wavelength of 0.811Å and a crystal-to-detector distance of 260mm with an oscillation width of 1.0° and 15 seconds exposure time.

*Table 6.3 Data processing and scaling statistics for derivative datasets*

| Dataset (soak time) | Completeness (last shell) % | $R_{sym}$ (last shell) % | Resolution (Å) | I/σI |
|---|---|---|---|---|
| Pt (45 min) | 84.0 (83.4) | 13.5 (42.9) | 3.1 | 7.0 (3.25) |
| Pt (50 min) | 67.8 (69.5) | 20.4 (68.1) | 3.5 | 5.44 (2.0) |
| Pt (~4 hrs) | 86.5 (88.3) | 15.4 (11.6) | 4.5 | 5.42 (7.8) |
| Pd (~2hrs) | 97.6 (94.8) | 7.7 (38.4) | 2.7 | 10.6 (2.1) |
| Os (50 min) | 92.1 (93.9) | 11.6 (40.9) | 3.0 | 6.06 (2.0) |

Programs from the CCP4 package (CCP4, 1994) as well as SHELXS-97 (Sheldrick, 1997) were used to locate and refine heavy atom positions. All these datasets were merged using the program CAD (CCP4, 1994). The derivative datasets were then scaled against the native 2.54Å dataset using SCALEIT (CCP4, 1994). The log file created by SCALEIT program analyses the derivative dataset against the native and outputs statistical values (*Table 6.4*) like the $R_{fac}$ and $D_{iso}$ (the isomorphous differences between the native and derivative dataset). The log file lists out, for each derivative to native scaling, $R_{fac}$ value for each resolution bin. A decrease in the $R_{fac}$ value around 4.0Å and subsequent increase at higher resolution gives a slight indication of whether or not the heavy atom is bound. This trend was seen only for the Osmium (Os) dataset. The log file also outputs the level acceptable differences for each derivative dataset, which is subsequently used as sigma level when plotting Difference Patterson maps for each derivative.

***Table 6.4*** *Statistics produced by SCALEIT for each derivative dataset*

| Dataset | Overall $R_{fac}$ | $D_{iso}$ |
|---|---|---|
| Pt (45 min) | 28.6% | 63.4 |
| Pt (~4 hrs) | 31.6% | 87.8 |
| Pd (~2hrs) | 50.6% | 87.2 |
| Os (50 min) | 19.3% | 39.7 |

Since the Osmium dataset seemed more promising than others, Patterson maps were calculated first for this dataset using FFT (CCP4, 1994). Possible heavy atom positions determined by manual deconvolution of the Patterson synthesis as well as cross verification of the peaks with those predicted by PEAKMAX (CCP4, 1994). Heavy atoms located were refined in MLPHARE (CCP4, 1994). A similar protocol was applied for other derivatives as well. Cross difference Fourier synthesis between the different derivatives were carried out to verify the heavy atom sites found independently. These sites were not confirmed by this procedure, perhaps because of very weak or no substitution at all.

Since we had initially found a zinc site from the anomalous dataset collected previously, the anomalous dataset and all the derivative datasets were scaled against the native data using SCALEIT. Heavy atom sites for Pt were determined and these were then used to calculate a difference Fourier to locate Zinc sites and confirm these sites with the ones found previously (independently). Two zinc sites and one platinum site thus identified were refined with their relative occupancies in MLPHARE. The statistical parameters output after heavy atom refinement revealed that the Pt sites identified had very low occupancies indicating perhaps a rather weak substitution. Overall refinement of the sites together produced a phasing power of just 1.10 and an $R_{cullis}$ of 0.92. The low phasing power and an $R_{cullis}$ of nearly 1 was not good enough to produce good model phases. Since a partial MR model from Molrep for the MMP-1 component of the complex was available a map with a combination of these phases was calculated. However, the peaks corresponding to the identified Pt and Zn sites could not be located in the resulting Fourier map.

## 6.4. DISCUSSION AND FUTURE WORK

It is not quite clear as to why the Molecular Replacement trials failed to result in structure solution. Past experiences of other scientists working in this field with MMP-TIMP complexes suggest that the TIMP molecule is highly flexible. The high solvent content of ~64% in our crystals also indicates that there must be regions in the protein that have very high thermal motion. Perhaps this may be one of the reasons why all the MR programs either failed or did not yield a correct solution. Inspection of the Fourier synthesis for the entire asymmetric unit, calculated using the Molrep solution for the MMP-1 component of the complex, revealed patchy difference density, which perhaps accounts for the missing portion of the asymmetric unit, namely the two TIMP-1 molecules. However, what is interesting is that this difference density is not in the region as would be expected from the MMP-3•TIMP-1 complex structure (Gomis-Ruth *et al.*, 1997). It can be possible that in our case, the orientation of the two protein molecules is different from what is expected based on other MMP-TIMP complexes, which may be another

reason why our search with the complex as a search model failed to yield any solution. Also the structure for the N-terminal domain of TIMP-1 (residues 1-126) was obtained from the full-length structure of TIMP-1 in the MMP-3•TIMP-1 complex (Gomis-Ruth *et al.*, 1997). Perhaps there is some difference in the orientation of the N-terminal domain of TIMP-1, when it forms a complex with the C-terminal domain and this variation may be another reason for the additional difficulty in finding a unique solution of thie structure.

The unsuccessful attempt with the SAD data can perhaps be attributed to the fact the data was not collected at the exact zinc edge. The wavelength at which the data was collected was 1.042Å whereas the exact wavelength for collecting anomalous zinc data is 1.2008Å. In other words, anomalous contribution or the strength of the anomalous signal from the zinc atoms in the observed data is perhaps very weak and probably does not contribute much to the observed structure factor. As a result, the quality of the phase estimates that were calculated on basis of single wavelength data collected away from the zinc edge, containing very weak anomalous signal, was poor and thus could not yield an interpretable map. However, due to limitation of the station, PX 14.2 at SRS, Daresbury, (UK), we could not optimise our data collection at the exact Zinc edge.

Recently, attempts were made to collect fresh anomalous data at the exact zinc edge at PX9.5, SRS, Daresbury (UK). However, non-availability of good quality, fresh crystals hindered the attempt. This was because the metalloenzyme, MMP-1 has a tendency to degrade over a period of time and thus crystallisation trials with old samples failed to produce good quality crystals. Crystallisation trials with a new batch of proteins are currently underway and we hope to collect a SAD dataset at the exact Zinc edge at ESRF, Grenoble (France) in February/March, 2003. We may also need to screen for more potential derivatives. Hopefully, a combination of isomorphous replacement and anomalous scattering methods should help in solving the structure of this complex.

## FUTURE WORK IN THE FIELD OF ANGIOGENESIS

The aim of angiogenesis research is to characterise the endothelial cells that originate from tumour tissues. Today, one of the major future directions for angiogenesis research is to continue to identify the biochemical targets and elucidate the three-dimensional structures of all the macromolecules involved in the process of angiogenesis. It is important to model and quantify the structure-functional relationship of these macromolecules in parametric terms and correlate the dynamics of the process with these parameters. This will help in incorporating the molecular properties of these targets in a cellular context, which will unravel the complex pathways that are integrated into the process of blood vessel formation. Also, it is necessary to develop new experimental models for angiogenesis so that the authenticity of the molecule being tested can be verified to a larger extent than now. For this we also need to evaluate angiogenesis in human tumours as opposed to animal tumours as a prognostic tool.

Every year at least one major event in cancer research takes place somewhere in the world. A vast array of research is being done by both basic and clinical researchers who are striving towards communicating the biology of their work into drugs and inhibitors. It is becoming increasingly important to design small molecule antagonists taking into account the macromolecular target in terms of its biological endpoint, especially since at least 50 different diseases (including all cancers) are caused by persistent blood vessel growth. The therapeutic applications that need to be developed should promote tumour regression by death of tumour cells and not normal, healthy cells. Also the need of the hour is to develop drugs that are easy to deliver, free of delivery risk and with low dosage requirements to combat poor immunogenicity, which is one of the major obstacles to the development of successful antagonists.

The structures of PlGF-1 and VEGF-B solved during the course of this thesis have laid a foundation for future work in the area of drug design based on their structures. However, further structural studies based on receptor-ligand complexes are required to gain useful insights into the desired and undesired

interactions for the key residues at the binding interface. Since both the molecules are close homologues of VEGF-A, the most potent inducer of angiogenesis, therapeutic approaches targeting these molecules will have a significant impact on the field of angiogenesis. Structural studies based on the ligand specificity of Flt-1, for PlGF-1 and VEGF-B, as opposed to the dual receptor specificity of VEGF-A. The structural basis behind the affinity of Flt-1 for PlGF-1 and VEGF-B may lead to the development of receptor/ligand specific therapeutics. PlGF-2, on the other hand, binds Flt-1, heparan sulphate proteoglycans (HSPGs) and the semaphorin receptors: neuropilins-1 and 2 (NP-1 and NP-2). It is known that the binding of heparin to PlGF-2 increases its affinity for the neuropilins. Also interesting is the fact that Flt-1 and NP-1 interact to form a receptor-receptor complex. Access to the three-dimensional macromolecular structures of these complexes is essential for the functional characterisation of the interactions between PlGF-2 and its binding partners. Therapeutics and drugs available to date for these angiogenic molecules, especially VEGF-A, are based on mutagenesis studies and the crystal structure of VEGF-A on its own and with its receptor, Flt-1. Since the activities of these growth factors, VEGF-A, PlGFs, VEGF-B, are all correlated in several tissues (e.g. heart), the next step would be to elucidate the crystal structure of all these complexes. This is important to understand the different modes of interaction between the molecules in order to minimize unwanted activities as well as enhance desired ones. These target structures would have a tangible effect on the production and optimisation of drug candidates for combating cancer and other angiogenesis related diseases like ischemia of the heart and arthritis.

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# APPENDIX

# APPENDIX A

The work described in this thesis involved the use of several computer programs. These invaluable programs have been developed by scientists with the aim to assist non-programmers in the process of structure determination. These different programs are used right from the time of data collection to the very end when a structure is analysed or even when the results need to be graphically represented. The following contains a brief description of all the programs that I have used in my Ph.D. work.

## A1. Data Collection, Processing And Scaling: THE HKL PACKAGE (Otwinowski and Minor, 1997)

In the present scenario data collection has become an interactive process. One can process data in tandem with data collection. Quick scaling of the first few images collected helps in planning the strategy for further data collection as well as making optimum use of the expensive synchrotron facilities, both in terms of the quantity of data collected as well as the quality time spent in doing so.

All the datasets presented in this thesis were collected at the Synchrotron Radiation Source, Daresbury, UK. The HKL package was used for the purpose of data processing and scaling. The programs in the HKL package were written by Zbyszek Otwinowski and Wladek Minor. This data processing suite for macromolecular crystallography comprises of three programs.

- ***XdisplayF***: This program enables visualisation of diffraction images.
- ***Denzo***: This program enables numerical analysis of the raw diffraction data. Denzo reduces the data to a set of *hkl* indices and the intensity for each recorded reflection.
- ***Scalepack***: This program provides the complete statistics on the collected dataset. It gives both the weighted ($\chi^2$) and the

unweighted ($R_{merge}$) statistical values, which help to establish the quality of the dataset.

**HKL2000 (Otwinowski and Minor, 1997):** The β-version of this program is installed in SRS, Daresbury, UK. This is a graphical user interface (GUI) based data processing suite, which includes all the various features of XdisplayF, Denzo and Scalepack.

## A2. Reflection Data Utilities: CCP4 suite (CCP4, 1994)

The reflection data file (the one produced by Scalepack) needs to be converted into a format that is suitable to input into the phasing program that will be used for structure solution *eg* all programs in the CCP4 suite use MTZ reflection files.

- ***Scalepack2mtz***: This program converts the scalepack reflection file into an mtz format.
- ***Truncate***: This program is used to calculate structure factor amplitudes from the observed intensities.
- ***Cad***: This program can be used to combine and sort reflections from several files into a single file or can be used to extend reflection data for space group P1 or simply place the reflection data in the CCP4 asymmetric unit and sort them out into a standard order.
- ***Uniqueify***: This program can be used to complete a dataset with reflections allowed for a given unit cell with a given symmetry for a given resolution limit, regardless of whether or not data have been measured for those reflections.

***Mtz2various***: This utility helps covert an mtz reflection file into formats that will be suitable for the different programs specified by the keyword "OUTPUT". The programs supported by this utility are SHELX, CNS/XPLOR, MULTAN, TNT, MAIN, CIF, SCAL. The OUTPUT type "USER" can be used to define a format specific for the user's needs. The user can specify which columns are to be output, how many and in what format.

***Mtz2utils***: This utility, like Cad, can be used to edit or re-arrange an mtz reflection file.

## A3. PHASING: Molecular Replacement (MR)

**A3.1 AMoRe (Navaza, 1994)**: This is the state-of-the-art program for structure solution and it comprises of 5 separate routines to run a complete molecular replacement. The five routines are:

1. ***SORTING***: In this routine the data is read, extended to cover a hemisphere of reciprocal space, sorts out the reflections according to the space group symmetry and reformats the mtz file into an hkl file for use by the next routines in AMoRe.
2. ***TABLING***: This routine reads in model coordinates and calculates structure factors for the model.
3. ***ROTING***: This routine calculates the rotation function and produces a list of several possible solutions by Patterson overlap.
4. ***TRAING***: This routine calculates the translation function for each of the possible rotation solution found in step 3
5. ***FITING***: This routine does a rigid-body refinement of all the solutions found by the rotation and translation search.
6. ***REORIENTATING***: This applies the MR solution on the initial coordinates of the search model. It uses the Eulerian angles and the Centre of Mass values from the tabling output as well as the rotation and translation search values from the fiting routine as input.

The better the Patterson overlap the higher is the correlation coefficient and lesser are the R-factors. Usually the top solution is the right one but NOT ALWAYS so. If there are several molecules in the asymmetric unit then one can find them by fixing the rotation and translation parameters of the first molecule in the second translation run and so on until all the molecules are found. As and when the right solutions are found the correlation coefficient increases with a simultaneous decrease in the R-factor. Nevertheless, it is important to check the packing of the solutions on the graphics.

**A3.2 BEAST (Read, 2001)**: This program, written by Randy J. Read, uses maximum-likelihood targets for calculating rotation and translation functions. Like AMoRe, Beast also facilitates searching for multiple molecules in the asymmetric unit by fixing ones already known. Beast makes it possible to use several possible search models in the same run.

**A3.3 CNS/XPLOR (Brünger, 1992b)**: Both CNS and XPLOR use Patterson correlation (PC) refinement based method for molecular replacement calculations. For rotation searches, a stationary Patterson map is calculated from the observed intensities. The Patterson map to be rotated in reciprocal space is calculated from either the observed intensities (for computing the self-rotation function) or from the search model (for computing the cross-rotation functions). The translation search can be performed for each rotation solution obtained from the PC refinement. CNS/XPLOR can also perform a packing analysis for each listed peak. The solution output is translated according to the highest translation function value.

**A3.4 EPMR (Kissinger *et al.*, 1999)**: This program by Charles R. Kissinger, Daniel K. Gehlhaar and Bradley A. Smith computes molecular replacement calculations using evolutionary search algorithms. It randomly generates rotation and translation parameters for the search model and computes correlation coefficient for each orientation. The solutions with correlation higher than a given threshold are kept. The program then makes random alterations to the rotation and translation parameters of these solutions and the correlation coefficient for each of the altered solutions is computed again. The high scorers of the round survive and the whole procedure is repeated for 'n' number of cycles. At the end of the specified no of cycles, the program performs a round of rigid-body refinement on the optimised solutions. These solutions are then checked on the graphics for their packing.

**A3.5 MOLREP (Vagin and Teplyakov, 1997)**: The authors of Molrep are Alex Vagin and A. Teplyakov. Molrep like AMoRe calculates overlapping functions between the observed and the calculated Pattersons. However, unlike AMoRe, Molrep calculates rotation function for three different orientations of

the model and averages them thus reducing noise level. Also, during the translation search, Molrep removes incorrect solutions on the basis of their bad packing by multiplying the translation function with the packing function for each peak computed.

## A4. PHASING: Multiple Isomorphous Replacement (MIR)/Anomalous Dispersion (MAD/SAD)

**A4.1 SCALEIT (CCP4, 1994)**: This program, written by Phil Evans, Eleanor Dodson and Richard Dodson, applies a derivative to native scaling function on the reflection dataset. This program is normally run after all the datasets, both native and derivative, have been merged into one file using CAD. The scaling factor is determined from the squared amplitudes and can be an overall scale factor, an isotropic or an anisotropic temperature factor. The program outputs a list of statistics that can be used to analyse the quality of data. It gives an estimate of the acceptable isomorphous and anomalous differences for each derivative and outputs a list of reflections that have abnormally high differences. These reflections can then be excluded while searching for heavy atom or anomalous sites during Patterson calculations.

**A4.2 FFT (CCP4, 1994)**: The program is an abbreviation for Fast Fourier Transform and was written by Lynn F. Ten Eyck. It is used to prepare different types of Fourier transforms from a binary reflection data file. These transforms include Fourier synthesis, double difference Fouriers, Patterson maps and difference Patterson maps.

**A4.3 PEAKMAX (CCP4, 1994)**: Phil Evans and Ian Clifton wrote this program to search the electron density map for peaks with heights above a given threshold. The program lists these peaks in descending order of their height. The top peak is compared with the difference Patterson to see if it appears there. This format of peaks is suitable for input to MLPHARE (a program for heavy atom refinement).

**A4.4 NPO (CCP4, 1994)**: This program by Sam Motherwell was originally written under the name of "pluto". Phil Evans and Eleanor Dodson later modified the program for plotting maps and protein molecules respectively. NPO can be used for plotting maps that are output from FFT, either as simple sections or as stacks.

**A4.5 XPLOT84DRIVER (CCP4, 1994)**: This program is used to view the plots created by NPO and was written by Jan Zelinka.

**A4.6 MLPHARE (CCP4, 1994)**: This program refines the heavy atom parameters and calculates phases from them. The refinement is based on maximum-likelihood function. MLPHARE allows for the use of cross difference Fouriers to find sites in other derivatives relative to the same origin as the sites from the first derivative. The results of the refinement are output in the form of statistical analysis as a function of Figure of Merit (FOM) or resolution range.

**A4.7 SHARP (de La Fortelle and Bricogne, 1997)**: SHARP was originally written by Gerard Bricogne and Eric de La Fortelle. It was converted into GUI based software for refinement of heavy atom parameters by John Irwin.

## A5. DENSITY MODIFICATION

Phasing programs are very closely associated with density modification programs as they aid in phase improvement and help in interpreting the electron density maps. Several programs have been written to facilitate density modification.

**A5.1 DM (CCP4, 1994)**: DM by Kevin Cowtan applies real space constraints based on the known features of an electron density map in order in order to improve the experimental phases.

**A5.2 SOLOMON (CCP4, 1994)**: This program written by J.P. Abrahams with the I/O sub-routines modified by Kevin Cowtan also performs density modification by solvent flipping instead of the solvent flattening mode

followed by DM. Solomon is now a feature of SHARP but it can be run on its own as well. Solomon also features the ability to include information from a partial structure in the form of Hendrickson-Lattman (HL) coefficients.

**A5.3 NCSMASK (CCP4, 1994)**: It is a mask manipulation program used for constructing masks in cases where non-crystallographic symmetry is present. This program performs operations on NCS symmetry masks constructed from a monomer. The masks generated by this program are ideal as input into DM when using NCS averaging.

**A5.4 CNS (Brünger, 1992b)**: CNS also uses the solvent flipping mode like Solomon for density modification based phase improvement. CNS can also be used for reducing model bias when using a MR solution. This program was written by Alex Brunger.

## A6. REFINEMENT AND MODEL BUILDING

**A6.1 O (Jones *et al.*, 1991)**: This software developed by T.A. Jones and Morten Kjeldgaard is used for displaying the electron density maps and the coordinates for the model after structure solution. It is used for model building between iterative refinement cycles.

**A6.2 CNS (Brünger *et al.*, 1998)**: CNS refinement is based on maximum-likelihood targets. It carries out simulated annealing refinement of crystal structures based on torsion-angle molecular dynamics. It automatically computes cross-validated $\sigma_A$ error estimates and the weighting factor between the X-ray refinement target function and the geometric energy function. CNS also refines bulk solvent and overall B-value by performing least-squares minimisation before refining atomic positions by simulated annealing protocol.

**A6.3 ARP/WARP (Lamzin and Wilson, 1993)**: Automated Refinement Procedure is a package developed by Anastassis Perrakis, Victor S. Lamzin,

Richard J. Morris and Petrus H. Zwart. This package can be used for automated tracing of the protein chain, improvement of experimental phases via map interpretation as well as refinement of MR solutions. ARP/WARP can also be used to build the solvent structure for the crystal structure. Like CNS, structure refinement is again a function of maximum-likelihood. Apart from requiring data to at least ~2Å, the other limitation of this program is that only P1 and acentric space groups are supported.

**A6.4 SHELXL (Scheldrick and Schneider, 1997)**: George Sheldrick's program, SHELXL, is based on conventional SF summations rather than the FFT based programs. For this reason it is slower but more accurate than the other standard macromolecular refinement programs. SHELXL can work with data up to 2.5Å or higher.

## A7. STRUCTURE ANALYSIS

**A7.1 BAVERAGE (CCP4, 1994)**: Eleanor Dodson's program, baverage, calculates the average B-factor values over the main chain and side chain atom.

**A7.2 CONTACT (CCP4, 1994)**: This CCP4 program was written by Tadeusz Skarzynski. It computes the contacts (both intra- and inter-molecular contacts) in a crystal structure. It lists out the contacts between the protein molecules, between protein and ligands/ions/prosthetic groups (if present) and between protein and waters.

**A7.5 MATTHEW'S COEFFICIENT (CCP4, 1994)**: Given the unit cell dimensions and the molecular weight of the protein, this program written by Misha Isupov calculates the Matthew's coefficient and the solvent content of the protein crystal. This helps compute the number of molecules in the asymmetric unit.

**A7.6 PROCHECK (CCP4, 1994)**: This program from CCP4 analyses the stereochemistry of the protein structures. It produces around 10 postscript files

detailing the stereo-chemical quality of the model, residue-by-residue. It also produces the RAMACHANDRAN PLOT.

**A7.7 SC (CCP4, 1994)**: Michael C. Lawrence's program SC analyses the shape complimentarity of two interacting molecular surfaces.

**A7.8 CNS (Brünger *et al.*, 1998)**: There are several scripts available in CNS for structure analysis. The ones used for the work presented in this thesis are as follows:

- ***model_stats.inp***: Calculates statistical information from the input PDB file and reflection data file. The information it produces is required for the PDB header.
- ***buried_surfaces.inp***: As the name suggests, it calculates buried surfaces in the molecular structure.
- ***xtal_pdbsubmission.inp***: Prepares the refined coordinates suitable for submission to the Protein Data Bank.

## A8. PICTURES

**A8.1 MOLSCRIPT (Kraulis, 1991)**: Molscript is a program used to display both schematic and detailed representations of the molecular structures. The version used for this work was Molscript v2.1.2. Information supplied by means of an input script that specifies the coordinates of desired molecule along with graphics parameters enables users to render graphics images.

**A8.2 ALSCRIPT (Barton, 1993)**: Alscript is a program that takes in a multiple sequence alignment file (files produced by the programs CLUSTAL, AMPS, GCG) and produces a PostScript file based on the set of formatting commands provided by the user via an input script. It does not align or edit sequences by itself. It only formats them according to user specifications.

# APPENDIX B

## *Figure of Merit*

Ambiguity in phase determination using Isomorphous Replacement Method or the Anomalous Scattering method arises from inaccuracy of intensity measurements, lack of isomorphism (for isomorphous replacement methods) and incorrect heavy atom positions. The total error, E, is given by

$$\langle E \rangle^2 = \langle \delta \rangle^2 + \langle e \rangle^2$$

where, $\langle \delta \rangle$ is the r.m.s. value of the structure factor amplitudes of the protein ($\mathbf{F_P}$) and those of the derivative ($\mathbf{F_{PH}}$) and $\langle e \rangle$ is the error in calculation of the heavy atom contribution, $\mathbf{F_{H(calc)}}$, to the derivative structure factors ($\mathbf{F_{PH}}$). If we are to assume that $\mathbf{F_H}$ is small compared to $\mathbf{F_P}$ and $\mathbf{F_{PH}}$, the magnitude of phase error introduced by errors in $\mathbf{F_P}$ is of the same magnitude as an error in $\mathbf{F_{PH}}$. This means that the magnitude of phase error is not affected by regarding the whole error to reside in $\mathbf{F_H}$.

Lack of closure (in relation to refinement of heavy atom parameters) is also based on the assumption that $\mathbf{F_H}$ is small compared with $\mathbf{F_P}$ and $\mathbf{F_{PH}}$. If a Gaussian distribution of errors was to be assumed then the total probability of the phase angle, $\alpha_P$. (for $\mathbf{F_P}$ when several heavy atom derivatives are used simultaneously) is given by the product of individual probabilities,

$$P(\alpha) = \prod_j P_j(\alpha) = \exp\left[-\sum_j (\epsilon_j(\alpha)^2 / 2E^2_j\right]$$

where, $\epsilon_j$ is the lack of closure of the phase triangle defined by

$$\epsilon_j = \mathbf{F_{PH(obs)}} - \mathbf{F_{PH(calc)}}$$

Blow and Crick (1959) showed that this product gives the joint probability curve which gives all available information about the phase of the reflection. This curve is then used to plot the probability map on an Argand diagram in order to find the centroid of the distribution. According to Blow and Crick, the centroid of the distribution is a better choice since it results in a synthesis with the least mean square error in the electron density over the unit cell. This takes into consideration both unimodal and bimodal probability

functions and thus doesn't give too much weight to uncertain phases with bimodal distribution.

If we consider the error in electron density arising from errors in one reflection, then the mean square error, over the unit cell of volume V, from this reflection is given by:

$$\langle \Delta\rho^2 \rangle = 1/V^2 \, (F_s - F_t)^2$$

where, $F_s$ is the coefficient used in the Fourier synthesis and $F_t$ if the true value. The probability of $F_t$ having a phase angle $\alpha$ can be expressed as:

$$F_t = F \exp i\alpha$$

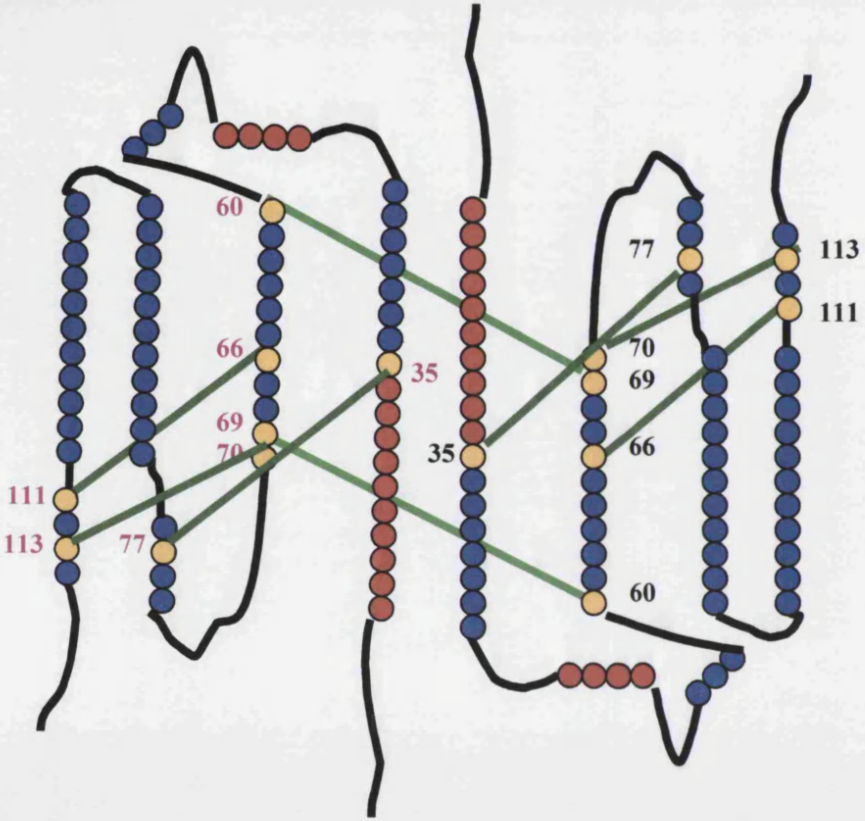
The mean square error can now be expressed as

$$\langle \Delta\rho^2 \rangle = 1/V^2 \int_{\alpha=0}^{2\Pi} (F_s - F \exp i\alpha)^2 \, P(\alpha) d\alpha \Big/ \int_{\alpha=0}^{2\Pi} P(\alpha) d\alpha$$

This integral will have a minimum value when $F_s$ is at the centre of gravity of the centroid distribution. Thus $F_{s(best)}$ or the *best Fourier* can be represented by

$$F_{s(best)} = mF \exp(i\alpha_{best})$$

Where, m is the magnitude equivalent to a weighting function and is defined as the '***figure of merit***'. The electron density map calculated using mF and $\alpha_{best}$ would have minimum mean square error when averaged over the entire unit cell.




Cys35A – Cys77A
Cys66A – Cys111A
Cys70A – Cys113A
Cys60A – Cys69B
Cys69A – Cys60B
Cys35B – Cys77B
Cys66B – Cys111B
Cys70B – Cys113B

*Schematic representation of the cystine-knot motif. The cysteine residues of monomer A have been shown in pink and those of monomer B in black. The text box on the right hand side lists the pairing of the cysteine residues involved in the knot, making a total of 8 disulphide bridges: 6 intra-chain bridges (3 each for monomers A and B: coloured dark green in the figure) and 2 inter-chain disulphide links (coloured light green in the figure).*

## LIST OF PUBLICATIONS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# The Crystal Structure of Human Placenta Growth Factor-1 (PlGF-1), an Angiogenic Protein, at 2.0 Å Resolution*

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**The angiogenic molecule placenta growth factor (PlGF) is a member of the cysteine-knot family of growth factors. In this study, a mature isoform of the human PlGF protein, PlGF-1, was crystallized as a homodimer in the crystallographic asymmetric unit, and its crystal structure was elucidated at 2.0 Å resolution. The overall structure of PlGF-1 is similar to that of vascular endothelial growth factor (VEGF) with which it shares 42% amino acid sequence identity. Based on structural and biochemical data, we have mapped several important residues on the PlGF-1 molecule that are involved in recognition of the fms-like tyrosine kinase receptor (Flt-1, also known as VEGFR-1). We propose a model for the association of PlGF-1 and Flt-1 domain 2 with precise shape complementarity, consider the relevance of this assembly for PlGF-1 signal transduction, and provide a structural basis for altered specificity of this molecule.**

Angiogenesis, the process of new blood vessel formation, is essential for development, reproduction, wound healing, tissue regeneration, and remodeling (1). It also plays a major role in tumor progression, diabetic retinopathy, psoriasis, and rheumatoid arthritis (2). Angiogenesis involves proliferation of endothelial cells (ECs)[1] in an organized fashion and is most likely regulated by polypeptide growth factors (3, 4) such as acidic and basic fibroblast growth factors (aFGF and bFGF, Ref. 5), vascular endothelial growth factor (VEGF, Refs. 6–10), and placenta growth factor (PlGF, Refs. 11–14). PlGF, VEGF (VEGF-A), VEGF-B (15), VEGF-C (16), VEGF-D (17), VEGF-E (18), and Fos-induced growth factor (FIGF, Ref. 19) are members of a family of structurally related growth factors. Intra- and interchain disulfide bonds among eight characteristically spaced cysteine residues are involved in the formation of these active dimeric proteins and hence termed as cysteine-knot proteins. They also share a number of biochemical and functional features (for a review, see Ref. 20) such that PlGF and VEGF can form heterodimeric molecules in cells in which both genes are expressed (21, 22).

Alternative splicing of the PlGF primary transcript leads to three forms of the mature human PlGF protein (22–24). The two predominant forms, PlGF-1 and PlGF-2 (also known as PlGF-131 and PlGF-152, respectively), differ only by the insertion of a highly basic 21-amino acid stretch at the carboxyl end of the protein. This additional basic region confers upon PlGF-2 the ability to bind to heparin (13, 23).

The exact role of PlGF in vascular development is yet to be established. However, purification of PlGF-1 from overexpressing eukaryotic cells and measurement of angiogenic activity of the purified PlGF-1 *in vivo* in the rabbit cornea and chick chorioallantoic membrane (CAM) assays showed induction of a strong neovascularization process that was blocked by affinity-purified anti-PlGF-1 antibody. In the avascular cornea, PlGF-1 induced angiogenesis in a dose-dependent manner and seemed to be at least as effective (if not more effective) as VEGF and bFGF under the same conditions and at the same concentration. PlGF-1 was shown to induce cell growth and migration of endothelial cells from bovine coronary postcapillary venules and from human umbilical veins (HUVECs). In these two *in vitro* assays, PlGF-1 seemed to have a comparable effect on the cultured microvascular endothelium (*e.g.* capillary venule endothelial cells, CVECs) to that of VEGF and bFGF. These results clearly demonstrate that PlGF-1 can induce angiogenesis *in vivo* and stimulate the migration and proliferation of endothelial cells *in vitro* (25). In the case of PlGF-2 it has been established that the recombinant, purified protein is able to stimulate bovine aortic endothelial cells (BAEC, Ref. 13) and HUVECs but not the ECs from hepatic sinusoids (26).

The VEGF homodimer binds to and induces autophosphorylation of two distinct kinase receptors: the fms-like tyrosine kinase, Flt-1 (also known as VEGFR-1) and the kinase insert domain-containing receptor/fetal liver kinase, KDR/Flk-1 (also known as VEGFR-2). Conversely, the PlGF-1 and -2 homodimer bind only to the Flt-1 receptor (22, 26–28). Likewise, VEGF-B also binds selectively to Flt-1 and hence appears to be

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*The atomic coordinates and structure factors (code 1FZV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).*

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[1] The abbreviations used are: ECs, endothelial cells; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor; FIGF, Fos-induced growth factor; PDGF-BB, platelet-derived growth factor-BB; TGF-β2, transforming growth factor-β2; NGF, nerve growth factor; Flt-1, fms-like tyrosine kinase; KDR/Flk-1, kinase insert domain-containing receptor/fetal liver kinase; VEGFR, VEGF receptor; HUVECs, human umbilical veins endothelial cells; MPD, 2-methyl-2,4-pentanediol; r.m.s., root mean square.

TABLE I
*Crystallographic statistics*

| Dataset | PlGF-1 |
|---|---|
| **Data collection statistics** | |
| Unit cell dimensions ($P4_3$, 1 homodimer/a.u.) | a = b = 62.6 Å, c = 84.1 Å |
| Resolution (Å) | 40–2.0 |
| Reflections measured | 161,044 |
| Unique reflections | 21,067 |
| $R_{sym}$ (%)[a] | 6.3 |
| I/σI (outermost shell) | 19.6 (3.6) |
| Completeness (outermost shell) (%) | 99.4 (98.8) |
| **Refinement statistics** | |
| $R_{cryst}$ (%)[b] | 21.6 |
| $R_{free}$ (%)[c] | 24.7 |
| Number of protein atoms (homodimer) | 1,546 |
| Number of solvent molecules (homodimer) | 132 |
| R.m.s. deviation in bond lengths (Å) | 0.010 |
| R.m.s. deviation in bond angles (°) | 1.5 |
| Average B-factor for protein atoms (Å$^2$) | 32.6 |
| Average B-factor for main-chain atoms (Å$^2$) | 32.6 |
| Average B-factor for side-chain atoms (Å$^2$) | 32.7 |
| Average B-factor for solvent molecules (Å$^2$) | 44.8 |
| B-factor (from Wilson plot) (Å$^2$) | 33.9 |

[a] $R_{sym} = \Sigma(|I_j - \langle I \rangle|)/\Sigma\langle I \rangle$ where $I_j$ is the observed intensity of reflection j, and $\langle I \rangle$ is the average intensity of multiple observations.

[b] $R_{cryst} = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes, respectively.

[c] $R_{free}$ is equal to $R_{cryst}$ for a randomly selected 4% subset of reflections not used in the refinement.

a closer homolog of PlGF in its receptor-binding profile (29). Purified heterodimeric VEGF/PlGF has been shown also to bind KDR/Flk-1 (22). The extracellular portion of both receptors consists of seven immunoglobulin (IgG)-like domains, and the receptors share 44% amino acid sequence homology. The IgG-like domain 2 of the Flt-1 receptor is responsible for the binding specificity of PlGF-1 and -2 (30–32). Furthermore, it has been reported that only PlGF-2 can recognize neuropilins-1 and -2, receptor molecules found at the endothelial surface, in a heparin-dependent fashion (33, 34).

Since PlGF has been shown to bind and induce autophosphorylation of Flt-1 but not KDR/Flk-1, it appears that PlGF should exert its mitogenic and chemotactic effects on ECs through the activation of the Flt-1 intracellular signaling pathway. PlGF induces DNA synthesis but not migration of porcine aortic ECs (PAE) overexpressing Flt-1 (28). However, recent findings that PlGF is mitogenic and chemotactic for CVECs and HUVECs *in vitro* (25) (discussed above), raise the question of whether PlGF induces Flt-1 directly to transduce mitogenic and chemotactic signals inside the cell or whether PlGF acts indirectly through a mechanism of decoy, as previously proposed by Park *et al.* (14).

The recent observation that Flt-1 is able to mediate signaling in HUVECs in response to both PlGF and VEGF, leading to distinct biological responses, suggests that Flt-1 does not act as

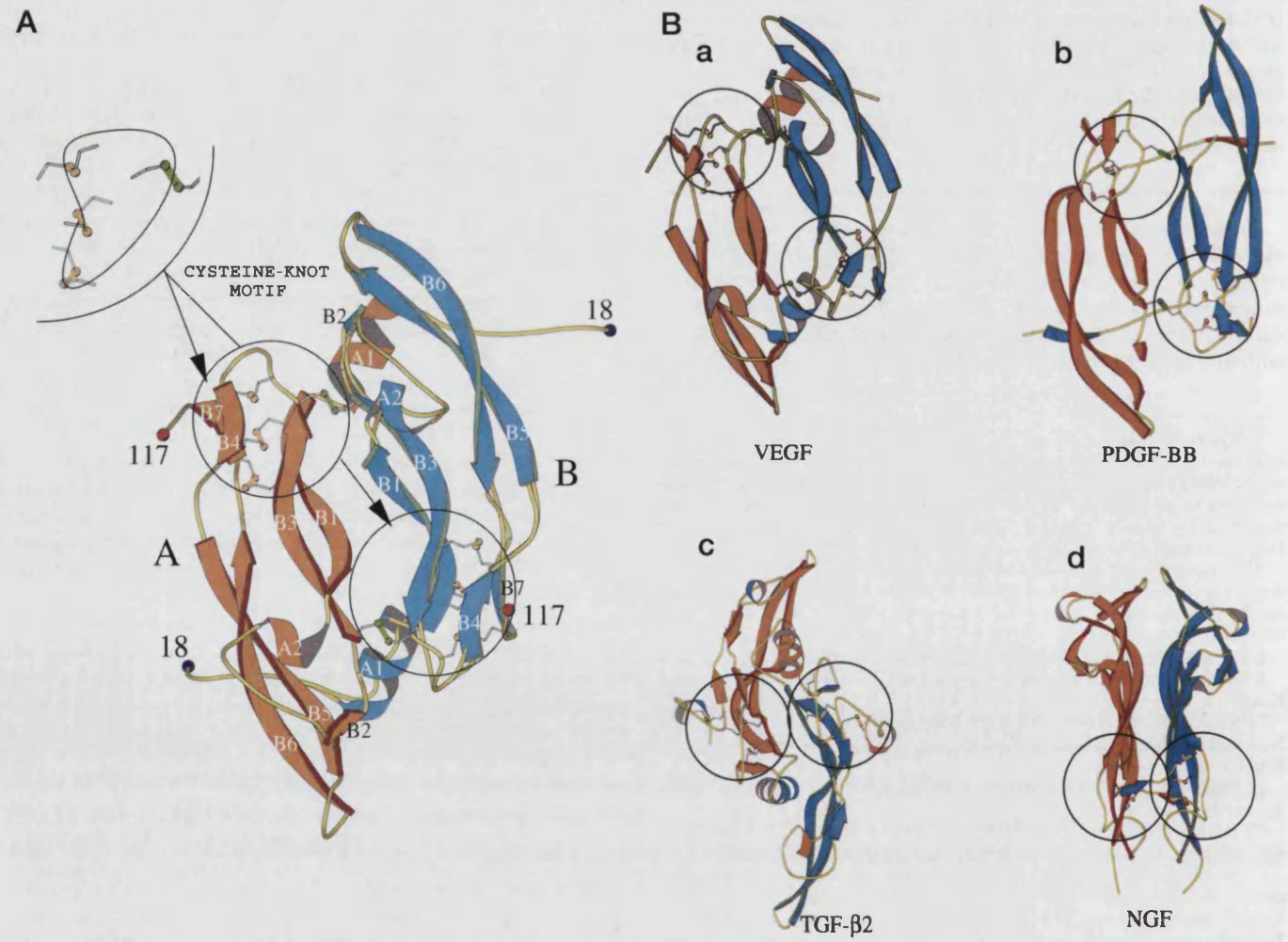


FIG. 1. **Structural comparison of PlGF-1 and other members of the cysteine-knot super family.** *A*, representation of the PlGF-1 homodimer structure. Disulfide bonds are shown in a *ball-and-stick* representation. The *inset* presents the organization of three intra- (in *yellow*) and one interdisulfide bridge (in *green*) in the cysteine-knot motif. Each monomer in the homodimer is colored differently to enhance clarity. *Orange*, monomer A; *cyan*, monomer B. *B*, representatives of known structures from the cysteine-knot protein family of dimeric molecules. *a*, VEGF (PDB code 2VPF, Ref. 39); *b*, PDGF-BB (PDB code 1PDG, Ref. 51); *c*, TGF-β2 (PDB code 1TFG, Ref. 52); and *d*, NGF (PDB code 1BTG, Ref. 53). With the exception of NGF, the homodimer 2-fold axis is perpendicular to the plane of the β-sheet. The cysteine knots are *highlighted*. *C*,

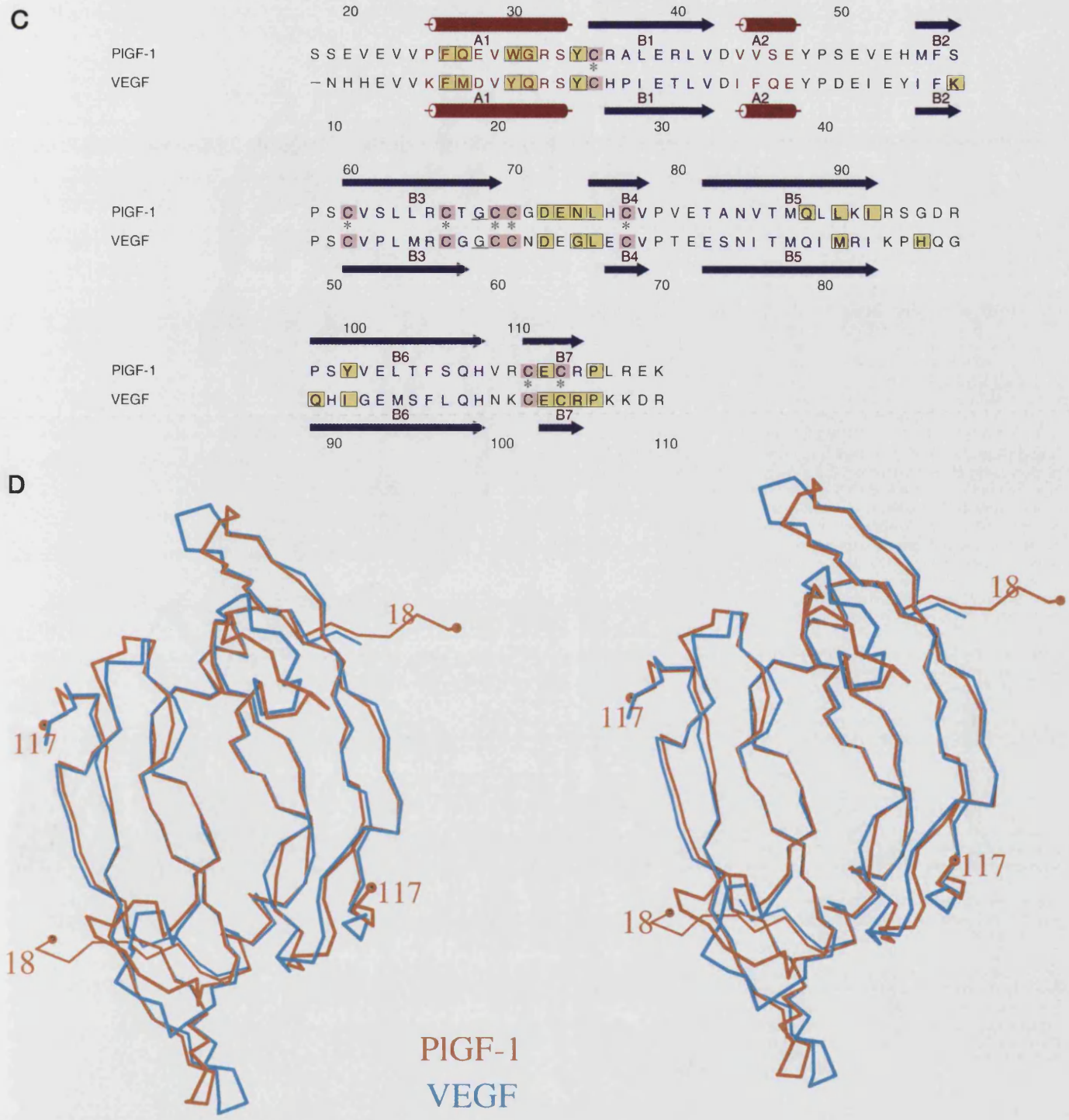


FIG. 1—*continued*

a decoy receptor but is indeed able to signal intracellularly (35). Inhibition of PlGF translation by antisense mRNA in the human dermal microvascular endothelial cells in culture results in the inhibition of cell proliferation under hypoxic conditions (36). These new findings assign a role to PlGF in the direct control of endothelial cell proliferation, probably competing with VEGF for binding to Flt-1 and thereby forcing the binding of VEGF to the KDR/Flk-1 and activating cell proliferation. In addition, both PlGF and VEGF are able to induce migration of 39% and 51% of monocytes, respectively, through activation of Flt-1 (35, 39). This suggests that PlGF may induce EC migration and proliferation through activation of Flt-1, although the existence of a yet unknown PlGF receptor cannot be ruled out.

A considerable amount of structural information is now

structure-based sequence alignment of PlGF-1 with VEGF (38, 39). Amino acid residues that form part of the secondary structural elements ($\beta$-strands and helices) as determined by DSSP (60) are shown in *blue* and *red*, respectively. The cysteine residues are shaded *pink*. VEGF residues involved in Flt-1 (VEGFR-1) binding (40), and the equivalent residues in PlGF-1 (based on a modeling study) are *boxed* and shaded in *yellow*. The conserved glycine residue in both structures is *underlined*. This figure was created with the program ALSCRIPT (61). *D*, stereo view displaying the C$\alpha$ traces of PlGF-1 (*orange*) and VEGF (*cyan*) (39) homodimers after alignment of the two structures with the program "O" (49). *A*, *B*, and *D* were created with the program MOLSCRIPT (59).

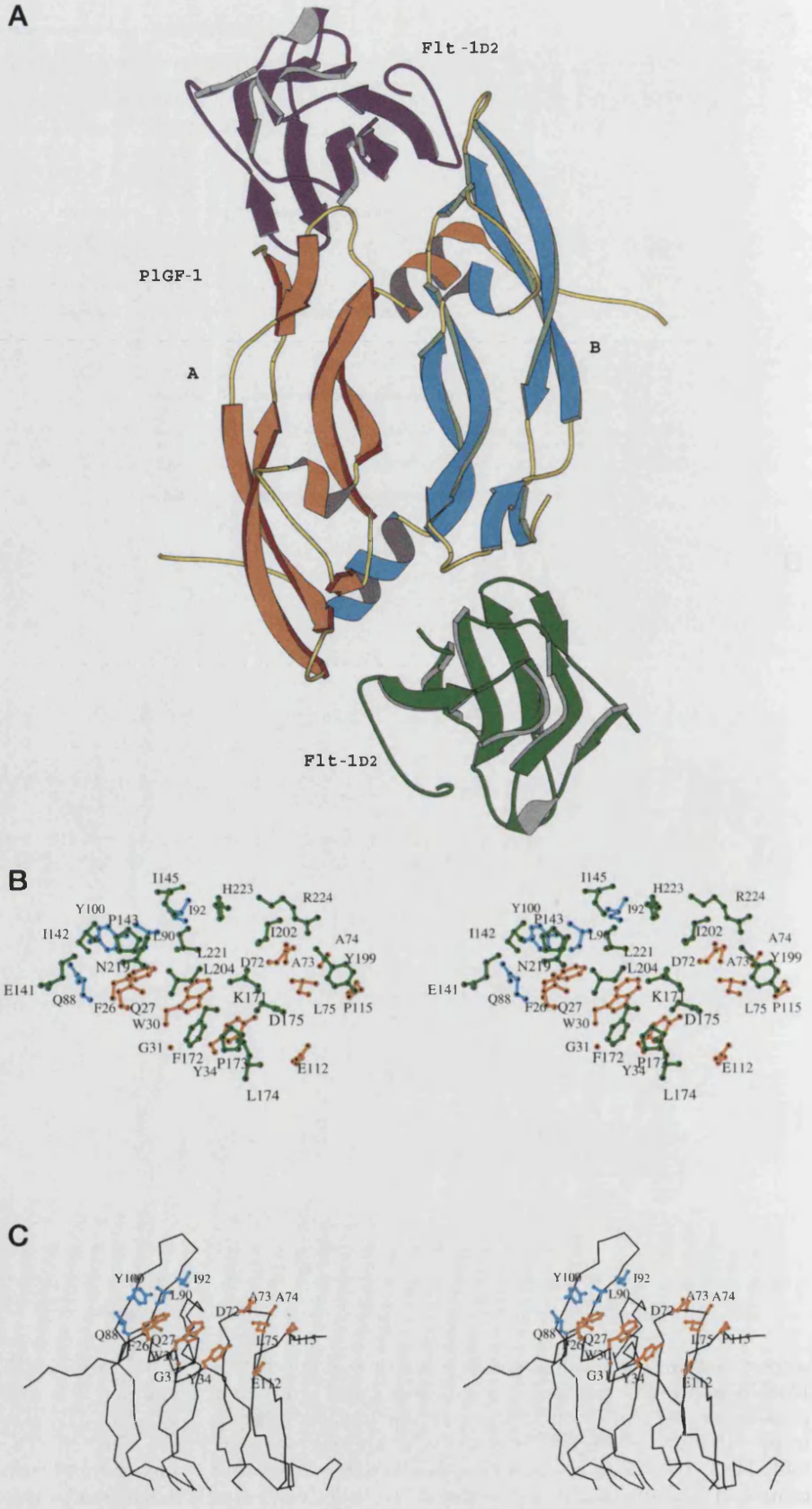


FIG. 2. **Proposed model for the PlGF-1·Flt-$1_{D2}$ complex based on the crystal structures of PlGF-1 (present study) and the VEGF·Flt-$1_{D2}$ complex (40), PDB accession code 1FLT.** *A*, PlGF-1 homodimer is shown in *orange* (molecule A) and *cyan* (molecule B), whereas the Flt-$1_{D2}$ molecules are shown in *purple* and *green*. *B*, stereo views of contact residues (Cα atoms plus sidechain atoms) at the putative PlGF-1·Flt-$1_{D2}$ interface. Residues from PlGF-1 monomers A and B are marked in *orange* and *cyan*, respectively. Residues from Flt-1 (figure based on model shown in *A*) are colored in *green*. The sidechains for $Glu^{73}$ and $Asn^{74}$ in free PlGF-1 are disordered and hence are treated as alanines. *C*, stereo views of contact residues (Cα atoms plus sidechain atoms) for PlGF-1. Residues from monomer A and B are marked in *orange* and *cyan*, respectively (figure based on model shown in *A*). The sidechains for $Glu^{73}$ and $Asn^{74}$ in free PlGF-1 are disordered and hence are treated as alanines. *D*, stereo views of contact residues (Cα atoms plus sidechain atoms) for Flt-$1_{D2}$ (figure based on model shown in *A*). *E*, stereo views showing the location of the groove in PlGF-1 (residues $Asp^{72}$, $Glu^{73}$, $Val^{52}$, $Met^{55}$, $Val^{45}$, $Asp^{43}$, and $Ser^{59}$) and VEGF ($Asp^{63}$, $Glu^{64}$, $Ile^{43}$, $Ile^{46}$, $Phe^{36}$, $Asp^{34}$, and $Ser^{50}$), implicated for recognition of domain 3 of Flt-1 (40). The figure also shows the difference in conformation for segment 90–95 in PlGF-1 and 81–86 in VEGF. Residues $Ile^{83}$, $Lys^{84}$, and $Pro^{85}$ are implicated in KDR recognition in VEGF (38), and the corresponding residues in PlGF-1 are $Ile^{92}$, $Ser^{94}$, and $Arg^{93}$. The sidechains for PlGF-1 and VEGF are shown in *orange* and *cyan*, respectively. $Ser^{94}$ and $Glu^{73}$ in the PlGF-1 structure are represented as alanines because of insufficient electron density beyond the Cβ atom. *A–E* was generated using MOLSCRIPT (59).

available for VEGF (VEGF-A). Muller *et al.* (38, 39) reported the crystal structures of the receptor binding domain of VEGF in different crystal forms and have identified the KDR binding site using mutational analysis. Also, Wiesmann *et al.* (40) have reported the crystal structure of VEGF in complex with domain 2 of Flt-1 (Flt-$1_{D2}$). To understand the specific molecular details of the receptor binding site and critical components of the homodimer, which will consequently help in understanding the differences in specificity and cross-reactivity among the VEGF homologs, we have embarked on a three-dimensional structural study of PlGF. Here we report the crystal structure of PlGF-1 at 2.0 Å resolution. As anticipated, the structure is

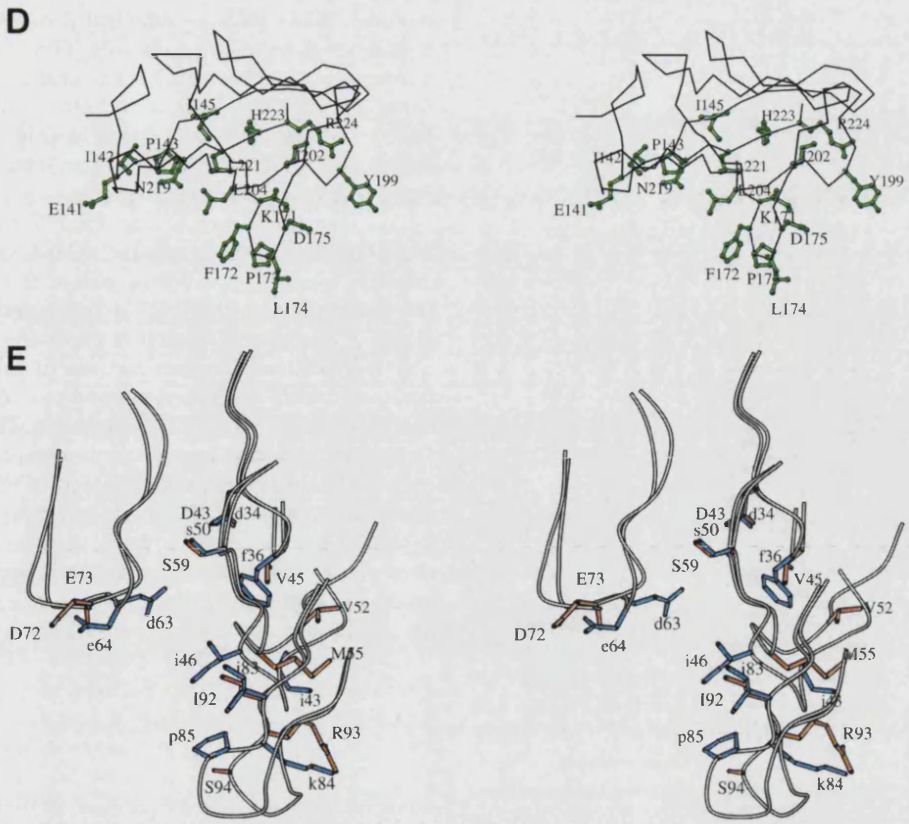


FIG. 2—*continued*

similar to that of VEGF. However, it shows subtle differences in molecular interactions at the receptor recognition site that appear to be relevant to signaling.

## EXPERIMENTAL PROCEDURES

*Protein Expression and Purification*—By polymerase chain reaction, the region of the human *PlGF-1* gene coding for the mature protein was cloned into a prokaryotic expression vector as described previously (11). The recombinant vector was used to transform a DE3 *Escherichia coli* strain, and the synthesis of PlGF-1 was induced by 1 mM isopropyl-2-D-thiogalactopyranoside. After preparation and refolding of the inclusion bodies, the PlGF-1 protein was purified first by anion exchange chromatography followed by reverse phase chromatography. Final recovery of the active protein was about 140 mg per liter of initial bacterial culture. The identity of the protein was checked by various assays such as immunoblotting, SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions, two-dimensional electrophoresis, reverse phase chromatography, and amino-terminal sequencing. The angiogenic activity was tested using a CAM assay (41); the purified bacterial-derived PlGF-1 was able to induce a strong and dose-dependent angiogenic response (42).

*Crystallization*—Crystals of recombinant PlGF-1 were grown using the hanging drop vapor diffusion method from drops containing 8 mg/ml protein at pH 6.0 in 0.05 M MES buffer, 10 mM $CaCl_2$ and 7.5% (v/v) 2-methyl-2,4-pentanediol (MPD) equilibrated against reservoirs containing 0.1 M MES buffer (pH 6.0), 20 mM $CaCl_2$ and 15% (v/v) MPD. Single crystals appeared after 5–6 days at 16 °C. These crystals could be flash-frozen at 100 K using a cryoprotectant solution containing 0.1 MES buffer (pH 6.0), and 30% (v/v) MPD. The systematic absences and symmetry were consistent with the tetragonal space group $P4_1$ or $P4_3$, with unit cell dimensions a = b = 62.6 Å, and c = 84.1 Å. There was one PlGF-1 homodimer per crystallographic asymmetric unit and ~50% of the crystal volume was occupied by solvent.

*Data Processing and Reduction*—X-ray diffraction data to 2.0 Å were collected at 100 K from a single crystal using the Synchrotron Radiation Source (station PX 9.5) at Daresbury (United Kingdom). Seventy images were collected ($\lambda$ = 1.0 Å, oscillation range of 1.5°, 45 s exposure time) using a MAR-CCD detector system. Data processing was performed with the HKL package (43). Data reduction was carried out using the program TRUNCATE of the CCP4 suite (44). Details of data processing statistics are presented in Table I.

*Phasing*—The structure of PlGF-1 was determined by molecular replacement with the program AMoRe (45) using a polyalanine (homodimer) model based on the structure of VEGF at 1.93 Å resolution (PDB code 2VPF, Ref. 39). Data in the range 15.0–3.0 Å were used for both the rotation and the translation function searches. No solution was found in space group $P4_1$. In space group $P4_3$, the best solution after FITING had a correlation coefficient of 56% and an *R*-factor of 51%. Rigid-body refinement with CNS version 0.9 (46) of this model corresponding to the highest peak using data in the range 40.0–2.0 Å, resulted in an $R_{free}$ and $R_{cryst}$ of 44.6 and 40.6%, respectively.

*Refinement*—All crystallographic refinement was carried out using the program CNS version 0.9 (46). Procedures carried out with CNS included simulated annealing using a maximum likelihood target function, restrained individual B-factor refinement, conjugate gradient minimization, and bulk solvent correction. The behavior of the $R_{free}$ value (811 reflections) was monitored throughout refinement. Several rounds of refinement (using all reflections) and model building were performed until the $R_{free}$ for the model could not be improved any further. During the final stages of refinement, water molecules were inserted into the model at positions where peaks in the $|F_o|-|F_c|$ electron density maps had heights greater than $3\sigma$ and were at hydrogen bond forming distances from appropriate atoms. $2\,||F_o|-|F_c||\,\phi_{calc}$ maps were also used to verify the consistency in peaks. Water molecules with a temperature factor greater than 65 $Å^2$ were excluded from the model and subsequent refinement. One bound MPD molecule per monomer from the crystallization medium was identified (interacting with the main-chain carbonyl oxygen atom of Thr-104 at one end and a water molecule at the other end) and was included in the final stages of the refinement. The details of refinement are presented in Table I. Map calculations were performed with CNS with the SigmaA protocol (47), using all the reflections in the resolution range 40.0–2.0 Å. The program PROCHECK (48) was used to assess the quality of the final structure. Analysis of the Ramachandran ($\varphi$-$\psi$) plot showed that all residues lie in the allowed regions. The program "O" (49) was used for map visualization and model building.

*Accession Number*—Final atomic coordinates of human PlGF-1 have been deposited with the RCSB Protein Data Bank under the accession code 1FZV.

TABLE II
*Hydrogen bond interactions between the two monomers at the dimer interface in the PlGF-1 structure*

| Molecule A | Molecule B | Distance (Å) |
|---|---|---|
| $Val^{24}$–N | $Thr^{86}$–O | 2.89 |
| $Val^{24}$–O | $Gln^{88}$–N | 2.89 |
| $Phe^{26}$–N | $Gln^{88}$–Oε1 | 3.06 |
| $Arg^{32}$–Nε | $Glu^{39}$–Oε1 | 2.79 |
| $Arg^{32}$–Nη2 | $Glu^{39}$–Oε2 | 3.22 |
| $Ser^{33}$–Oγ | $Cys^{60}$–O | 2.78 |
| $Arg^{36}$–Nη2 | $Glu^{39}$–Oε1 | 2.45 |
| $Glu^{39}$–Oε1 | $Arg^{32}$–Nε | 3.02 |
| $Glu^{39}$–Oε1 | $Arg^{36}$–Nη1 | 2.77 |
| $Glu^{39}$–Oε2 | $Arg^{36}$–Nη2 | 3.26 |
| $Cys^{60}$–O | $Ser^{33}$–Oγ | 2.78 |
| $Thr^{86}$–O | $Val^{24}$–N | 2.89 |
| $Gln^{88}$–N | $Val^{24}$–O | 2.88 |
| $Gln^{88}$–Oε1 | $Phe^{26}$–N | 2.86 |

## RESULTS AND DISCUSSION

*Quality of the Structure*—The crystal structure of PlGF-1 was determined at 2.0 Å resolution. Details of the data collection and refinement statistics are shown in Table I. The protein crystallizes as a homodimer in the asymmetric unit. As in the VEGF structure (38), the first 17 amino-terminal residues of both monomers are not visible in the electron density map and were excluded from crystallographic refinement. Both monomers A and B contain residues 18–117. Also, residues $Ser^{18}$, $Glu^{51}$, $Glu^{73}$, $Asn^{74}$, and $Ser^{94}$ in both molecules and residues $Glu^{53}$ and $Arg^{117}$ in molecule A have been modeled as alanines because of lack of sufficient density beyond Cβ atoms. The arrangement of the homodimer and the nomenclature used throughout the text are shown in Fig. 1*A*. The final model (homodimer) includes 1,546 non-hydrogen protein atoms, 132 water molecules, and two MPD molecules with a crystallographic *R*-factor ($R_{cryst}$) of 21.6% in the resolution range 40.0–2.0 Å. The $R_{free}$ value is 24.7% with 4% of the reflections excluded from the refinement (50). The mean coordinate error calculated from a plot of ln $\sigma_A$ *versus* $(\sin\theta/\lambda)^2$ is 0.3 Å. The root mean square (r.m.s.) deviation in Cα atoms between each monomer of the pair is 0.43 Å (for 100 Cα atoms). Regions that deviate most include residues 18–19 from the amino-terminal tail, part of the loop connecting strands β3 and β4 (residues 72–73), and the carboxyl-terminal residue 117. Excluding these residues improves the r.m.s deviation to 0.17 Å (for 94 Cα atoms). Examination of the Ramachandran plot shows 91.5% of the residues in most favorable regions and no residues in disallowed regions.

*Overall Structure*—The crystal structure of PlGF-1 consists of a homodimer, organized in an antiparallel arrangement with the 2-fold axis perpendicular to the plane of the β-sheet (Fig. 1*A*). The homodimer is covalently linked by two interchain disulfide bonds between $Cys^{60}$ and $Cys^{69}$. The most prominent feature of the structure is the presence of a cysteine-knot motif, positioned symmetrically opposite at one end of each monomer. This motif is found in other closely related growth factors such as VEGF (38, 39), platelet-derived growth factor-BB (PDGF-BB, Ref. 51), transforming growth factor-β2 (TGF-β2, Ref. 52) and nerve growth factor (NGF, Ref. 53) (Fig. 1*B*). The knot consists of an eight residue ring formed by one interchain ($Cys^{60}$–$Cys^{69}$) and three intrachain ($Cys^{35}$–$Cys^{77}$, $Cys^{66}$–$Cys^{111}$, $Cys^{70}$–$Cys^{113}$) disulfide bonds (Fig. 1*A*). The ring structure is formed between two adjacent β-strands, β3 and β7, with the third intrachain disulfide bond penetrating the covalent linkage and connecting strands β1 and β4. The cysteine ring contains a conserved glycine residue at position 68, which seems to be important in optimizing the conformation of the sidechains in the knot. As in the VEGF structure (38, 39), this residue adopts positive dihedral φ angles of 141 and 149° in monomers A and B, respectively. Thus the cysteine-knot motif appears to be important for the stabilization of the dimer as there are only a few contacts between the β-strands (β1 and β1′) at the dimer center. One peptide bond in the PlGF-1 structure adopts a *cis* conformation: that connecting $Ser^{57}$ and $Pro^{58}$ in both monomers.

The structural core of the PlGF-1 monomer consists of a four-stranded, highly irregular, solvent-accessible β-sheet (Fig. 1*A*). The total buried surface area at the interface between the two monomers is 2,627 Å$^2$. A considerable proportion of this (1,830 Å$^2$ or 69%) is accounted for by the extensive intermolecular hydrophobic core interactions at the interface on the opposite end of the cysteine-knot and provides additional stability to the central portion of the structure. The hydrophobic core is formed by residues from both monomers and is known to be part of the receptor binding region of PlGF-1 (see under "Receptor Recognition"). Fourteen potential hydrogen bond interactions were observed between the two monomers (Table II). Two water-mediated hydrogen bonds between $Glu^{39}$ from each monomer forms a bridge between two strands (β1 and β1′) across the center of the dimer interface.

*Comparison with VEGF Structure*—Overall, the structure of PlGF-1 exhibits remarkable topological identity with that of VEGF (38, 39) (with which it has 42% amino acid sequence identity) despite significant functional diversity (Fig. 1, *B—D*, r.m.s deviation of 1.47 Å using 95 Cα atoms). The mode of dimerization for PlGF-1 is similar to that of VEGF. Conformational differences between PlGF-1 and VEGF are observed at the amino-terminal residues (18–25), some residues from loop regions (loops connecting β3–β4, β5–β6, and α2–β2) and the carboxyl-terminal residues (116–117). Interestingly, these loop regions appear to be part of the receptor-binding face in both molecules (see below). Approximately 70 water molecules are conserved in PlGF-1 and VEGF and appear to be important for the structural integrity of the homodimer in both molecules.

*Receptor Recognition*—The extracellular domain of both KDR and Flt-1 receptors consist of seven immunoglobulin domains. Mutational analysis of VEGF has revealed that symmetrical binding sites for KDR are located at each pole of the VEGF homodimer (38). Each site appears to contain two functional regions composed of binding determinants presented across the intermolecular interface. This experimental evidence suggested that only a small number of VEGF residues are important for binding to KDR, and the binding epitope for KDR contains two hot-spots, each of which extends across the dimer interface (39, 54–56). Furthermore, analysis of the conformational variability of VEGF (based on the high resolution structure of VEGF, Ref. 39) showed that the loop connecting strands β5 to β6 undergoes a concerted movement. This loop is important for binding to both Flt-1 and KDR, suggesting that these receptor molecules have overlapping binding sites on the target molecule. It has also been established that minimally domains 2 and 3 of Flt-1 are necessary and sufficient for binding VEGF with near native affinity, and domain 2 alone binds to VEGF (60-fold less tightly than wild-type, Ref. 38). Similar results have been found for deletions in the KDR (56).

Recently, the crystal structure of VEGF in complex with Flt-$1_{D2}$ (at 1.7 Å) has revealed that domain 2 is predominantly involved in hydrophobic interactions with the poles of the VEGF dimer (40). Based on this structure and previous mutagenesis data, Wiesmann *et al.* (40) have proposed a model of VEGF bound to the first four domains of Flt-1. In the case of PlGF, it has been shown that binding of PlGF to human ECs revealed a high affinity site and a low affinity site (35, 37). The high affinity site is for Flt-1 and PlGF can displace VEGF from

TABLE III
*Putative intermolecular contacts*

Putative intermolecular contacts between PlGF-1 and Flt-$1_{D2}$ at the receptor interface in the modeled complex (left) and intermolecular contacts between VEGF and Flt-$1_{D2}$ at the receptor interface (right) as observed from the crystal structure of VEGF · Flt-1 complex (40).

| PlGF-1 | Flt-$1_{D2}$ Polar (< 3.4 Å) | Distance | VEGF | Flt-$1_{D2}$ Polar (< 3.4 Å) | Distance |
|---|---|---|---|---|---|
| | | Å | | | Å |
| Molecule A | | | Molecule A | | |
| $Gln^{27}$–Oε1 | $Asn^{219}$–Nδ2 | 3.15 | $Asp^{63}$–Oδ1 | $Arg^{224}$–Nη2 | 2.54 |
| $Asp^{72}$–Oδ1 | $Arg^{224}$–Nε | 3.33 | $Asp^{63}$–Oδ2 | $Arg^{224}$–Nε | 2.85 |
| $Asp^{72}$–Oδ1 | $Arg^{224}$–Nη2 | 3.18 | $His^{86}$–Nδ1 | $Gln^{225}$–Nε2 | 2.84 |
| $Ala^{74}$–N | $Arg^{224}$–Nη2 | 3.12 | $Gln^{89}$–Nε2 | $His^{147}$–Nε2 | 3.40 |
| | | | $Arg^{105}$–Nη1 | $Tyr^{199}$–OH | 3.31 |
| Molecule B | | | Molecule B | | |
| $Tyr^{34}$–OH | $Lys^{171}$–Nζ | 3.33 | $Tyr^{25}$–OH | $Lys^{171}$–Nζ | 3.35 |
| $Asp^{72}$–Oδ1 | $Arg^{224}$–Nε | 3.04 | $Lys^{48}$–Nζ | $His^{223}$–Nδ1 | 3.22 |
| $Tyr^{100}$–OH | $Pro^{143}$–O | 3.19 | $Arg^{105}$–Nη1 | $Tyr^{199}$–OH | 3.23 |
| $Glu^{112}$–Oε2 | $Asp^{175}$–Oδ1 | 3.03 | $Arg^{105}$–Nη2 | $Tyr^{199}$–OH | 3.00 |
| | | | $Asp^{63}$–Oδ1 | $Arg^{224}$–Nη2 | 2.74 |
| | | | $Asp^{63}$–Oδ1 | $Arg^{224}$–Nε | 3.33 |
| | | | $Asp^{63}$–Oδ2 | $Arg^{224}$–Nε | 2.68 |

| PlGF-1 | Flt-$1_{D2}$ van der Waals contacts[a] | Contacts | VEGF | Flt-$1_{D2}$ van der Waals contacts[a] | Contacts |
|---|---|---|---|---|---|
| | | Å | | | Å |
| Mol A | | | Mol A | | |
| $Phe^{26}$ | $Pro^{143}$(2), $Leu^{221}$(2)[b] | 4 | $Phe^{17}$ | $Ile^{142}$, $Pro^{143}$(3) | 4 |
| $Gln^{27}$ | $Pro^{143}$(2), $Leu^{204}$(3), $Asn^{219}$(3) | 8 | $Met^{18}$ | $Glu^{141}$, $Leu^{204}$ | 2 |
| $Trp^{30}$ | $Leu^{221}$ | 2 | $Tyr^{21}$ | $Gly^{203}$, $Leu^{204}$(6) | 7 |
| $Tyr^{34}$ | $Lys^{171}$, $Phe^{172}$(4), $Pro^{173}$(2) | 7 | $Gln^{22}$ | $Phe^{172}$ | 4 |
| $Asp^{72}$ | $Tyr^{199}$, $Ile^{202}$(8), $Arg^{224}$ | 10 | $Tyr^{25}$ | $Lys^{171}$, $Phe^{172}$, $Pro^{173}$(2) | 4 |
| $Ala^{73c}$ | $Arg^{224}$ | 2 | $Lys^{48}$ | $Leu^{221}$, $His^{223}$ | 2 |
| $Ala^{74c}$ | $Arg^{224}$ | 3 | $Asp^{63}$ | $Ile^{202}$(2), $Arg^{224}$(8) | 10 |
| $Leu^{75}$ | $Tyr^{199}$ | 5 | $Gly^{65}$ | $Arg^{224}$ | 1 |
| $Gln^{88}$ | $Ile^{142}$ | 1 | $Leu^{66}$ | $Tyr^{199}$ | 1 |
| $Leu^{90}$ | $Ile^{145}$ | 1 | $Met^{81}$ | $Leu^{221}$ | 1 |
| $Tyr^{100}$ | $Ile^{142}$ | 3 | $His^{86}$ | $Gln^{225}$ | 6 |
| $Glu^{112}$ | $Leu^{174}$ | 1 | $Gln^{89}$ | $His^{147}$ | 1 |
| $Pro^{115}$ | $Tyr^{199}$ | 12 | $Ile^{91}$ | $Ile^{142}$ | 2 |
| | | | $Cys^{104}$ | $Tyr^{199}$ | 2 |
| | | | $Pro^{106}$ | $Tyr^{199}$ | 7 |
| Mol B | | | Mol B | | |
| $Phe^{26}$ | $Pro^{143}$(4), $Leu^{221}$(2) | 6 | $Phe^{17}$ | $Ile^{142}$, $Pro^{143}$(2), $Leu^{221}$ | 4 |
| $Gln^{27}$ | $Glu^{141}$(3), $Ile^{142}$, $Pro^{143}$(9), $Leu^{204}$, $Asn^{219}$(3) | 17 | $Met^{18}$ | $Asn^{219}$ | 1 |
| $Trp^{30}$ | $Leu^{204}$ | 1 | $Tyr^{21}$ | $Gly^{203}$(2), $Leu^{204}$(6), $Leu^{221}$ | 9 |
| $Gly^{31}$ | $Phe^{172}$ | 2 | $Gln^{22}$ | $Phe^{172}$ | 8 |
| $Tyr^{34}$ | $Phe^{172}$(10), $Pro^{173}$(2) | 12 | $Tyr^{25}$ | $Phe^{172}$ | 1 |
| $Asp^{72}$ | $Ile^{202}$, $Arg^{224}$(9) | 10 | $Asp^{63}$ | $Arg^{224}$ | 8 |
| $Asn^{74}$ | $Arg^{224}$ | 3 | $Gly^{65}$ | $Arg^{224}$ | 2 |
| $Leu^{75}$ | $Tyr^{199}$ | 3 | $Leu^{66}$ | $Arg^{224}$ | 2 |
| $Gln^{88}$ | $Ile^{142}$ | 2 | $Met^{81}$ | $Pro^{143}$ | 1 |
| $Leu^{90}$ | $Ile^{145}$ | 2 | $Gln^{89}$ | $Ile^{145}$, $His^{147}$ | 2 |
| $Ile^{92}$ | $His^{223}$ | 5 | $Ile^{91}$ | $Ile^{142}$ | 5 |
| $Tyr^{100}$ | $Ile^{142}$(10), $Pro^{143}$(2) | 12 | $Glu^{103}$ | $Lys^{200}$ | 1 |
| $Glu^{112}$ | $Asp^{175}$ | 5 | $Cys^{104}$ | $Tyr^{199}$ | 2 |
| $Pro^{115}$ | $Tyr^{199}$ | 9 | $Arg^{105}$ | $Tyr^{199}$, $Lys^{200}$(2) | 3 |
| | | | $Pro^{106}$ | $Tyr^{199}$ | 3 |

[a] van der Waals distances are the maximum allowed values of C–C, 4.1Å; C–N, 3.8Å; C–O, 3.7Å; O–O, 3.3Å; O–N, 3.4Å; and N–N, 3.4Å.
[b] Numbers in parentheses represent the number of contacts made with the indicated Flt-1 residue. Hydrogen bond parameters and van der Waals contacts were calculated with the program CONTACT (44).
[c] $Glu^{73}$ and $Asn^{74}$ are modeled as alanines due to insufficient electron density for the sidechain atoms in the free PlGF-1 structure.

both truncated and full-length Flt-1 receptors. However, at the present time it is yet be established whether both PlGF-1 and VEGF bind identically to Flt-1.

*Flt-1 (VEGFR-1) Receptor Interactions*—The structure reported here for PlGF-1 is an unliganded structure and hence it is not possible to establish the precise nature of the interaction of PlGF-1 with Flt-1. However, using the structural data on the VEGF·Flt-$1_{D2}$ complex, we have been able to construct a model to visualize the binding mode between PlGF-1 and Flt-1. The PlGF-1·Flt-$1_{D2}$ complex was modeled by superimposing the atomic coordinates of the VEGF·Flt-$1_{D2}$ complex (Ref. 40, PDB code 1FLT) onto the PlGF-1 model followed by energy minimization using the program X-PLOR (57). The resultant model showed a reasonable fit between PlGF-1 and Flt-1 without any obvious stereochemical impediments between the two proteins (Fig. 2*A*). The interface of the putative PlGF-1·Flt-$1_{D2}$ complex appears to include some 22 amino acids from the PlGF-1 molecule: residues from the α1 helix, β3-β4 loop, and β7 strand of one monomer, and residues from strands β5, β6, and the β5-β6 loop of the second monomer. In the modeled complex, nineteen residues from the Flt-$1_{D2}$ segments 141–147, 171–175, 199–204, and 219–226 form part of this contact surface. Modeling studies based on the VEGF·Flt-$1_{D2}$ complex structure (40) predict that binding between PlGF-1 and Flt-$1_{D2}$ might also be mediated through hydrophobic interactions involving planar surfaces from both the ligand and the receptor (Table III). Such

shape complementarity is energetically favorable for maximizing the contribution of van der Waals contacts.

Based on the PlGF-1·Flt-$1_{D2}$ model, we speculate that both PlGF-1 and Flt-$1_{D2}$ form extensive contacts through sidechain interactions (Table III, Fig. 2*B*). The contact residues from the two individual components of the modeled complex are shown in Fig. 2, *C* and *D*. $Asp^{72}$ in PlGF-1 appears to be the only residue to make direct H-bond interactions with $Arg^{224}$ of Flt-$1_{D2}$. (In the VEGF·Flt-$1_{D2}$ complex structure, the conserved VEGF residue $Asp^{63}$ makes similar interactions with Flt-1, Ref. 40, Table III.) PlGF-1 residues $Gln^{27}$, $Tyr^{34}$, $Ala^{74}$, $Tyr^{100}$, and $Glu^{112}$ in one molecule (either A or B) are predicted to make both polar and van der Waals interactions with Flt-1, whereas residues $Phe^{26}$, $Trp^{30}$, $Gly^{31}$, $Glu^{73}$, $Leu^{75}$, $Gln^{88}$, $Leu^{90}$, $Ile^{92}$, and $Pro^{115}$ seem to participate in van der Waals interactions with Flt-1 residues (Table III). Additional PlGF-1 residues $Pro^{25}$, $Cys^{70}$, $Gly^{71}$, $Pro^{98}$, $Cys^{111}$, $Cys^{113}$, and $Arg^{114}$ also appear to be part of the interface.

A general mechanism of Flt-1 recognition by PlGF-1 can be postulated based on the proposed dimeric model of the receptor binding domain of VEGF in complex with domains 1–4 of Flt-1 (40). A similar picture may be visualized for PlGF-1·Flt-1 recognition with domain-1 pointing away from PlGF-1, the domain 2–3 linker region occupying the groove (6.8 Å wide) between the two monomers, and domain 3 making contact with its bottom face, which would bring domain 4 into direct inter-receptor contacts and hence involved in dimer formation. In the PlGF-1 structure, the walls of the groove are formed by residues $Asp^{72}$, $Glu^{73}$, $Val^{52}$, $Met^{55}$, $Val^{45}$, $Asp^{43}$, and $Ser^{59}$. The corresponding groove in VEGF was formed by residues $Asp^{63}$, $Glu^{64}$, $Ile^{43}$, $Ile^{46}$, $Phe^{36}$, $Asp^{34}$, and $Ser^{50}$, which was implicated for recognition of domain 3 of Flt-1 (40). This comparison illustrates a high degree of conservation of residues in this region between the two molecules (Fig. 1*C*). However, at the structural level, one can visualize significant changes in conformation (Fig. 2*E*), which could be one of the contributing factor for the distinct receptor specificity for PlGF-1 (see below).

*Lack of KDR (VEGFR-2) Recognition*—A detailed mutagenesis study of VEGF by Muller *et al.* (38) identified eight amino acid residues forming part of the KDR binding site. These were grouped as major and minor hot-spots for receptor recognition. The major hot-spot consists of two important residues $Ile^{46}$ and $Ile^{83}$ (VEGF numbering) and four additional residues $Ile^{43}$, $Glu^{64}$, $Lys^{84}$, and $Pro^{85}$ with slightly lesser importance. The minor hot-spot contains residues $Phe^{17}$ and $Gln^{79}$ (38). Furthermore, recently a variant of VEGF, which had amino acids 83–89 replaced with the analogous region of the related PlGF demonstrated significantly reduced KDR binding compared with wild-type VEGF emphasizing the point that this region is important for VEGF-KDR interaction (58). Amino acid sequence alignment (Fig. 1*C*) shows that of the six most important KDR binding determinants (the major hot-spot) of VEGF, only two residues from VEGF ($Glu^{64}$ and $Ile^{83}$) are conserved in PlGF-1 ($Glu^{73}$ and $Ile^{92}$) and both residues from the minor hot-spot are conserved in the two structures. Observation of the PlGF-1 structure indicates significant conformational rearrangement corresponding to regions 43–45 and 83–85 (the major hot-spot residues for KDR recognition in VEGF, Fig. 2*E*). This provides a possible structural explanation for the inability of PlGF-1 to recognize KDR. Based on the amino acid sequence alignment of VEGF-A, PlGF-1, and VEGF-B, similar arguments can be put forward for VEGF-B, where considerable changes have appeared in the KDR binding determinants and hence may not recognize this receptor.

We have also performed modeling studies on the PlGF-1·VEGF heterodimer in complex with Flt-$1_{D2}$ (in a similar way to that described above for the PlGF-1·Flt-$1_{D2}$ complex). From this model, it appears that the putative contact residues in the heterodimer are similar to those listed in Table III for Flt-1 recognition.

*Concluding Remarks*—Recent structural studies on polypeptide growth factors in complex with their receptors have provided a wealth of information in the area of protein-receptor signaling. In the case of cysteine-knot proteins, the target molecule (*e.g.* VEGF, PDGF, or NGF) seems to form complexes with one or two domains of the receptor molecule. In this report, we have tried to address this question with another member of this family, PlGF-1, referring to the molecular details of a closely related molecule, VEGF. As in the case of VEGF, PlGF-1 appears to use only a small number of residues in receptor recognition. These details would be the starting point for the design of small mimics of PlGF. Such agonists could be useful for the design of PlGF antagonists, which prevent the interaction with the receptor, and may serve to be important for the treatment of pathological disorders involved in neovascularization during tumor growth.

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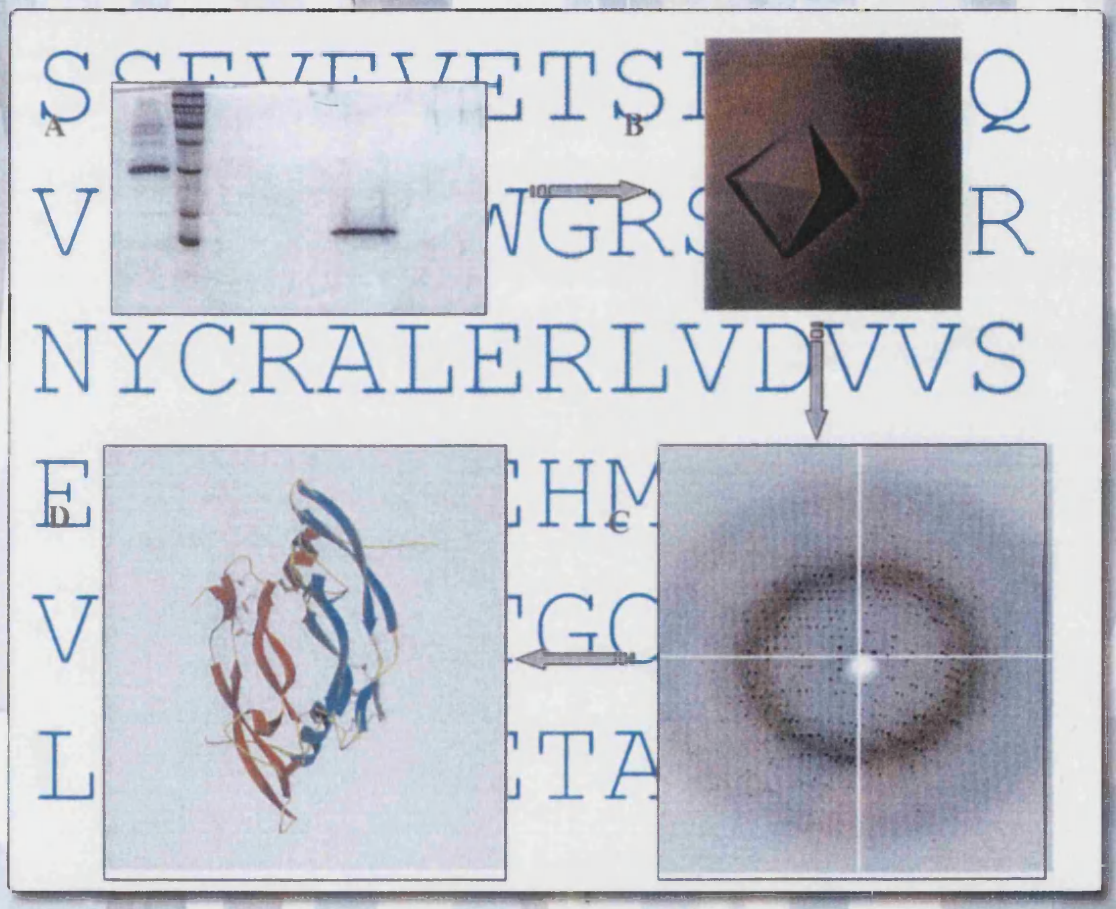
## Placenta Growth Factor: Protein Sequence to Crystal Structure



## IN THIS ISSUE

# ROLE OF PLACENTA GROWTH FACTOR IN CARDIOVASCULAR HEALTH

by

SHALINI IYER and K. RAVI ACHARYA

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# Role of Placenta Growth Factor in Cardiovascular Health

Shalini Iyer and K. Ravi Acharya*

*Placenta growth factor (PlGF), a member of the cysteine-knot family, is an angiogenic protein. The PlGF gene has been conserved across different species of the animal kingdom. It is expressed primarily in the placenta, especially in the later stages of gestation. PlGF expression is upregulated during pathological conditions such as ischemia of the heart and myocardial infarction. It is now known that PlGF can modulate the activity of vascular endothelial growth factor, the most potent of all angiogenic inducers and hence makes it an attractive target for therapeutic strategies. Recent structural studies on different isoforms of PlGF promise to reveal important topological and molecular details of these proteins that may be of potential use in the design of effective small molecule inhibitors to combat pathological angiogenesis.* (Trends Cardiovasc Med 2002;12:128–134) © *2002, Elsevier Science Inc.*

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The development of a hierarchical vascular network by remodeling of the primary capillary plexus is known as angiogenesis. This process is essential for many physiological as well as pathological conditions, including tumor progression (Folkman 1995). Several intra- and intercellular signals control the mechanisms that initiate the sprouting of new vessels or pruning of the pre-existing ones. Vascular endothelial growth factor (VEGF) and related growth factors are important regulators of this complex molecular phenomenon. VEGF is the most potent angiogenic inducer and is known to initiate two major steps in blood vessel formation: proliferation and migration of the endothelial cells (Conn et al. 1990, Connolly et al. 1989a, and 1989b, Dvorak et al. 1995, Ferrara 1995, Ferrara and Henzel 1989).

Members of the VEGF family belong to the cysteine-knot superfamily of growth factors. These bioactive proteins display a common structural architecture (based on a cyclic-knot of cysteines) despite little homology in their amino acid sequence. This cysteine-knot motif gives stability to the three-dimensional structure of these proteins (McDonald and Hendrickson 1993, Murray-Rust et al. 1993). It also supports the solvent-exposed, receptor-binding surface of these polypeptides and brings the key residues involved in receptor recognition into spatial proximity. The VEGF family consists of six structurally related proteins: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PlGF). PlGF shares ~40% amino acid sequence identity with VEGF.

## • PlGF: Genomic Structure and Splice Variants

The human PlGF gene is located on chromosome 14 of the genome (Maglione et al. 1993) and consists of seven exons. PlGF-1 was first isolated from the human placenta and was characterized as being highly homologous to VEGF (Maglione et al. 1991). This was followed by the discovery of PlGF-2, an isoform of PlGF-1. The two isoforms (PlGF-1/$PlGF_{131}$ and PlGF-2/$PlGF_{152}$) are generated by differential splicing of the PlGF mRNA (Maglione et al. 1993). A third isoform of the PlGF gene (Figure 1) was characterized recently with the use of cDNA from the human terminal placental tis-

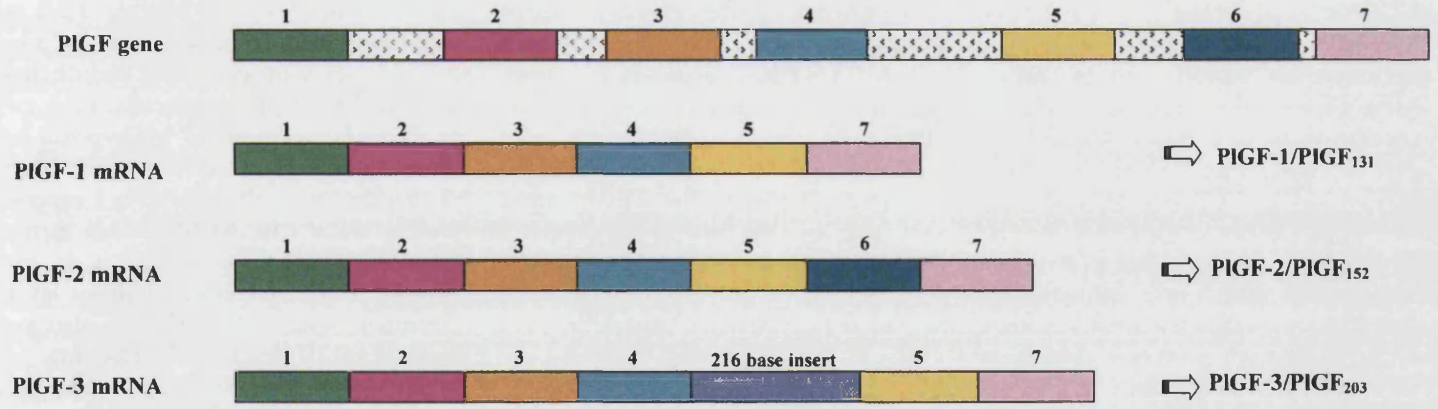


**Figure 1.** A schematic representation of the three spliced variants of the PlGF gene. The gene has 7 exons (each colored differently) separated by introns (shaded). PlGF-1 and PlGF-3 mRNAs lack exon 6. PlGF-3 mRNA has a 216 base insert between exon 4 and 5. The length of the amino acid sequence indicated for each isoform corresponds to that of the mature protein (after cleavage of the signal peptide).

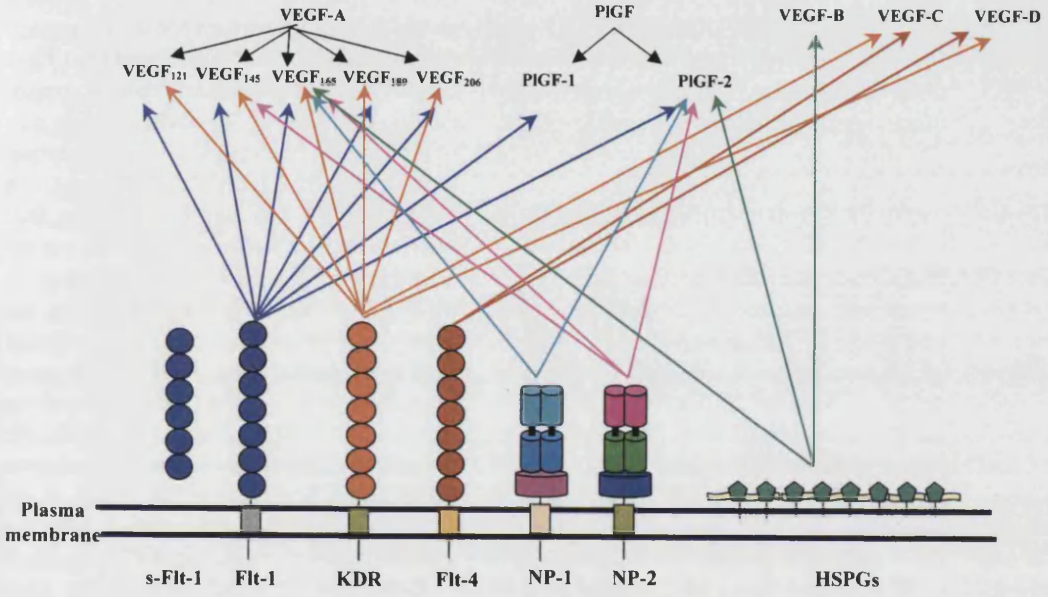


**Figure 2.** A schematic representation of the various receptors characterized for the members of the cysteine-knot family of growth factors. Flt-1, KDR, and Flt-4 are tyrosine kinase receptors. sFlt-1 is the soluble truncated form of Flt-1. NP-1 and NP-2 (neuropilins) belong to the family of semaphorin receptors. HSPGs represent heparan sulfate proteoglycans that act as binding partners for some of the growth factors. The receptors have been colored individually. The arrows point to the ligands that bind each of the receptors and these have been colored according to the receptor they represent.

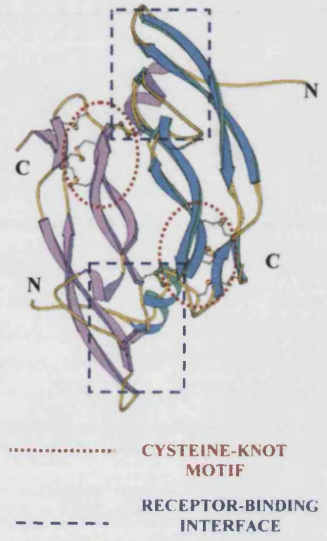


**Figure 3.** The three-dimensional crystal structure of PlGF-1. The structure has been color-coded to differentiate between the two subunits: Monomer A has been colored purple and Monomer B is in cyan. The cysteine-knot motif (consists of 3-intra and 1 inter-chain disulfide bridge passing through the 8-membered cysteine ring) and the receptor-binding region have been highlighted at each pole of the dimer.

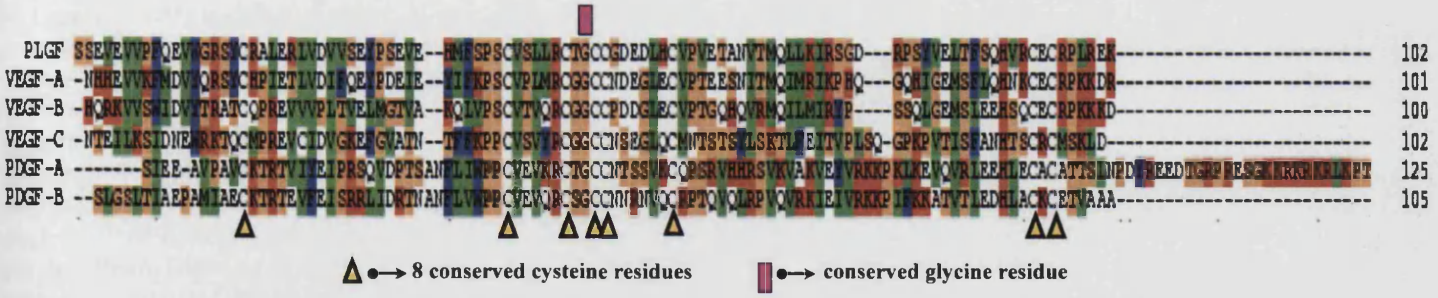


**Figure 4.** Amino acid sequence alignment of the members of the cysteine-knot superfamily of growth factors. The Swissprot databank accession codes for PlGF, VEGF-A, VEGF-B, VEGF-C, PDGF-A, and PDGF-B are P49763, P15692, P49765, P49767, P04085, and P01127 respectively.

sue. This was termed as PlGF-3. The PlGF isoforms, apart from their size, differ in their secretion properties as well as their binding affinities. Different experiments were conducted to characterize the differential distribution of the mRNAs of the PlGF isoforms. Results from these studies showed that the human PlGF-cDNA hybridizes to genomes from species across the animal kingdom, indicating that the gene has been sustained through evolution (Maglione et al. 1991). PlGF is expressed in the placenta during different stages of pregnancy. PlGF-mRNA is also present in very small amounts in heart, lung, thyroid, goiter, and skeletal muscle. It is not expressed, however, in kidney and pancreas (Maglione et al. 1993, Ziche et al. 1997). It has also been observed that PlGF expression corresponds more often with pathological conditions than with normal conditions.

## • PlGF: Molecular Characterization

PlGFs are biologically active as homodimers. PlGF-1 has a molecular mass of ~46 kDa (dimer) and is composed of 131 amino acid residues per monomer. It has been shown that PlGF-1 is a secretory protein and undergoes N-glycosylation. PlGF-2 consists of 170 amino acid residues prior to signal peptide (18 amino acid residues in length) cleavage. Compared to PlGF-1, PlGF-2 that is associated with the membrane has a highly cationic 21 amino acid insert at the carboxy-terminal of the protein (Cao et al. 1997). The insert corresponds to exon 6 of the PlGF gene and contributes to the ability of PlGF-2 to bind polyanionic substances such as heparan sulfate proteoglycans (HSPG; Hauser and Weich 1993). PlGF-3, which is the longest isoform of the three, has an extra 72 amino acid residues that correspond to the 216 base insert between exon 4 and 5 of the PlGF gene. Like the first isoform, it also lacks amino acids that provide HSPG binding affinity. In the mature protein the PlGF monomers are N-glycosylated at Asn84, although it has been demonstrated that glycosylation is not essential for maintaining the biological activity of the protein. In both PlGF and VEGF, the eight cysteine residues that are characteristic of members of the cysteine-knot growth factor superfamily are conserved. As a result, the structural folds of these two proteins are very similar. Heterodimers of PlGF and VEGF occur naturally and have been shown to be mitogenic toward endothelial cells. Although the potency of these heterodimers as mitogens is lower than that of the VEGF homodimers, it seems that VEGF expression and thus its activity is negatively regulated by heterodimerization (Disalvo et al. 1995, Park et al. 1994).

## • PlGF: Receptors and Their Expression

The distinct patho-physiological roles of the PlGF isoforms are mediated by binding to the *fms*-like tyrosine kinase receptor-1 (Flt-1/VEGFR-1; Figure 2; Park et al. 1994). It has been shown that PlGF-1 binds to Flt-1 with high affinity, transphosphorylates Flt-1, and thus activates the signal transduction cascade. PlGF-1 does not induce autophosphorylation of kinase-insert domain receptor (KDR/VEGFR-2; Cao et al. 1996, Landgren et al. 1998, Sawano et al. 1996, Terman et al. 1994). VEGF, on the other hand, is a functional ligand for both Flt-1 and KDR. Both Flt-1 and KDR belong to the FLT-subfamily of endothelial cell receptors (for a review see Mustonen and Alitalo, 1995) and share some 44% amino acid sequence identity. Mutation of the critical Flt-1 binding sites in VEGF (receptor-selective variants) significantly reduced the affinity of VEGF for KDR, suggesting that the binding determinants for KDR and Flt-1 overlap (Li et al. 2000).

The mature form of Flt-1 consists of 1316 amino acids. The receptor is made up of seven immunoglobulin-like extracellular domains, one transmembrane domain and a cytoplasmic, split tyrosine kinase domain (De Vries et al. 1992, Fournier et al. 1997, Jussila et al. 1998, Matthews et al. 1991, Shibuya et al. 1990, Terman et al. 1992). Flt-1 is also produced as a soluble (sFlt-1) form, which lacks the seventh immunoglobulin-like extracellular domain (Kendall and Thomas 1993). The occurrence of the soluble form is thought to be a physiological means to downregulate the activity mediated by binding of ligands to the Flt-1 receptor. Also, it is believed that sFlt-1 binds to VEGF with very high affinity and probably forces VEGF to form non-functional heterodimers with KDR. The second immunoglobulin-like domain of the receptor is mainly responsible for recognition of the ligands. Flt-1 is expressed on both endothelial cells (proliferating as well as resting endothelial cells) and their precursor cells. Flt-1 expression is upregulated by hypoxia in vivo (Thieme et al. 1995). It has been shown that in adult tissues Flt-1 expression is highest in the lungs and in the developing placenta, where it is expressed in the spongiotrophoblast layer (Dumont et al. 1995). sFlt-1 is also expressed at high levels in the placenta.

Flt-1 is primarily responsible for endothelial cell morphogenesis (Fong et al. 1995) rather than mitogenesis, which is characteristic of KDR-induced signal transduction (Millauer et al. 1993, Terman et al. 1992, Waltenberger et al. 1994). Gene knock-out studies have shown that mouse embryos lacking Flt-1 do not survive; i.e., it is important for development (Fong et al. 1995). It is believed that Flt-1 might play an important role in maintaining vascular organisation and this role of Flt-1 might be dependent on its interactions with other receptors (Karunagaran et al. 1996). It has also been shown that Flt-1 mediates chemotactic monocyte recruitment and activation in response to PlGF-1 binding. This correlates with the overexpression of Flt-1 on the endothelial cells and induction of PlGF-1 in human keratinocytes during wound healing (Clauss et al. 1996, Failla et al. 2000). Although it is known that Flt-1 stimulation leads to $Ca^{2+}$ signaling (De Vries et al. 1992), it is unclear as to which cellular responses follow the binding of PlGF to Flt-1.

Cross-linking experiments show that another class of receptors called the semaphorin receptors act as binding partners to the heparin-binding form of PlGF (Figure 2). Semaphorin receptors, the neuropilins (np-1 and np-2), are involved in axonal guidance. Sequences corresponding to exons 6 and 7 of the PlGF gene contribute to the functional np-1 binding domain on PlGF-2 (Midgal et al. 1998). Binding of PlGF-2 to np-1 is heparin dependent. It has been shown that sulfation of glucosamine-O-6 and/or iduronic acid-O-2 groups of heparin is required to augment the binding of PlGF-2 to np-1. However, this does not modulate the migratory responses induced by binding of PlGF-2 to Flt-1 as seen for VEGF-$A_{165}$-KDR mediated response. Experimental evidence indicates that *np-1* gene disruption leads to abnormalities in the peripheral nervous

system as well as anomalies in the development of the cardiovascular system (Midgal et al. 1998).

## • PlGF: Protein Structure

The three-dimensional crystal structure of recombinant human PlGF-1 was recently elucidated at 2.0 Å resolution (Iyer et al. 2001; Figure 3). More recently, the resolution was extended to 1.55 Å (Iyer et al., unpublished results). Here we shall review the functional implications of PlGF-1 corresponding to the structural details at this higher resolution.

PlGF-1 structure is remarkably similar to VEGF-$A_{121}$ (Figure 3). It occurs as a biological homodimer in which the monomers initially associate via hydrophobic interactions with the dimer axis perpendicular to the plane of the β-sheet. Each monomer consists of two α-helices and seven β-strands. There are very few contacts between the two monomers at the central, highly irregular and solvent accessible β-sheet region. The dimeric structure is stabilized by the cysteine-knot motif and a hydrophobic core region (one each per monomer). The motif consists of one inter-chain disulfide bond that holds the two monomers together. Conservation of a glycine residue at the position that precedes the cysteine residue involved in the formation of an inter-chain disulfide bond emphasizes the importance of the motif in maintaining structural integrity of the proteins belonging to this superfamily (Figure 4). Also, since the glycine residue is devoid of a side chain, the structural conformation of this motif is optimal. The side-by-side, head-to-tail orientation of the monomers places the receptor-binding interface at each pole of the PlGF-1 dimer. This facilitates receptor dimerization, which is essential for initiating the signal transduction cascade. Residues from both the monomers contribute to the receptor-binding interface (Figure 3).

## • PlGF: Structure and Drug Design

Structure-based drug design of small molecule antagonists or agonists requires a detailed three-dimensional structural and functional characterization of the target molecule. The structure of PlGF-1 shows that although it has a structural fold very similar to that of VEGF, it displays important conformational differences, especially in the flexible loops that form part of the receptor-binding region of PlGF-1. It has been known that the solvent-exposed loops undergo concerted movements, which decide the receptor-specificity for VEGF. In PlGF-1, the flexibility of the loop connecting strands $\beta_5$ and $\beta_6$ is somewhat restricted compared to the corresponding loops in VEGF. This is because of amino acid changes that involve two prolines that affect the movement of the loop vis-à-vis their physical properties. There are some other changes in the sequence of PlGF-1 at the receptor-binding interface that probably correspond to the inability of PlGF-1 to induce proliferation of endothelial cells despite its ability to bind Flt-1 like VEGF. These changes include $Arg^{93}$→Lys, $Gly^{95}$→His and $Tyr^{100}$→Ile and might also explain why PlGF-1 has a lower affinity for Flt-1 than VEGF and is unable to displace VEGF from Flt-1. A single change from $Ile^{46}$ in VEGF to $Met^{55}$ in PlGF-1 significantly affects its binding affinity for KDR. Structurally, it was seen that unlike $Ile^{46}$ in VEGF, the side chain of $Met^{55}$ is facing away from the region where Flt-1/KDR is proposed to bind the ligand (Keyt et al. 1996, Muller et al. 1997).

The structure of the Flt-1·PlGF-1 complex is yet to be elucidated. However, based on the structure of the VEGF·Flt-1 complex (Wiesmann et al. 1997), it can be postulated that Flt-1 binds at the monomer-monomer interface of PlGF-1 and not exclusively to each of the subunits in the dimer (Figure 5). Domain deletion experiments had revealed that domain 2 and 3 of Flt-1 contain the key determinants required for signaling. These experiments have mapped the ligand-binding residues to the second domain and those important for receptor dimerization have been characterized as domain 3 residues (Davis-Smyth et al. 1996). Mutagenesis studies and the crystal structure of the complex reveal the binding determinants on Flt-1 for VEGF. Davis-Smyth et al. (1996) have identified $Glu^{137}$, $Arg^{159}$, $Lys^{171}$, $Glu^{208}$, $His^{223}$, and $Arg^{224}$ as critical residues that contribute towards PlGF-1 binding. Modeling studies (Iyer et al. 2001) showed that $Gln^{27}$, $Tyr^{34}$, $Asp^{72}$, $Tyr^{100}$, and $Glu^{112}$ could make both hydrogen bonding and van der Waals contacts with the residues implicated in PlGF-1 binding. However, these modeling data need to be substantiated with the complex structure of PlGF-1 with Flt-1 that will unravel the exact orientation of PlGF-1 when it binds Flt-1. This would be very useful, as the modeling study is based on the assumption that both PlGF-1 and VEGF bind to Flt-1 in an analogous fashion.

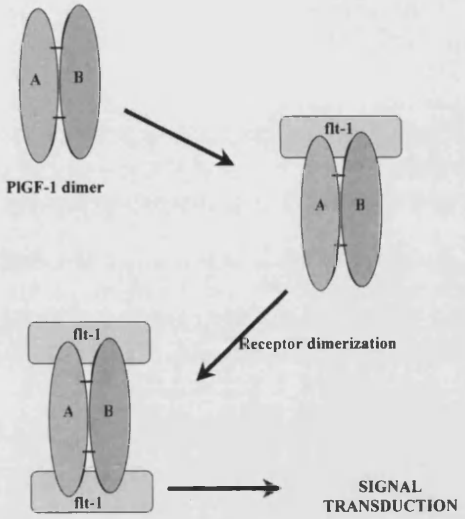


**Figure 5.** A cartoon representation of the possible events leading to receptor dimerization and formation of the PlGF-1·Flt-1 complex via inter-subunit binding of Flt-1 to the PlGF-1 dimer.

The unliganded structure of PlGF-1 is a starting point for designing drug molecules that can either activate Flt-1 and therefore potentiate VEGF stimulation or inhibit Flt-1 stimulation by binding either to Flt-1 or PlGF-1 itself. The drugs can either be small molecule activators/inhibitors or peptides that target the protein–protein interaction sites. Such peptides have a great pharmacological value and are possible candidates that can be developed further as potential therapeutics.

## • Role of PlGF-1 and Its Receptors in Angiogenesis

Initial experiments on PlGF-1 were rife with contradictions. There were two main schools of thought: one considered PlGF-1 to be angiogenic and the other opposed this view. Although in vitro and in vivo experiments by Ziche et al. (1997) showed that PlGF-1 induces formation of blood vessels and is chemotactic as well as mitogenic on certain types of endothelial cells (postcapillary venule ECs), these results were questioned for a long time. It is now generally believed that PlGF does play an important role in angiogenesis and placental development. Its expression during the development

of the placenta suggests a role for the protein during the invasiveness of the placental trophoblasts into the maternal decidua (Vuorela et al. 1997). Expression of the PlGF mRNA and the protein increases during the third trimester of human pregnancy. This period is characterized by non-branching type villous angiogenesis and formation of the terminal villi. This is brought about by the binding of PlGF to Flt-1 (Kingdom et al. 2000). It has been shown that PlGF-1–stimulated Flt-1 signaling induces the production of tissue factor in monocytes. This leads to activation and chemotactic recruitment of monocytes and macrophages, which are critical players in the process of blood vessel formation and tumor progression (Clauss et al. 1996). It was also shown that there is a constitutive expression of PlGF mRNA and the protein in human keratinocytes during wound healing (Failla et al. 2000). The microenvironment within the tissue is responsible for the effect of PlGF on blood vessels, indicating that it has a tissue-specific role.

PlGF is produced only by certain types of tumors, namely thyroid tumors and germ cell tumors, in contrast to VEGF, which is produced by all tumor types. PlGF is expressed in these tumors and in inflamed tissues (Kodama et al. 1997, Takahashi et al. 1994), and this expression is probably a consequence of angiogenic factors being released from the extracellular matrix. The mast cells that are subsequently activated during tumor angiogenesis provide an exogenous source of heparin that potentiates the binding of PlGF-2 to neuropilin-1. This increased binding, however, does not lead to tumor progression or neovascularization of the tumors (Midgal et al. 1998, Viglietto et al. 1995 and 1996).

Most of the earlier experiments were done with recombinant human PlGF-1, which was angiogenic only under certain conditions. These raised several doubts and questions regarding the role of endogenous PlGF-1 in angiogenesis. The recent results reported by Carmeliet et al. (2001), based on gene inactivation experiments in mice, answer some of these queries. Their experiments showed that loss of PlGF correlated with impaired angiogenesis. Although PlGF deficiency hardly affects vascular development, the absence of the protein affected VEGF-dependent endothelial cell survival, migration and proliferation. Their results indicate that even though PlGF on its own is not an effective inducer of angiogenesis, it does potentiate the activity of VEGF in physiological conditions. Also, the expression of PlGF in pathological conditions indicates that it might induce the angiogenic switch by increasing the bioavailability of VEGF.

Another important aspect that links PlGF and VEGF is the ability of the two cytokines to form heterodimers. It is hypothesized that PlGF binds to VEGF and either reduces the bioavailability of VEGF (Cao et al. 1996) or, at the other extreme, augments the activity of low concentrations of VEGF (Park et al. 1994). PlGF/VEGF heterodimers are somewhere between PlGF and VEGF homodimers vis-à-vis their mitogenic ability. Chick chorio-allantoic membrane assays show that the heterodimers, unlike the PlGF homodimers, can bind to KDR, through which they exert their stimulatory effects on endothelial cell proliferation (Kurz et al. 1998).

## • PlGF and Cardiovascular Diseases

The frontiers of cardiovascular health have expanded ever since scientists, worldwide, have taken an interest in the therapeutic use of VEGF in cardiovascular diseases (Zachary et al. 2000). VEGF is the angiogenic cytokine that has been shown experimentally to be responsible for initiation of angiogenesis. It is believed that VEGF is a promising candidate in therapeutic angiogenesis because of its ability to induce vasodilation and enhance vascular permeability. Therapeutic angiogenesis, as opposed to anti-angiogenesis therapy, involves stimulation of blood vessel formation in diseases like ischemia, atherosclerosis, thrombosis, restenosis, etc. Studies based on VEGF-mediated angiogenesis therapy show mixed results (Isner 1999), and as a consequence it is not possible to draw any substantial conclusion from them. Also, such a therapy is shrouded by risks of unwanted neovascularization (Maglione et al. 2000, Zachary et al. 2000).

Recently, however, PlGF seems to be making waves not only because of its similarity to VEGF but also because of its ability to augment the activity of VEGF. A fundamental reason that makes PlGF such a promising factor is that on its own, PlGF cannot initiate endothelial cell stimulation. Relatively speaking, PlGF-1 has no permeability activity compared to VEGF (Maglione et al. 2000). So, in effect, this growth factor should not have any adverse effects on the blood vessels, both near and far from the site of administration. PlGF would only enhance the effects of the available low concentrations of endogenous VEGF (Park et al. 1994). Another important factor that goes in favor of PlGF is that it can stimulate the recruitment and activation of monocytes via the Flt-1 receptor (Clauss et al. 1996). Monocyte recruitment is yet another essential step in the process of formation of mature vascular networks, especially collateral growth of blood vessels. It is not known as yet whether VEGF alone is sufficient for collateral vascular growth.

It was shown in animal models that recombinant PlGF-1 explicates protection from isoprenaline-induced ischemic lesions in the heart without influencing the heart beat rate (Maglione et al. 2000). Although these findings need to be confirmed with human-based experiments, it is safe to suggest that PlGF-1 could be considered as a potential therapeutic agent for ischemic myocardial infarction. Also, evidence and reasoning seem to suggest that since PlGF acts synergistically with VEGF (Carmeliet et al. 2001), a combination therapy of VEGF and PlGF would probably be more effective in the treatment of cardiovascular diseases.

## • Future Prospects

Conformational differences at the receptor-binding interface between the structures of PlGF-1 and VEGF-$A_{121}$, despite a high degree of sequence homology, have uncovered crucial structural insights that are valuable in the rational design of novel angiogenesis inhibitors. Further structural studies on PlGF-1 are required, studies that are largely based on receptor-ligand complexes. The structural basis behind Flt-1 receptor specificity of PlGF-1 and the other isoforms may lead to the development of receptor/ligand specific therapeutics. This needs to be complemented with the characterization of the entire sequence of events that occur during PlGF-1–induced signal transduction. The next step then would be to correlate the signaling events with the topological details of the ligand and the receptor at the binding interface. Also, the elucidation of the three-dimensional structure of PlGF-2, the heparin binding isoform, will take our understanding of these proteins

a step further. Although PlGF-2 has a highly basic insert of 21 amino acids at the C-terminal end of the molecule, the cysteines involved in the formation of the ring structure are all conserved and hence the structural core of PlGF-2 should be very similar to PlGF-1. These two isoforms do not have any other differences in their sequence apart from the insert. It would be interesting to see how different PlGF-2 is from its isoform, and mapping of the binding determinants for heparin as well as np-1 should enhance our understanding of molecular details involved in PlGF recognition.

## • Conclusion

Researchers have spent the last three decades trying to bring the totality of the complex phenomenon of blood vessel formation into perspective. Three-dimensional structures of the various proteins involved in angiogenesis have given the researchers great headway into realizing this goal and simultaneously in the design of small molecule inhibitors to fight diseases related to angiogenesis. The recent work by Carmeliet et al. (2001) has introduced a new thought process. The structure of PlGF-1, along with insights into the mechanism of action of PlGF in angiogenesis as an important VEGF consort, have made PlGF isoforms very promising targets for both anti- and pro-angiogenesis therapy.

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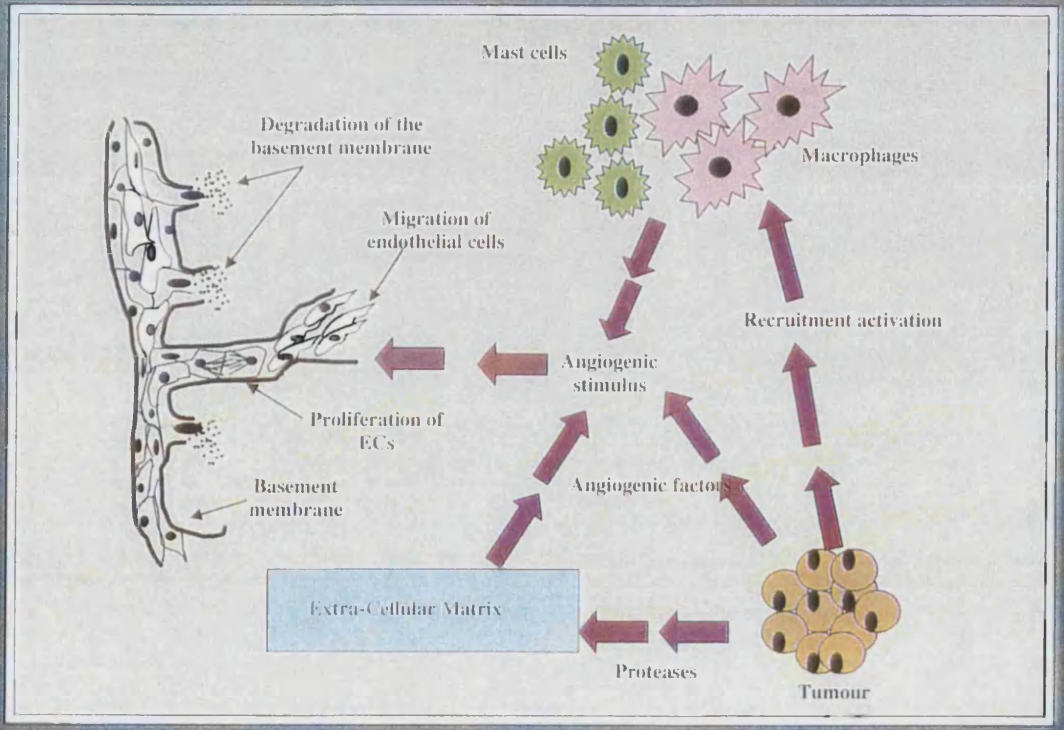


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# Angiogenesis: What We Know and Do Not Know

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Angiogenesis is the process of formation of blood vessels from pre-existing blood vessels. It differs from vasculogenesis, which means differentiation of angioblasts into blood islands. It occurs in a series of orchestrated network of cooperative interactions. This process plays a vital role in reproduction, growth and healing. It was in 1971 that the hypothesis made by Judah Folkman first implicated vascular growth in tumour progression. Since then, the need to address questions that pertain to the control of blood vessel growth has helped this concept evolve each year with increasing new discoveries. The local equilibrium between positive and negative regulators of angiogenesis is the key determinant to the state of the capillary networks. Integration of growth factors, cytokines, proteases, adhesion molecules and endogenous modulators is central to the correct outcome of the process. Switch to malignancy, which is characterised by onset of angiogenesis, occurs when this balance tips in favour of the inducers of the process. During the last decade numerous angiogenic genes and gene products have been isolated and purified from both normal and neoplastic tissues. These regulatory molecules induce or inhibit blood vessel formation by both paracrine and autocrine mechanisms. Comprehensive pre-clinical research activities have translated the various advances in basic angiogenesis research into possible clinical therapies by using a number of *in vitro*, *in vivo* and transgenic animal models. Although lead compounds that proved to be successful in animal models are less promising in treating human cancers, there are about 30 compounds now in clinical trials that target angiogenesis. This review discusses the different effectors and inhibitors of angiogenesis, especially in context with tumour angiogenesis. The review also addresses the therapeutic approaches to control blood vessel formation and the compounds in clinical trials.

**Key Words: Angiogenesis, Tumors, Growth factors, Hypoxia, Proteases, Angiotensins, Angioproteins, Angioproteins, Endogenous inhibitors**

## Introduction

Angiogenesis is the process of formation of vascular sprouts from pre-existing microvasculature. Physiological angiogenesis occurs only during foetal development, a woman's endometrial cycle, wound healing and muscular growth. The process of vascularisation is also essential for the progression of several diseases. Pathological conditions pertaining to this process can be a function of either uncontrolled angiogenesis as seen in psoriasis, rheumatoid arthritis, atherosclerosis and many cancers (Folkman 1971, Folkman 1995, Norrby, 1997) or dysfunctional angiogenesis that can be associated with ischemia of the heart, poorly healing wounds and fractures, ulcers and infertility (Folkman 1998, Arnold et al. 1987).

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The local release of an angiogenic stimulus from, for example, a tumour, tissue injury or inflammatory cells, triggers off a highly regulated sequence of events leading to the formation of blood vessels. A vessel wall is composed of an endothelial cell lining, a basement membrane, and a layer of cells called pericytes that partially surrounds the endothelium. The construction of a vascular network requires different sequential steps including:

- the disappearance of the pericytes
- the release of proteases from the endothelial cell lining of the vessel wall
- the breakdown of the basement membrane surrounding the existing vessel
- binding of the angiogenic factors to their receptors on the endothelial cells

- proliferation of the endothelial cells
- migration of the endothelial cells into the interstitial space
- the reorganisation of the endothelial cells
- lumen formation
- generation of a new basement membrane
- fusion of the newly formed vessels
- initiation of blood flow

Overall, angiogenesis can be viewed as the result of an extensive interplay between oncogenes and suppressor genes, stimulatory and inhibitory molecules, proteases and endogenous inhibitors and environmental factors such as the oxygen level or copper ion (Suri et al. 1996). Angiogenesis is such a complex phenomenon that a clear-cut distinction between angiogenic factors (table 1) as being either inducers or inhibitors would be an oversimplification. Some angiogenic factors act as both direct and indirect inducers of the process while some function contextually; sometimes as inducers and sometimes as inhibitors. In other words, angiogenesis is a process tightly regulated by the spatially and temporally coordinated activities of a number of key angiogenic regulators, both environmental and genetic.

During physiological angiogenesis, there exists a dynamic balance between the positive and the negative regulators of the process while on the other hand the net balance is usually tipped in favour of angiogenesis during pathological circumstances (figure 1). It has been experimentally established that the loss of control of termination and stabilisation of the blood vessels is due to the up-regulation of the positive regulators as well as due to the exhaustion of the endogenous inhibitors of the process of blood vessel formation.

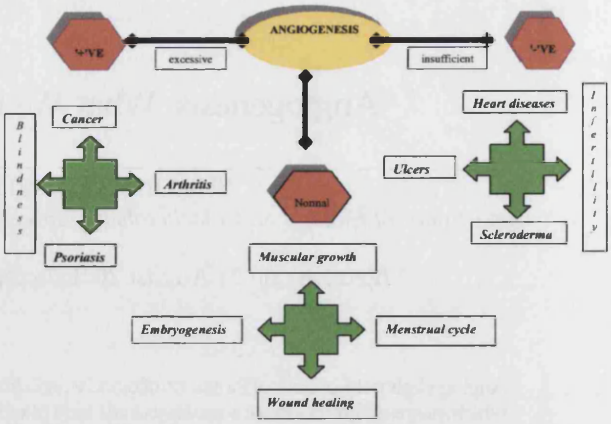


**Figure 1** A schematic showing the consequences of both physiological and pathological angiogenesis

### *1.1. Tumour Angiogenesis*

More often than not, tumour growth starts with uncontrolled cell proliferation. The rapidly dividing tumour mass soon reaches a steady state due to lack of nutrients and oxygen. The tumour may remain dormant for several months or years yet when the circumstances are beneficial it can switch to an angiogenic phenotype. This event can be triggered off by environmental stress factors such as hypoxia, low *p*H and pressure generated by proliferating endothelial cells (ECs), immune responses, and genetic factors such as activation of oncogenes or deletion of tumour-suppressor genes that control the production of factors regulating the process of angiogenesis (figure 2).

Some tumours and metastases in particular do not initiate in an avascular manner (Holash et al. 1999, Pezzella 1997). They rather grow by homing in on a host vessel and start off as a vascular tumour (figure 3). Soon after tumour co-option the host

*Abbreviations:* ***ACE***, Angiotensin Converting Enzyme; ***Ang-1***, Angiopoietin-1; ***Ang-2***, Angiopoietin-2; ***Ang II***, Angiotensin-II ; ***ARNT***, Arylhydrocarbon Receptor Nuclear Translocator; ***CAM***, Chick Chorioallantoic Membrane ; ***COX***, Cyclooxygenase ; ***ECs***, Endothelial Cells ; ***ECM***, Extra-cellular Matrix ; ***EGF***, Epidermal Growth Factor; ***EGFR***, Epidermal Growth Factor Receptor ; ***FGF***, Fibroblast Growth Factor; ***FGFR***, Fibroblast Growth Factor Receptor ; ***FGF-BP***, Fibroblast Growth Factor-Binding Protein ; ***FPP***, Farnesyl Pyrophosphate ; ***GGPP***, Geranylgeranyl Pyrophosphate ; ***HGF/SF***, Hepatocyte Growth Factor/Scatter Factor; ***HIF-1α***, Hypoxia Inducible Factor-1; ***HSPGs***, Heparan Sulphate Proteoglycans; ***HUVECs***, Human Umbilical Vein Endothelial Cells; ***IL***, Interleukin; ***MAPK***, Mitogen-Activated Protein Kinase; ***MCP-1***, Macrophage Chemoattractant Protein-1; ***MMP***, Matrix Metallo-Proteinases; ***L-NAME***, N-Nitro-L-Arginine Methyl Ester; ***NF-κB***, Nuclear Factor-kappaB; ***NO***,Nitric Oxide; ***NOS***, Nitric Oxide Synthase; ***PD-ECGF***,Platelet-Derived Endothelial Cell Growth Factor; ***PFT***, Protein Farnesyl Transferase ; ***PDGF***, Platelet-Derived Growth Factor; ***PDGFR***, Platelet-Derived Growth Factor Receptor; ***PGGT-1***, Protein Geranylgeranyl Transferase-1; ***PlGF***, Placenta Growth Factor; ***RAS***, Renin-Angiotensin System; ***SMCs***, Smooth Muscle Cells; ***TAMs***, Tumour-associated Macrophages; ***TGF-α*** , Transforming Growth Factor-alpha; ***TGF-β***, Transforming Growth Factor-beta; ***TNF-α***, Tumour Necrosis Factor-alpha; ***TSP***, Thrombospondin; ***VEGF***, Vascular Endothelial Growth Factor; ***VEGFR***,Vascular Endothelial Growth Factor Receptor.

**Table 1** Different classes of angiogenic regulators

**Growth factors**
1. Vascular Endothelial Growth Factor (VEGF)
2. Placenta Growth Factor (PlGF)
3. Platelet Derived Growth Factor (PDGF)
4. Transforming Growth Factors (TGFs)
5. Fibroblast Growth Factors (FGFs)
6. Epidermal Growth Factor (EGF)
7. Angiotropin
8. Hepatocyte Growth Factor/Scatter Factor (HGF/SF)
9. Platelet Derived-Endothelial Cell Growth Factor/ Thymidine Phoshphorylase (PD-ECGF/TP)
10. Angiogenin

**Proteases and protease inhibitors**
1. Cathepsins
2. Gelatinase A, B
3. Stromelysins
4. Urokinase-type Plasminogen Activator (uPA)
5. Plasminogen Activator Inhibitor-1 (PAI-1)

**Signal transduction enzymes**
1. Farnesyl transferase (FT)
2. Geranylgeranyl transferase (GGT)

**Cytokines**
1. Interleukins (IL)
2. Tumour Necrosis Factor-α (TNF-α)

**Oncogenes**
1. c-myc
2. ras
3. c-src
4. v-raf
5. c-jun
6. p53 Rb

**Environmental factors**
1. Hypoxia
2. Low *p*H
3. Hypoglycaemia
4. Copper
5. Zinc
6. Nitric Oxide

**Endogenous modulators**
1. Angiostatin
2. Endostatin
3. Integrins
4. Angiopoietins (Ang 1 and Ang 2)
5. Angiotensins (Ang I and Ang II)
6. Endothelin (ET)
7. Nitric Oxide Synthase (NOS)
8. Thrombospondin (TSP)
9. Prolactin (PRL)

vessels begin to die as a result of apoptosis and the tumours become secondarily avascular and hypoxic. This leads to a definitive increase of tumour derived VEGF. The induced VEGF leads to a new burst of angiogenic sprouting from the regressing blood vessels. Thus the onset of a robust new angiogenesis allows the tumour to survive and grow further.

### *1.2. Models to Study the Process of Angiogenesis*

Considerable insight in the molecular and cellular biology of angiogenesis has been obtained by *in vitro* and *in vivo* studies. However, comparison between different studies is rather difficult because of the great diversity in the models being used. Also, any given compound that is angiogenic *in vitro* may not show any response *in vivo*, where it might be activated or inactivated by other molecules and thereby show results opposite to that of the *in vitro* model. Likewise, substances with no obvious effects, chemotactic and/or mitogenic, *in vitro* may play a crucial role *in vivo* in angiogenesis. Hence, both *in vitro* and *in vivo* models must be employed in order to evaluate the efficacy and potency of various angiogenic and/or anti-angiogenic compounds. Here we have made an attempt to give a brief overview of the various models that are available to us to study the process of angiogenesis.

#### *1.2.1. In vitro Models*

Most steps in the angiogenic cascade can be understood by using endothelial cells to analyse processes such as proliferation, migration and differentiation. The advantages of using *in vitro* models are that the spatial and temporal concentrations of the various angiogenic mediators involved in the angiogenic cascade can be controlled and each step of the cascade can be studied individually.

**Chemotaxis** can be examined in a Boyden Chamber. The chamber consists of two wells, an upper well and a lower well, separated by a membrane filter (figure 4). The chemotactic solution is placed in the lower well and the cells in the upper well and after a period of incubation, the cells on the lower surface of the membrane are counted which gives an indication of the number of cells that have migrated towards the chemotactic stimulus (Fan et al. 1993).

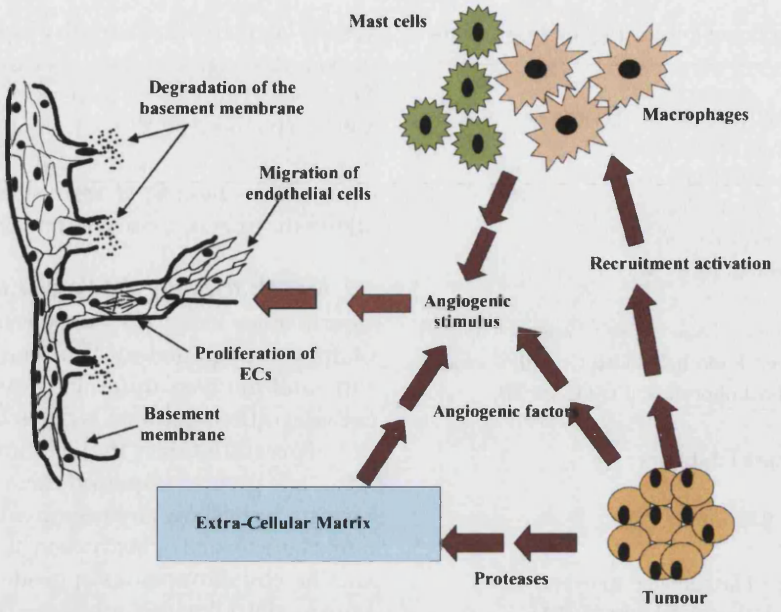


**Figure 2** A schematic of events that can trigger off the angiogenic switch in dormant tumours

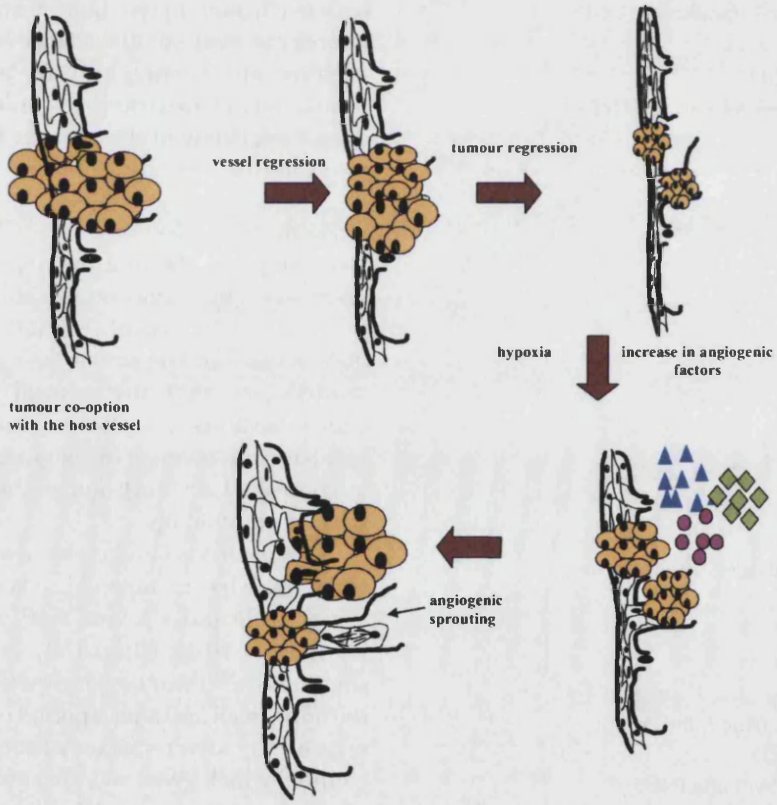


**Figure 3** A schematic showing tumour angiogenesis initiated by tomour co-option with host vessels

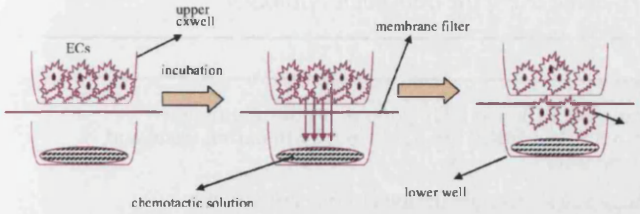


**Figure 4** Chemotaxis in a Boyden Chamber

**Proliferation** studies are based on cell counting, thymidine incorporation, or immuno-histochemical staining. The increase in the number of cells is calculated by measuring Proliferating Cell Nuclear Antigen (PCNA; Hall et al. 1990) or by measuring apoptosis (by the Tunel Assay, Mori et al. 1994).

**Differentiation** can be studied by culturing the ECs in different extra-cellular matrix (ECM) components, including two- and three-dimensional fibrin clots, collagen gels and matrigel (Benelli & Albini 1999).

*1.2.2. In vivo Models*

Classical *in vivo* angiogenic assays include the Chick Chorioallantoic Assay (CAM), the rabbit cornea assay, sponge implant models, matrigel plugs and conventional tumour models. The first evidence of tumour induced angiogenesis used a syngeneic combination of tumour and mouse cornea, without involvement of inflammation (Muthukkaruppan et al. 1982, Muthukkaruppan & Auerbach 1979).

**CAM assay**: The CAM assay is a relatively simple, inexpensive and most widely used assay for large-scale screening as the early chick embryo lacks a mature immune system (Auerbach et al. 1974). The major disadvantage of the model is the presence of a developed network of blood vessels that makes it difficult to differentiate between the newly developed capillaries and the already existing ones.

**Rabbit cornea assay**: This assay is more reliable than the CAM assay as it represents an *in vivo* avascular site and thus makes it easier to differentiate between newly developed blood vessels and already existing ones. Computer image analysis after perfusion of the cornea with India ink helps to measure the vascular response. It is not a practical model for large-scale screening as it is technically very demanding and very expensive as well.

**Implantation models**: Subcutaneous implantation of various artificial sponges like gelatin and polyvinyl alcohol (incorporated/injected with the compounds to be evaluated) provides another means to study the process of angiogenesis. However, the implantation of these sponges results in some non-specific immune responses which in turn may result in a vascular response even in the absence of exogenous growth factors in the sponge (Andrade et al. 1987).

**Matrigel**: Matrigel generated from Engelbreth-Holm-Swarm (EHS) murine sarcoma contains basement membrane components (collagens, laminin, and proteoglycans) as well as matrix degrading enzymes, their inhibitors and growth factors. Role of the ECM receptors and enzymes in tumour progression has been well characterised using a matrigel incorporated with tumour cells (Passaniti et al. 1992).

**Transgenic models**: Transgenic models are fast becoming the gold standard for manipulating the angiogenic candidate molecules in a controllable manner (table 2). In the transgenic approach, a particular candidate disease gene is chosen and re-engineered using molecular tools, confirmed as the desired gene, and is then introduced into the model so it becomes a part of that model's genome. The model then possesses the new trait. Both homologous and site-specific recombination, in embryonic stem (ES) cells, have allowed us to study the consequences of deficiencies, mutations and conditional expression of the different gene products in the transgenic models. The breakthrough in the novel gene technologies has enabled researchers to study and analyse the pathophysiological function of disease genes in a conclusive manner. Apart from this, gene transfer technologies provide us with the potential possibility of utilising the gene products in therapeutic approaches.

These various models for studying the process of angiogenesis along with the pivotal role played by the three-dimensional structures of these angiogenic regulatory molecules have broadened our understanding of this complex process. What follows now is a brief overview of the role played by each of these different regulatory molecules (table 1) in the process of blood vessel formation.

**Table 2** *Some major breakthroughs in angiogenesis research using the transgenic approach*

| References | Findings |
|---|---|
| (Carmeliet et al. 2001) | Specific synergism between VEGF and PlGF potentiates the angiogenic response to VEGF and not FGF-2; PlGF has a role to play in pathological and not physiological angiogenesis |
| (Sacco et al. 2000) | Liposome-delivered angiostatin strongly inhibits tumor growth in a transgenic model of spontaneous breast cancer |
| (Yokoyama et al. 2000) | Endostatin is effective in delaying spontaneous tumour development and growth |
| (Gao et al. 2000) | Inhibition of Iκ-B alpha degradation abolishes the induction of NF-κB-dependent gene expression and up-regulation of VCAM-1 and ICAM-1 |
| (Yamamoto et al. 2000) | Advanced glycation endproducts participate in the development of diabetic microangiopathy |
| (Lira 1999) | Understanding the biological role of the chemokines and their receptors in leukocyte trafficking, angiogenesis, immunoregulation and development |
| (Karasek 1999) | Biology of psoriasis |
| (Bourdeau et al. 1999) | Endoglin, an accessory protein of the TGF-β receptor superfamily, is critical for both angiogenesis and heart valve formation |
| (Wang et al. 1999c) | TGF-β1 has been implicated in wound healing and skin carcinogenesis |
| (Asahara et al. 1999) | VEGF plays a complementary role to angiogenesis in postnatal neovascularisation |
| (Sariola & Sainio 1998) | Identification of the first signalling genes acting in the inductive interactions in the kidney for differentiation of the metanephros |
| (Wong et al. 1999) | VEGF is up-regulated in the early pre-malignant stages of colorectal tumour progression |
| (Kowal et al. 1999) | EVEC, a new EGF-like repeat containing protein is up-regulated in embryonic and adult vasculature |
| (Tyner et al. 1999) | Role of p53 loss independent of other exogenous oncogenic influences |
| (Achen & Stacker 1998) | VEGF family of proteins and their receptors are critical for angiogenesis, EC differentiation and vasculogenesis |
| (Bergers et al. 1998) | Interplay between apoptosis and proliferation in pancreatic carcinomas and squamous cell carcinomas of the skin |
| (Edelberg et al. 1998) | Environmental induction of PDGF-AB α receptor interaction is central to the regulation of cardiac microvascular EC hemostatic and angiogenic activity |
| (Thurston et al. 1998 ) | Angiogenic ECs bind and internalise cationic liposomes and liposome-DNA complexes but no other types of liposomes in tumours and chronic inflammation |
| (Calmels et al. 1997) | Ets-1 transcription factor gene is expressed in ECs during blood vessel formation under normal or pathological conditions |
| (Visvader et al. 1998) | SCL/tal-1 (transcription factor) is essential for the angiogenic remodeling of the yolk sac capillary network into complex vitelline vessels |
| (Hicks et al. 1996) | Trafficking and compartmentalisation of FGF-1 during cellular transformation |
| (Dubois-Stringfellow et al. 1994) | Pathobiology of hemangiomas as well as investigation of angiogenesis inhibitors |
| (Christofori & Hanahan 1994) | Multiple genetic events are responsible for the genesis of malignant tumours (molecular characterisation of tumour cell proliferation and tumour angiogenesis) |

both in physiological and pathological settings. Although the therapeutic approaches for controlling angiogenesis have been discussed towards the end of this review, we have highlighted some of the strategies undertaken, for treatment of various diseases, while dealing with each individual class of regulators.

## 2. Oncogenes and Angiogenesis

Cellular oncogenes have been shown to induce developmental and tumour angiogenesis, leading to the enhanced activity of molecules that stimulate angiogenesis. However, activated oncogenes have also been known to facilitate angiogenesis by down-regulating the inhibitors of angiogenesis. Several oncogenes have been implicated in the process of angiogenesis and tumour progression. Most common of them are the c-myc, ras, jun, raf, src and p53. Oncogenes and various oncoproteins especially associated or related with cell cycle regulation have provided important prognostic information. Oncogenes are a means for molecular substaging of different types of cancer according to age, histology, gender etc. For instance, p53 and Rb are available as markers for decreased cancer-specific survival in women whereas among men erb-2 is the only available marker for lung cancer (D'Amico et al. 2000).

Experiments by Li et al. (1999) show that de-novo methylation of Thrombospondin-1 (TSP-1) potentially serves to inactivate its expression in human cancers and tumours. Wild type p53 down-regulates the endogenous VEGF mRNA level as well as the VEGF promoter activity in a dose-dependent manner (Mukhopadhyay et al. 1995a). VEGF expression in colon tumours is also regulated by the Src family members, c-src, Lyn and Rac. Signalling pathways dependent on the src members are major mediators of factor VIIa/TF effects in pathophysiology (Versteeg et al. 2000). It is believed that the hypoxic induction of VEGF occurs on account of c-Src activation (Mukhopadhyay et al. 1995b). Indirectly this induction of VEGF brings about the activation of phospholipase C$\chi$1 (PLPC-$\chi$1) and the formation of Inositol 1, 4, 5-triphosphate ($IP_3$; figure 5).

Disturbances of the cellular signalling cascades such as the ras-raf Mitogen Activated Protein (MAP) kinase cascade, due to oncogenic gene mutations, leads to an abnormally high level expression of eicosanoid-forming enzymes, *e.g.* cyclooxygenases and lipoxygenases during cancer/tumour development (Marks et al. 2000). Mutation of the c-k-ras gene is a prerequisite of colon cancer progression, apoptosis, growth suppression and alteration of angiogenic factor expression (Tokunaga et al. 2000). Significantly elevated levels of microvessel density in both *chronic pancreatitis* and *pancreatic adenocarcinoma* further indicate that ras mutations potentiate activities that promote angiogenesis and thereby tumour progression (Banerjee et al. 2000). Ras oncogenes in their active state affect gene expression globally by coupling extracellular signals with transcription factors and transducing signals necessary for angiogenesis, invasion and metastasis via pathways involving cytoplasmic kinases, small GTP-binding proteins such as rho and rac (Zuber et al. 2000) This leads to the paracrine and cell-dependent stimulation of the potent VEGF indicating a link between the oncogenes and tumour angiogenesis.

Another family of oncogenes that are sometimes called the *immediate early genes* is

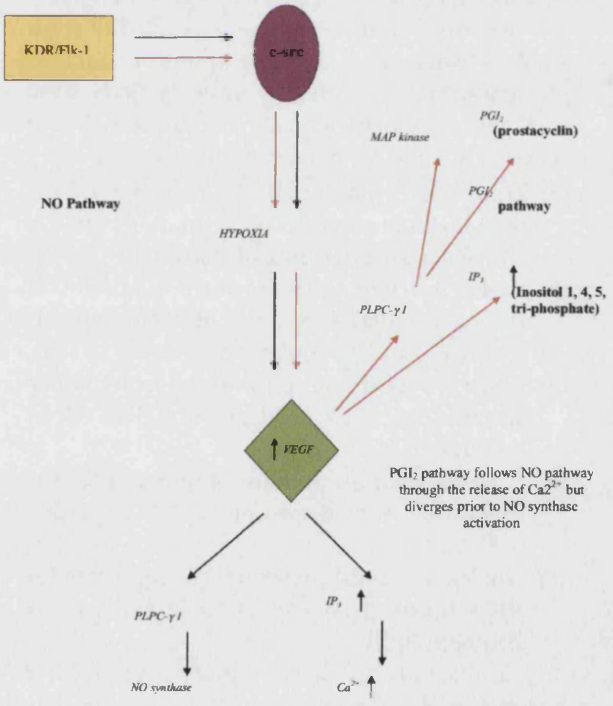


**Figure 5** Indirect activation of PLPC-$X^1$ by hypoxia

indicative of signal transduction. Induction of c-fos, c-jun is followed by the VEGF/VPF interaction with the cultured endothelium (Mukhopadhyay et al. 1998). Oxidative stimuli and the degradation products of hyaluronan induce a rapid transient up-regulation of c-jun and thus promote pathological angiogenesis in diseases like rheumatoid arthritis, endometrial cancers and stimulate overexpression of inter-cell adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) in human endothelial cells (Wang et al. 1999b, Fujimoto et al. 1998, Deed et al. 1997, Shono et al. 1996). The protooncogene, c-myc regulates cell growth, differentiation, and apoptosis, and its aberrant expression is frequently observed in several human cancers. Sustained activation of c-Myc is sufficient to induce angiogenesis and pre-malignant changes.

### *2.1. Role of p53 in Tumour Suppression*

p53, the tumour suppressor gene, plays a central role in protecting cells against DNA damage and numerous other types of physiological stress. This protection is mediated by inducing apoptosis and by arresting cell cycle. It has been experimentally shown that the loss of p53 function leads to genomic instability, inactivation of growth regulatory functions and tumorigenesis (Finlay et al. 1989). Human p53 is a five domain, nuclear phosphoprotein with 393 amino acids. Each of the five evolutionarily conserved domain has a specific function, which are as follows:

(a) Transactivation domain (N-terminus): This domain interacts with (Seto et al. 1992; Chao et al. 2000) the transcriptional machinery
(b) Proline rich region: This region is important for (Venot et al. 1998, Prives & Hall 1999) apoptosis It also negatively regulates p53
(c) Central core domain: Involved in sequence specific (Levine 1997, el-Deiry et al. 1992) DNA binding
(d) Oligomerisation domain: Important for the formation (Friedman et al. 1993) of p53 tetramers
(e) Nuclear Localisation Sequence: Important for RNA binding (Wu et al. 1995, Cadwell & Zambetti 2001)

Normal cells have low levels of p53 protein in a latent state. However, a variety of stress factors like UV rays, DNA damaging drugs, hypoxic conditions etc induce an increase in the levels of p53 protein. The biological activities of p53 are regulated by a variety of different mechanisms (Cadwell & Zambetti 2001):

(a) Autoregulatory, negative feedback loop created by binding of Mdm2 protooncogene to the N-terminus of p53 (Wu et al. 1993, Honda et al. 1997).
(b) Phosphorylation of p53 at N-terminus by DNA damage activates p53 (Giaccia & Kastan 1998).
(c) Acetylation of p53 at C-terminus also by DNA damage activates p53 (Sakaguchi et al. 1998, Gu & Roeder 1997).

## 3. Signal Transduction Enzymes and the Importance of Prenylation for Angiogenesis

Small G-proteins are anchored to cellular membranes by means of a post-translationally added lipophilic (iso)prenyl group (figure 6).

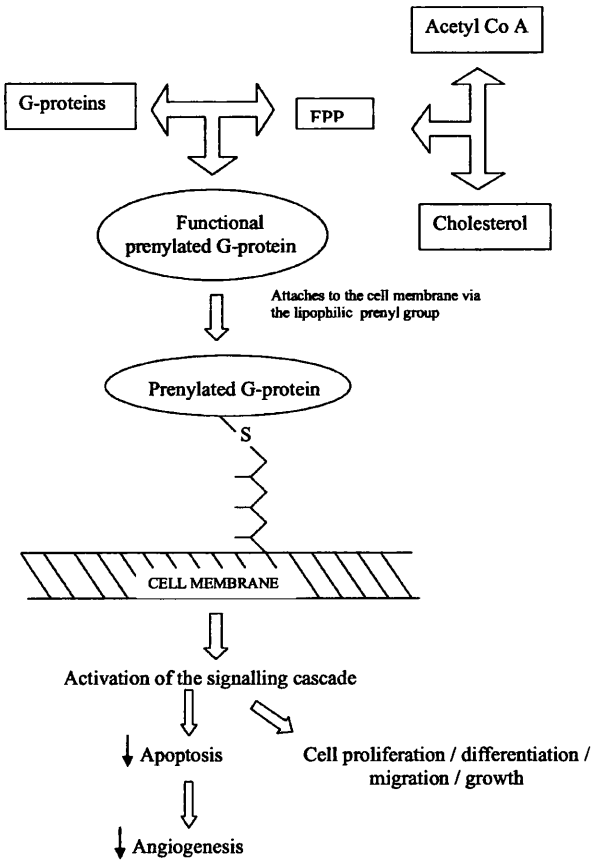


**Figure 6** A flow diagram indicative of prenylation-mediated down-regulation of angiogenesis

Prenylation is important for the G-proteins to mediate their signal transduction function (Maltese & Sheridan 1987). The prenyl groups, for instance the farnesyl group in Ras and the geranylgeranyl group in Rac and Rho, are derived from the farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) respectively (Casey 1992). Protein farnesyl transferase (PFT) and Protein geranylgeranyl transferase-1 (PGGT-1) mediate the covalent interaction of the G-proteins to the cholesterol biosynthesis pathway intermediates (FPP & GGPP). Prevention of prenylation of the G-proteins renders them inactive and hence there occurs inhibition of growth, proliferation, differentiation and migration of cells.

Farnesylated Ras activates growth factor signal transduction. Ras mutations are known to correlate with the carcinoma of the colon and the pancreas. This provided the incentive to target the prenylation enzymes (PFT, PGGT-1) for the search of new drugs (Gibbs et al. 1994, Leonard 1997, Sebti & Hamilton 1997, Cox & Der 1997). Since protein prenylation is associated with cell growth, prenylation inhibitors (figure 7) provide a novel approach to therapeutics in the treatment of various diseases.

These peptidomimetics are highly vulnerable to protease degradation. Also the presence of many negatively charged phosphate groups impairs their cellular uptake. So, their charge is masked by a covalently bound Pivaloyl-Oxo-Methyl (POM) group (Freeman & Ross 1997). Endogenous hydrolases remove the POM group after entering the cell where the prodrug gets converted to active form (Dickson et al. 1996). These peptidomimetics are the mimics of the carboxy-terminal tetrapeptide of the unprocessed G-protein, designated as CAAX (for FPT) or as CAAL (for PGGT-1). The prenyl group is bound to the cysteine residue of CAAX / CAAL. In the consensus sequence A stands for any aliphatic residue, L for leucine and X for methionine or serine.

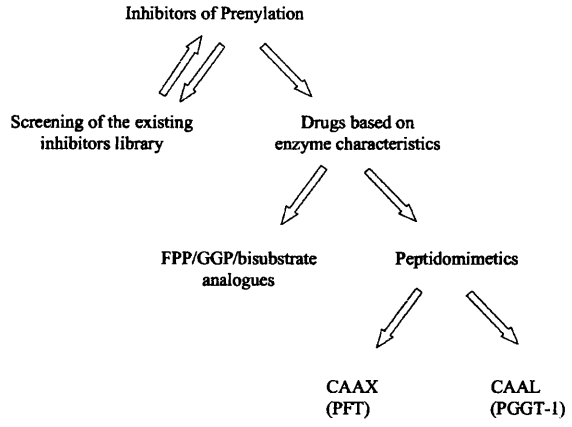


**Figure 7** A flow diagram to show how the various inhibitors of prenylation evolved

Many investigations support that for non-transformed cells geranylgeranylation is more important than farnesylation. For instance, co-addition of GGPP completely suppresses statin-induced inhibition of the growth of smooth muscle cells (SMCs). The FPP have only a partial effect (Terano et al. 1998, Laufs et al. 1999). The normally geranylgeranylated Rho has to be inactivated to arrest the transition of G1 to S phase during cell cycle (Guijarro et al. 1998, Olson et al. 1995). Since, Rho and Rac are integral to cell cycle, it becomes obvious that both these kinds of prenylation must be suppressed. This led to the development of inhibitor compounds that are effective against both PFT and PGGT-1.

The intracellular mechanism of action of prenylation inhibitors is still not exactly predictable. Their efficacy depends upon the type of G-protein mutations that occur in different tumours. For example, the Ki-ras mutation tumours are blocked by a combination of PFT and PGGT-1 inhibitors since Ki-ras can also be geranylgeranylated apart from being farnesylated (Mazet et al. 1999). Specificity of the signalling pathways involved in different cells and the sensitivity of the G-proteins to the various prenylation inhibitors compound the uncertainity associated with their efficacy and potency. However, despite all this some of the inhibitors designed to inhibit farnesylation have reached clinical trials for cancer treatment.

## 4. Growth Factors in Angiogenesis

The most important and perhaps the most extensively studied class of angiogenic regulators are the Growth Factors. These growth factors and the cytokines present in the tumour's microenvironment regulate a variety of genes associated with malignant properties of tumour

cells such as growth, migration, invasion, and metastatic capacities.

### *4.1. Cysteine-knot Superfamily of Polypeptide Growth Factors*

Several of the angiogenic polypeptide growth factors belong to the cysteine-knot superfamily of growth factors. These bioactive proteins exemplify the development of distinct biological functionalities during the process of blood vessel formation. These polypeptides display a common structural architecture despite little sequence homology. The crystal structures of these growth factors demonstrate that all have a similar topology based on a cyclic-knot of cysteines involved in both intra-chain and inter-chain disulphide bonds. This cysteine connectivity stabilises the Cα framework of these growth factors for elaboration of the solvent exposed loop regions that form the receptor-binding surface on these polypeptides. The members of this family occur as either homo- or hetero-dimers. These molecules differ on the basis of the number of disulphide bonds, the orientation of the monomers relative to the dimer axis and the extent of buried surface, thus varying the residues that are involved in the dimer formation. The disulphide bonds spatially bring the key residues involved in receptor recognition into close proximity of each other. The cysteine-knot members, although topologically similar, act via distinct cell-surface receptors with which they form complex signalling systems. The following section will review the role of the different members of this superfamily in the process of angiogenesis.

#### *4.1.1. Vascular Endothelial Growth Factor*

**VEGF-A**

Vascular endothelial growth factor (VEGF) is the most potent and pivotal regulator of angiogenesis and vasculogenesis. A highly specific mitogen for vascular endothelial cells, VEGF promotes extravasation of proteins from tumour-associated blood vessels. Disruption of genes encoding VEGF results in severe defects and abnormalities in the development of the cardiovascular system (Ferrara et al. 1996). VEGF induces cell signalling in different ECsdifferent ECs as for example in HUVECs and thus initiates a variety of biological effects (figure 8).

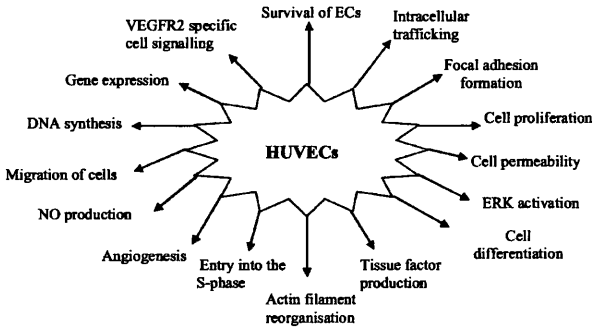


**Figure 8** VEGF-A induced biological effects in different ECs as a result of various signalling responses

Hypoxia is one of the major up-regulators of VEGF expression and is thought to drive angiogenesis during organogenesis. In developing organs, increasing distance between the migrating cells and the existing blood vessels creates hypoxic conditions. This stimulates the formation of new vasculature towards the VEGF producing cells (Stone et al. 1995, Pierce et al. 1995).When cells are no longer able to synthesize VEGF, angiogenic diseases ensue. Besides hyper-proliferation, blood vessels undergo unregulated and excessive fusion leading to formation of vessels with exceptionally large lumens. On the other hand, limited/reduced VEGF supply to the tissues leads to inhibition of organ development (Drake & Little 1995, Ferrara et al. 1998, Folkman & Hanahan 1991).

Crystal structure of VEGF-A at 1.93 Å resolution (Muller et al. 1997b) showed that each protomer consists of three intra-chain disulphide bonds and one inter-chain disulphide bond (figure 9). The monomers are perpendicular to the dimer axis such that there are two disulphide bonds related to each other by the dimer axis. Receptor dimerisation via a VEGF bridge activates signal transduction events that lead to a variety of mitogenic and chemotactic responses that are specific to VEGF-A depending upon where it is expressed.

VEGF is expressed in spatial and temporal association with physiological as well as tumour angiogenesis *in vivo* (Shweiki et al. 1993, Jakeman et al. 1993, Kim et al. 1993, Millauer et al. 1994). Expression of VEGF induces the formation of vesiculo-vacuolar organelles that channelise the blood borne proteins into the tumours. This forms an extravascular fibrin gel that stimulates ECs and

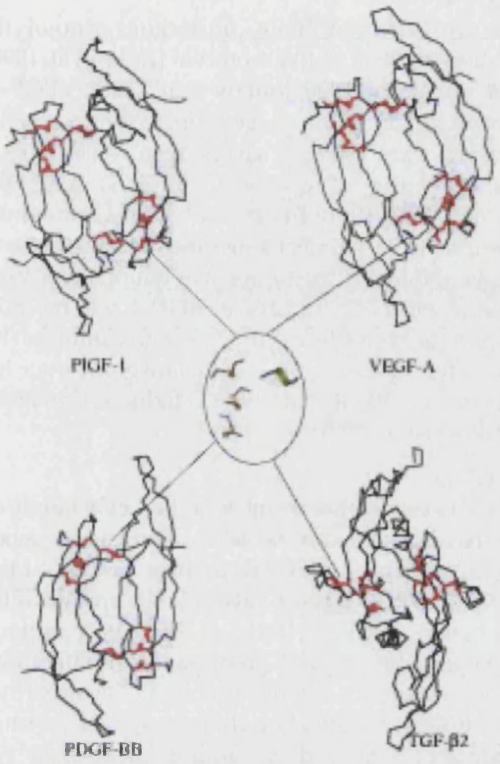


**Figure 9** The angiogenic members of the cysteine-knot superfamily of growth factors

tumour cells to proliferate and migrate and also supports the invasion of stromal cells into the growing tumours (Dvorak et al. 1995). The similarity of mechanism of VEGF induction during physiological as well as tumour angiogenesis explains why VEGF plays a central role in so many types of diverse tumours. A plethora of cytokines, growth factors and other external factors regulate VEGF production and thus indirectly stimulate/inhibit angiogenesis. These include FGF, PDGF, KGF, TGF-β, TNF-α, IL-6, IL-10 and IL-13.

Like hypoxia, hypoglycaemia is also a major VEGF stimulant (Shweiki 1992, Dantz et al. 2002). VEGF expression can be stimulated by several other factors even in the presence of oxygen. Activated oncogenes that are part of the ras/MAP kinase signal transduction pathway, potentiate VEGF mRNA expression (Grugel et al. 1995, Rak et al. 1995). Hypoxia independent production of VEGF (Richard et al. 2000) can also be brought about by inactivation of tumour suppressor genes like von Hippel Landau (vHL) and p53 genes at both transcriptional and post-transcriptional level (Mukhopadhyay et al. 1997, Iliopoulos et al. 1996, Pal et al. 1997). The loss of p53 gene has been associated with increased angiogenesis in tumours although some reports contradict these findings.

Alternative splicing of VEGF mRNA produces 5 isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. These isoforms differ in their heparin and heparan sulphate binding ability. Binding of VEGF to ECM-associated heparan sulphate proteoglycans (HSPGs) releases angiogenic growth factors such as the basic-FGF (Jonca et al. 1997). Heparin and not heparin like molecules enhance the binding of $VEGF_{165}$ to vascular endothelial growth factor receptor-2 (VEGFR-2). HSPGs, like the Glypican, act as a chaperone and restore the binding of $VEGF_{165}$ to VEGFR-2 after oxidative damage. The same however is not true for $VEGF_{121}$ (Gitay-Goren et al. 1992, Weksberg et al. 1996).

Apart from binding to cell surface HSPGs/heparin, VEGF binds two receptors belonging to the tyrosine kinase receptor family: VEGFR-1 and VEGFR-2 (figure 10). These two receptors are, predominantly, expressed by the ECs, monocytes, trophoblasts and tumourigenic cell types like melanomas. The binding domains of the two receptors are located at the opposite ends of the VEGF monomer such that in the active VEGF dimer, the main VEGFR-1/VEGFR-2 binding domains are at the opposite ends of the molecule (Keyt et al. 1996, Muller et al. 1997a). There is some evidence that the two receptors can heterodimerise

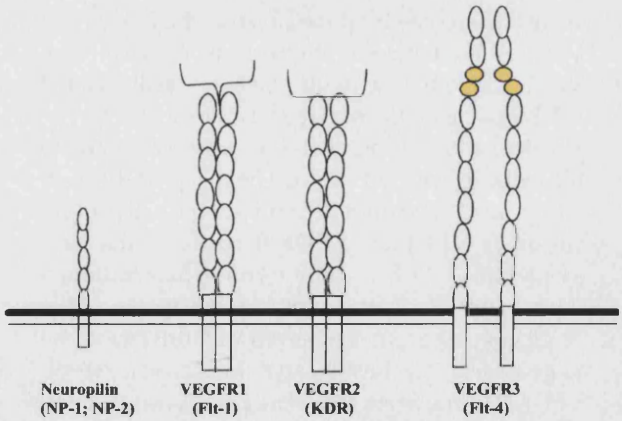


**Figure 10** The tyrosine kinase receptors and their ligands. The yellow region in VEGFR3/Flt-4 depicts the hinge region of the receptor

via a VEGF bridge (Wiesmann et al. 1997). Activation of VEGFR-2 by VEGF results in a mitogenic response while that of VEGFR-1 does not induce cell proliferation. Some isoforms of VEGF namely the $VEGF_{145}$ and $VEGF_{165}$ bind another class of receptors called the neuropilins-1 and 2 (Soker et al. 1998). The binding of the VEGF isoforms to these receptors is mediated by the exon 7 of the VEGF gene (Soker et al. 1997) that is absent in $VEGF_{121}$. Gene disruption studies indicate that neuropilins may act as co-receptors of $VEGF_{165}$ because a dysfunctional/ absent neuropilin leads to an impaired development of cardiovascular system leading to the death of mouse embryos.

Inhibition of VEGF signalling inhibits the development of many tumours. Impaired VEGF function abrogates tumour metastasis (Millauer et al. 1996, Skobe et al. 1997). Production of antagonistic VEGF mutants (Siemeister et al. 1998), VEGF receptor inhibitors (Strawn et al. 1996), antisense mRNA expressing constructs (Cheng et al. 1996), inhibitory soluble receptors (Kendall & Thomas 1993, Lin et al. 1998), humanised monoclonal antibodies against human VEGF (Presta et al. 1997) are some of the strategies that are being undertaken to treat VEGF induced tumour angiogenesis. VEGF is also being used to develop therapeutics for the treatment of diseases related with dysfunctional angiogenesis (Walder et al. 1996, Magovern et al. 1997, Baumgartner & Isner 1998).

**VEGF-B**

One amongst the recently discovered novel VEGF family members, VEGF-B also like its counterpart occurs in alternately spliced forms: VEGF-B167 and VEGF-B186 (Olofsson et al.1996, Paavonen et al.1996). VEGF-B is both structurally and functionally related to VEGF-A and Placenta Growth Factor (PlGF). The smaller isoform is highly basic and is associated to the cell surface via HSPGs. The receptor through which VEGF-B stimulates endothelial cell proliferation is VEGFR1. VEGF-B controls the bioavailability of VEGF-A by forming heterodimers with it. Both the isoforms of VEGF-B are devoid of N-glycosylation sites whereas VEGF-B186 has an O-glycosylation site instead (Olofsson et al. 1996). VEGF-B is known to stimulate DNA synthesis and has been characterised as an angiogenic protein like VEGF-A (Olofsson et al. 1996).

*PlGF belongs to the PDGF family of growth factors.

**VEGF-C**

The pro-peptide VEGF-C undergoes proteolytic cleavage to form a 21 kDa protein (Kukk et al. 1996, Paavonen et al. 1996, Joukov et al. 1996). VEGF-C autophosphorylates a tyrosine kinase receptor Flt-4/ VEGFR3 and VEGFR2 (Paavonen et al. 1996). It has a higher binding affinity for VEGFR3 than VEGFR2 (Joukov et al.1996).In adults, VEGF-C is mainly restricted to the lymphatic endothelium and has been implicated in the development of lymphatic vessels (Kaipainen et al.1995, Oh et al.1997). It was recently shown that soluble VEGFR3 potently inhibits the foetal lymphangiogenesis in transgenic mice by suppressing VEGF-C &VEGF-D induced signalling (Makinen et al. 2001).

**VEGF-D**

VEGF is yet another member of the VEGF family of proteins. It shares around 48% amino acid sequence identity with VEGF-C (Yamada et al. 1997). Like VEGF-C, VEGF-D also binds VEGFR2 and VEGFR3 (Achen & Stacker 1998). VEGF-D is a tumour angiogenesis factor and promotes EC proliferation. Experiments with a mouse tumour model reveal that VEGF-D stimulates lymphangio-genesis within tumours. It promotes tumour metastasis via development of the lymphs by activating VEGFR3 and not VEGFR2 (Stacker et al. 2001).

*4.1.2. Placenta Growth Factor*

The human term placenta seems to be a source of several angiogenic factors. A human cDNA coding for one such angiogenic protein was isolated from a placental cDNA library and named as the Placenta Growth Factor (PlGF*). The gene encoding this protein is located on chromosome 14 of the human genome (Maglione et al.1993). DNA sequence analysis of the PlGF gene showed that an alternative splicing mechanism is responsible for the production of the different forms of PlGF. PlGF-mRNA is abundantly expressed in the placental tissue and is also present in very small amounts in heart, lung, thyroid, goitre and skeletal muscle. No expression of PlGF has been detected in kidney and pancreas (Ziche et al. 1997). The PlGF coding sequence is encoded by seven exons spanning approximately an 8000-kb long DNA interval. The PDGF-like domain exhibited by the PlGF protein is encoded by exons 3 and 4. There are three isoforms of PlGF, namely PlGF-1, PlGF-2 and PlGF-3, which arise from alternative splicing of the PlGF-mRNA (Maglione et al. 1993).

Until recently the biological significance or the relevance of the different forms of PlGF with different composition was not known, but hybridisation studies reveal that PlGF is well conserved in the mammalian, bovine, chicken, amphibian and insect genomes (Maglione et al.1992, Maglione et al. 1991). This suggests that the PlGF gene has specific and indispensable functions. The importance of this protein is emphasised by the fact that a dysfunc-tional/absent gene in a mouse embryo leads to impaired blood vessel network and subsequently the death of the embryo. PlGF occurs in the placenta, thyroid and lungs. It is important for maintaining the network of blood vessels between the mother and the growing baby. The development and cell-specific regulation of the process of alternative mRNA splicing may have important consequences for physiological and pathological processes. Studies reveal a preferential expression of PlGF-2 in the placental tissue and cell lines, suggesting that this long form of PlGF may be involved in the growth and maintenance of pregnancy (Maglione et al. 1993).

Recently the crystal structure of PlGF-1 was elucidated at 2.0 Å resolution (Iyer et al. 2001, Iyer & Acharya 2002). The structure showed that PlGF-1 bears a remarkable topological identity to VEGF-A with which it shares around 42% sequence identity (figure 9). Interestingly it was observed that there are conformational differences in the receptor binding loop regions of the two molecules as well as the side chain orientations of the key residues involved in receptor recognition. Based on modelling studies with Flt-1/VEGFR1, the receptor for PlGF-1, (figure 10) and the ligand it was hypothesised that these structural differences perhaps render PlGF-1 unable to recognise VEGFR2/KDR despite high degree of conservation of amino acids at the receptor binding regions of PlGF-1 and VEGF-A.

Purification of PlGF-1 from overexpressing eukaryotic cells and measurement of angiogenic activity of the purified PlGF-1 *in vivo* in the rabbit cornea and CAM assays showed induction of a strong neovascularisation process that was blocked by affinity-purified anti-PlGF-1 antibody. In the avascular cornea, PlGF-1 induced angio-genesis in a dose-dependent manner. PlGF-1 has been shown to induce comparable cell growth and migration of endothelial cells from bovine coronary post-capillary venules and from human umbilical veins (HUVECs) *in vitro* (Ziche et al. 1997).

Experiments reveal that PlGF is induced in human keratinocytes during wound healing (Failla et al. 2000). PlGF expression is regulated by key cytokines released during an injury or a wound. It has also been shown that melanoma progression in humans is accompanied by deregulated, constitutive PlGF expression (Graeven et al. 2000). PlGF, however, serves no apparent autocrine role in melanoma proliferation. In case of PlGF-2 it has been established that the recombinant, purified protein is able to stimulate bovine aortic endothelial cells (BAECs) (Hauser & Weich, 1993) and HUVECs (Hauser et al.1993),but not the ECs from hepatic sinusoids (Sawano et al.1996). PlGF isoforms have very little mitogenic or permeability-enhancing activity. However, they significantly potentiate the action of low concentrations of VEGF *in vitro* and more strikingly *in vivo* (Park et al. 1994). The credibility of these findings were further cemented by recent experiments with knock out mice (Carmeliet et al. 2001). Synergistic cooperation between PlGF and VEGF in pathological conditions is specific. Upregulation of PlGF by ECs leads to displacement of VEGF from VEGFR1. As a result, increased amounts of VEGF are available to bind to the mitogenic response inducing receptor, VEGFR2. These experiments have implicated PlGF in pathological angiogenesis unlike VEGF that is essential for both physiological as well as pathological angiogenesis (figure 11). This makes PlGF a novel target for therapeutic treatment.

*4.1.3. Platelet-Derived Growth Factor*

Platelet-Derived Growth Factor (PDGF) is a potent inducer of growth and motility in several cell types, especially the mesenchymal cells. PDGF occurs in three different forms: PDGF A-chain homodimer (PDGF-AA), PDGF B-chain homodimer (PDGF-BB) and PDGF-AB (the heterodimer). PDGF elicits its biological responses via a tyrosine kinase receptor, Platelet-Derived Growth Factor Receptor (PDGFR). The receptor also occurs in different isoforms: α-PDGFR and β-PDGFR. PDGF-AA activates α-PDGFR only whereas PDGF-BB activates both the receptor forms. α-PDGFR is mainly involved in cell-

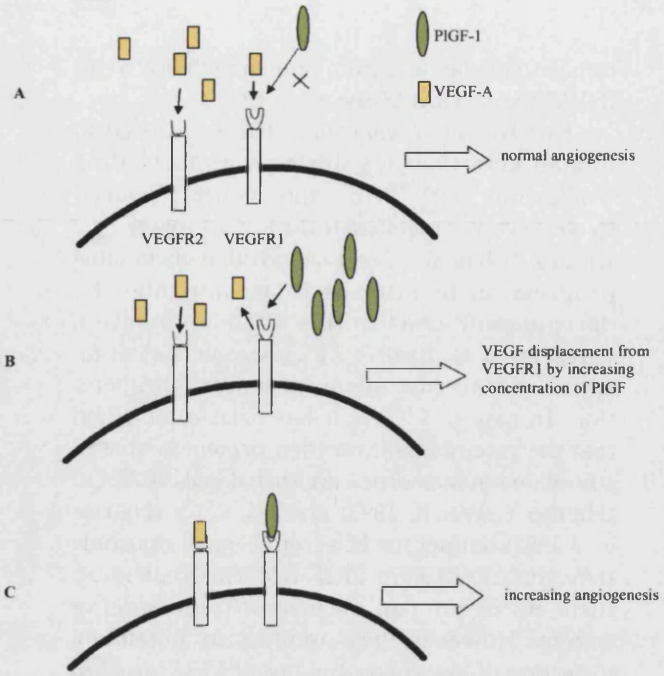


**Figure 11** Modification of the EC response to VEGF by PIGF during pathological conditions (A) low concentration of PIGF, (B) increasing concentration of PIGF

type specific signalling that is critical for the regulation of cell migration. On the other hand, β-PDGFR is involved in promoting cell migration (Yu et al. 2001). Like many other growth factors, PDGF can function both as a paracrine and an autocrine factor. The crystal structure of the homodimeric BB isoform of human recombinant platelet-derived growth factor (PDGF-BB) was determined by X-ray crystallography at 3.0Å resolution. The polypeptide chain is folded into two highly twisted antiparallel pairs of β-strands and contains an unusual knotted arrangement of three intramolecular disulfide bonds (figure 9). Dimerisation leads to the clustering of three surface loops at each end of the elongated dimer, which most probably form the receptor recognition sites (Oefner et al. 1992). The important findings in the field of PDGF research have been listed in table 3.

#### *4.1.4. Transforming Growth Factors*

Transforming Growth Factors (TGFs) are polypeptides that are produced by the transformed and tumour cells, and that can confer phenotypic properties associated with the transformation of normal cells in culture. These angiogenic factors are of two types, TGF-α and TGF-β. TGF-α is a member of the epidermal growth factor (EGF) family with which it shares the same receptor, the EGFR or erbB1. TGF-α is widely distributed in the central nervous system. Experiments have pointed out that TGF-α is a multifunctional factor in the CNS. It is a principal molecule in the normal and neoplastic development of the mammary gland. TGF-α overexpression can induce hyperplasia, hyperproliferation and occasional carcinoma.

Transforming Growth Factor–β(TGF-β) belongs to a family of multifunctional proteins that inhibit the growth of most cell types, and these proteins induce the deposition of extracellualr matrix. TGF-β inhibits the growth and migration of endothelial cells *in vitro* but induces angiogenesis *in vivo*. The other members of this family include the activins and bone morphogenetic proteins. TGF-β is produced as latent high molecular weight complexes from the producer cells and is then activated by plasmin or thrombospondin. Latent TGF-β binds covalently to latent TGF binding protein (LTBP) that targets it to the ECM. TGF-β binds several cell-surface receptors, including type III receptor (betaglycan), endoglin, type II and type I receptor (ALK-1). Type III receptor and endoglin are indirectly involved in signal transduction. The type II and type I receptors have intracellular serine/threonine kinase domains. They form a hetero-dimeric complex after ligand binding and are most important for signal transduction. The type II receptor transactivates the type I receptor which in turn transduces various signals (Miyazono 1996). The three -dimensional structures of the different isoforms have been elucidated using NMR and X-ray crystallography (Hinck et al. 1996). These structures showed several notable differences in structure and flexibility of the β-sheet loops that run parallel to the dimer axis (figure 9). It is believed that these differences are recognised by the TGF-β receptors which thus accounts for the different cellular responses (figure 12).

### *4.2. Other Important Angiogenic Growth Factors*

#### *4.2.1. Fibroblast Growth Factors*

One of the broadest spectrums of growth factors characterised so far are the fibroblast growth factors (FGFs). Since the discovery of the FGF in 1975 (Gospodarowicz 1976), about 20 members, that

Table 3 Important findings in the field of PDGF research

| References | Conclusions |
|---|---|
| (Wunderlich et al. 2000) | The expression pattern of Connective Tissue Growth Factor (CTGF) in retinal vascular ECs is not regulated by PDGF-BB |
| (Takehara 2000) | PDGF and CTGF stimulate chemotaxis of skin fibroblasts |
| (Westphal et al. 2000) | The vascular density of melanoma xenografts *in vivo* relates to the expression of VEGF, FGF-2, IL-8, PDGF and angiostatin |
| (Lafuente et al. 1999) | VEGF (development of secondary neoplastic change) and β-PDGFR (correlates to proliferation indexes) are expressed in human gliomas |
| (Harwood et al. 1999) | PDGF-BB and b-FGF regulate avβ3 and avβ1 integrin receptors in intrasynovial flexor tendon cells |
| (Somjen et al. 1999) | PDGF inhibits DNA synthesis in vascular smooth muscle cells |
| (Godden et al. 1999) | PDGF has a role to play in melanoma-cell derived factor stimulation of fibroblast glycosaminoglycan |
| (Asakawa & Kobayashi 1999) | An imbalance in the effects of co-culture with smooth muscle cells on ECs may lead to pathological events |
| (Gurski et al. 1999) | PDGF is expressed in the placenta and it may have a possible role in pre-eclampsia |
| (Wang et al. 1999a) | PDGF induces VEGF expression in ECs by activating phosphatidylinositol 3-kinase |
| (Nakamura et al. 1998) | PDGF gene transfer *in vivo* leads to healing of patellar ligament |
| (Bird et al. 1998) | PDGF induces calcium transients in cultured human peritoneal mesothelial cells |
| (Edelberg et al. 1998) | PDGF mediates cardiac microvascular communication |
| (Anand-Apte et al. 1997) | Rac and extracellular signal-regulated pathways differentially regulate PDGF and fibronectin-stimulated migration |
| (Schneller et al. 1997) | avβ3 integrin associates with activated insulin and β-PDGFR to potentiate the biological activity of PDGF |
| (Finkenzeller et al. 1997) | PDGF-induced gene expression requires the Sp1 recognition sites in the proximal promoter of the human VEGF gene |
| (Thommen et al. 1997) | PDGF-BB increases EC migration on cord movements during angiogenesis *in vitro* |
| (Krupinski et al. 1997) | PDGF might have a functional role in angiogenesis and neuroprotection after ischemic stroke in humans |
| (Tolonen et al. 1997) | Both VEGF and PDGF are expressed in disc herniation tissue |
| (Barnhill et al. 1996) | PDGF-A, PDGF-B and α-PDGFR are expressed in human malignant melanoma |
| (Kobayashi et al. 1996) | PDGF-BB released by diabetic-state modified macrophages in GK rat are involved in tube formation of the aortic ECs |
| (Horner et al. 1996) | A rapidly forming human bone expresses PDGF-A chain mRNA, the protein and α-PDGFR |
| (Landgren et al. 1996) | PDGF-BB induces the expression of both FGFR-1and the protein |
| (Zhang & Lo 1995) | Fibronectin expression in cultured rat thoracic aortic adventitial fibroblasts is regulated by PDGF-BB |
| (Kuwabara et al. 1995) | aFGF, FGF-2 and PDGF induced by hypoxia in mononuclear phagocytes stimulate the growth of hypoxic ECs |
| (Phillips & Stone 1994) | PDGF-BB induced chemotaxis is impaired in aged capillary ECs |
| (Liu et al. 1994) | Human brain abscess expresses FGF-2, nerve growth factor (NGF), PDGF and transforming growth factor-β (TGF-β) |
| (Holmgren et al. 1991) | Angiogenesis during human extra-embryonic development involves spatio-temporal control of PDGF and PDGFR gene expression |

share about 55% identity at the amino acid level, have been added to this family of proteins. Acidic fibroblast growth factor (a-FGF/FGF-1) and basic-fibroblast growth factor (b-FGF/FGF-2) have been well characterised (Maciag et al. 1984); (Shing et al. 1984). The b-FGF exists in four different isoforms resulting from alternative initiations of translation (Prats et al. 1989). All the four isoforms differ in their biological functions and occur in different intracellular compartments.

FGFs are involved in a number of pathophysiological processes that range from morphogenesis to tumour angiogenesis (figure 13). The FGF signalling is mediated via cell membrane spanning tyrosine kinase receptors FGF receptors (FGFRs) and their variants (Jaye et al. 1992, Wilkie et al. 1995). a-FGF is the only FGF known to bind all the FGFRs with high affinity (Chellaiah et al. 1994). Both a-FGF and b-FGF are potent mitogens for many cell types like the fibroblasts, ECs, smooth muscle cells and glial cells (Gospodarowicz et al. 1987). Signal transduction requires association of FGF with its

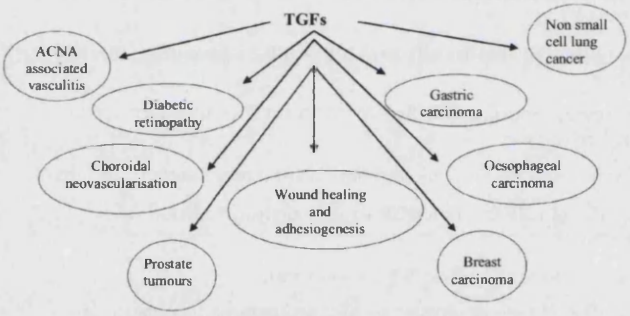


**Figure 12** The various pathophysiological processes in which the TGFs are involved

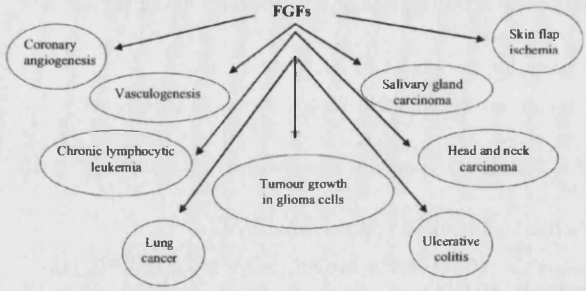


**Figure 13** The various pathophysiological processes in which the FGFs are involved


**Figure 14** The crystal structures of acidic FGF and basic FGF (Blaber et al. 1996, Eriksson et al. 1991)

receptor (FGFR) and heparan sulphate proteoglycan (HSPG) in a specific complex on the cell surface (Pellegrini et al. 2000). HSPGs modulate the presentation of FGF-2 to the FGFR on the cell surface via low-affinity interactions and regulate the diffusion rate of FGF-2 (Faham et al. 1996, Ornitz et al. 1995, Kan et al. 1993, Dowd et al. 1999). There is conflicting biochemical and biophysical evidence making it unclear whether or not FGF oligomerisation facilitated by the HSPGs are important for signalling through the high affinity interactions with the FGFR. The three-dimensional crystal structures of both a-FGF and b-FGF show that the region where the high affinity receptors, FGFRs, bind to the two ligands are structurally very similar to each other (figure 14). On the other hand, the site where the HSPGs bind on the FGFs is structurally very different. X-ray crystallography has thus provided a structural explanation for the role of these low affinity receptors, HSPGs, in discriminating between the FGFRs (Blaber et al. 1996).

### *4.2.2. Epidermal Growth Factor*

The maintenance of the integrity of the gastrointestinal mucosa and the healing of the gastric mucosal lesions are controlled by growth factors, especially the epidermal growth factor (EGF) which originates from the salivary glands and the transforming growth factor-α (TGF-α). TGF-α is released in the gastric mucosa itself, particularly when the mucosa is exposed to topical irritants and noxious substances.

EGF inhibits gastric acid secretion and stimulates duodenal bicarbonate secretion (Szabo & Vincze 2000). Apart from being an important growth factor in the process of ulcer healing, EGF and EGFR

are believed to be important for regulating angiogenesis and vascular function in the human endometrium (Moller et al. 2001). There exists a consistent relation between EGF activation and VEGF production. Several human glioma cell lines demonstrate this relation. This secretion of bioactive VEGF by the glioma cells accounts for the key histopathological features of glioblastoma multiforme, namely hypervascularity, focal necrosis, persistent cerebral oedema and rapid cellular proliferation(Goldmanetal.1993). Phosphorylation of EGFR leads to biphasic induction of VEGF following ultraviolet B irradiation. UVB also initiates synthesis of TGF-α. Both these growth factors (VEGF and TGF-α) enhance cutaneous angiogenesis in the chronically sun-exposed skin (Blaudschun et al. 2000). EGFR provides prognostic information to determination of tumour angiogenesis and microvessel density in women with invasive breast carcinoma (Weidner & Gasparini 1994). It is thought that the oncogenic properties of EGFR are mediated by up-regulating the potent angiogenic molecules such as the VEGF. These are probably enhanced by environmental factors that maximally stimulate tumour angiogenesis (figure 15; Petit et al. 1997).

### *4.2.3. Angiotropin*

Angiotropin is a potent angiogenesis factor secreted from the activated peripheral macrophages. It is directly involved in monocyte-induced angiogenesis. It activates microvascular ECs and thus triggers off inflammatory and proliferative reactions during wound healing (Hockel et al. 1988). *In vitro* it stimulates migration, phenotypic differentiation, and tube formation (figure 16). It does not induce proliferation of capillary ECs. Intradermal administration of angiotropin induces angiogenesis associated hyperplasia of the skin (Hockel & Burke 1989). Angiotropin-induced

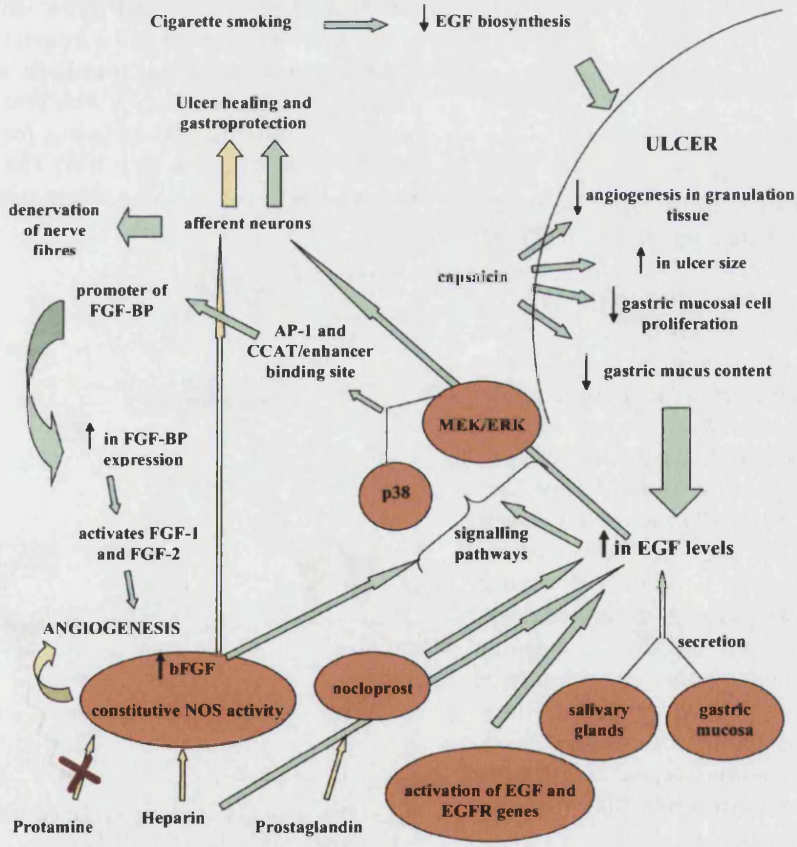


**Figure 15** An overview of the various *in vivo* effects of Epidermal Growth Factor

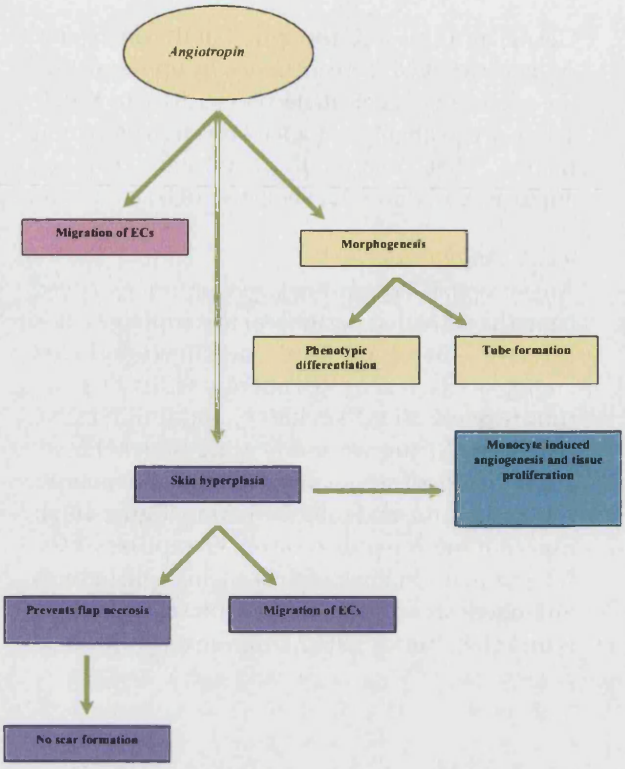


**Figure 16** A brief overview of the in vitro and in vivo properties of Angiotropin

angiogenesis is not inhibited by local dexamethasone. Transient proliferation occurs in the absence of tissue necrosis and does not lead to scar formation (Hockel et al. 1988). Exper-iments have revealed that local pre-treatment of angiotropin prevents flap necrosis and augments dermal regeneration after wounding in rabbit skin (figure 16). These results imply that angiotropin might be useful as an adjuvant to healing in surgery. Hockel et al(1987) have also shown that angiotropin does not stimulate proliferation of capillary endothelial cells and 3T3 cells. However, it was seen that in concentrations less than 1ng/ml, it enhances random migration of capillary endothelial cells but not of 3T3 cells. Angiotropin leads to defined dose-dependent and reversible changes of cellular morphology on confluent monolayers of capillary ECs. In the presence of angiotropin, capillary endothelial cells rapidly form tube-like structures on gelatinised plates. These results indicate that the biological action of angiotropin is different from that of other growth factors.

*4.2.4. Hepatocyte Growth Factor/Scatter Factor*

Hepatocyte Growth Factor (HGF) is an 85kDa heparin binding protein that is secreted by the mesenchymal cells and affects epithelial cell proliferation, motility and morphology (Matsumoto & Nakamura 1992, Vigna et al. 1994, Gherardi & Stoker 1991, Montesano et al. 1991). HGF is an important paracrine mediator of epithelial-mesenchymal cell interactions (Sonnenberg et al. 1993, Rosen et al. 1994). HGF binds and activates a high affinity tyrosine kinase receptor called the c-Met through which it mediates its diverse biological activities (Bottaro et al. 1991, Vigna et al. 1994). HGF also has a low-affinity binding site for heparin and HSPGs. Both the size and the charge of the HSPGs determine the binding affinity. HSPGs regulate cellular signalling (figure 17) by modulating the stability, diffusability and the biological activity of HGF. Zioncheck et al. (1995) showed that heparin or HSPGs can potentiate HGF mitogenesis but they are not required for the growth factor to bind to c-Met.

Depending upon cell type and culture conditions HGF can act as a mitogen (increasing DNA synthesis and cell number), a motogen (causing colonies of cells to scatter into individual cells) or a morphogen (causing formation of capillary like tubules *in vitro*). The HGF acts through the tyrosine kinase receptor encoded by

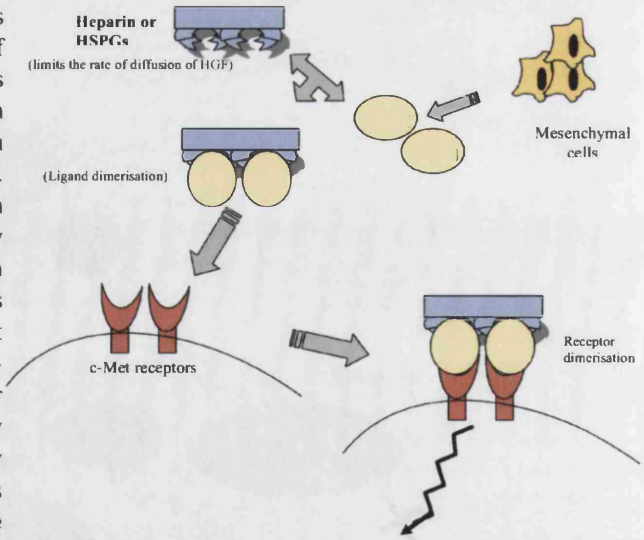


**Figure17** A schematic showing regulation of HGF induced cellular signaling by heparin/HSPGs

the c-Met proto-oncogene and induces a variety of cellular responses in various pathological conditions (figure 18). The inactive HGF/SF precursor comprises an N-terminal domain, four kringle (K1, K2, K3 and K4) domains and a C-terminal domain. The C-terminal domain is cleaved off to give the active molecule. The N-terminal domain, on the other hand, plays a critical role in receptor binding. Alternate splicing of the primary transcript produces two truncated forms of HGF/SF, namely, NK1 and NK2. Both fragments retain the ability to bind and activate the c-Met receptor *in vivo*. The three dimensional structure of the entire HGF/SF is not known although the crystal structure of the NK1 fragment was elucidated sometime ago (Chirgadze et al. 1999). The structure provides a basis for rationalisation of its agonistic activity and shows that the receptor binding sites comprise of residues from both the N-terminal domain and the kringle domain, K1, thus have implications for the mechanism of receptor-ligand interactions.

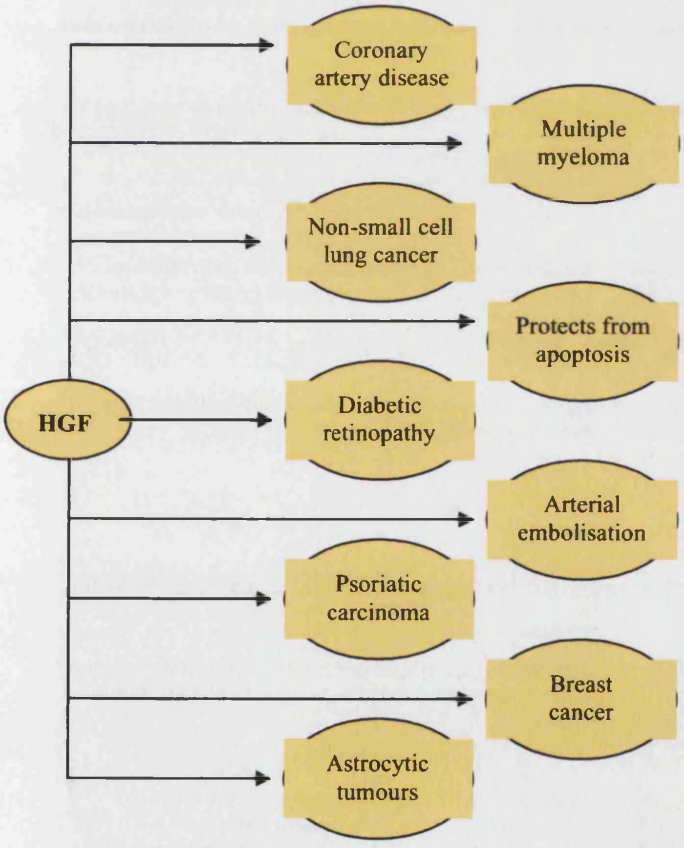


Figure 18 The different pathological processes in which HGF/SF is involved

*4.2.5. Platelet-Derived Endothelial Cell Growth Factor / Thymidine Phosphorylase*

Platelet Derived Endothelial Growth Factor (PD-ECGF) or Thymidine Phosphorylase (TP) is a 45kDa single chain polypeptide that stimulates DNA synthesis and chemotaxis of endothelial cells *in vitro* and angiogenesis *in vivo*. TP/PD-ECGF is found in the human platelet cells and the placenta. It lacks a hydrophobic leader sequence and most of the protein remains inside the producer cells. TP contains nucleotide covalently bound to the serine residues (Miyazono et al. 1991). The gene encoding human PD-ECGF is composed of 10 exons dispersed over a 4.3kb region on chromosome 22. This gene is conserved phylogenetically among vertebrates and contains six copies of potential Sp-1 binding sites just upstream of transcription start sites (Hagiwara et al. 1991). Amino acid sequencing of PD-ECGF showed that the placental form of the protein has additional 5 amino acids at the N-terminus as compared to the platelet form (Miyazono & Takaku 1991). PD-ECGF has thymidine phosphorylase activity and shares around 40% amino acid sequence similarity over 439 residues with the TP of *E.coli*, hence PD-ECGF is also known as Thymidine Phosphorylase. Human TP occurs as a 90 kDa biological homodimer. The *in vitro* and the *in vivo* effects of TP may occur by an indirect mechanism through its enzymatic activity (Usuki et al. 1992). Immunohistochemical staining on sections of human placenta from each trimester of pregnancy reveals that TP is expressed in the trophoblast and in the centre of the villous core during early pregnancy. This indicates an active role for the protein in influencing the development of the villous vascular network (Jackson et al. 1994). TP catalyses the reversible phosphorylation of thymidine to deoxyribose-1-phosphate and thymine. TP has an important role to play in cellular metabolism and in angiogenesis(table 4). Western Blotting and immuno-histochemical studies using a monoclonal antibody against TP have revealed that the expression of this protein in normal tissues is tightly regulated and that the cellular thymidine pools may serve different functions. For instance, in the nucleus it might modulate the pool for DNA synthesis. The high level expression of TP in macrophages and skin may be important for total body thymidine homeostasis (Fox et al. 1995). It has been observed that TP activity and expression levels in many tumours are higher from

**Table 4** *Some important insights into the role of TP/PD-ECGF in angiogenesis*

| References | Experiments | Biological significance |
|---|---|---|
| (Relf et al. 1997) | Correlation of the expression of TP with that of Estrogen receptor and vascular density in primary breast cancers in humans. | Angiogenesis may involve a coordinated regulation of some vascular growth factors. Implications for a broad spectrum therapy using agents that inhibit EC migration or are directly toxic to EC by blocking features common to the vascular growth factors. |
| (Imazano et al. 1997) | Spectrophotometry and immunoblotting revealed that the enzymatic activity of TP in renal carcinoma cells was 9 times higher than that in the non-neoplastic kidney tissues | There is no significant association between TP expression and other clinicopathological characteristics and thus TP expression is an unfavourable prognostic factor in human renal cell carcinoma. |
| (Creamer et al. 1997) | Ribonuclease Protection Assay and Immunohistochemical studies reveal overexpression of TP/PD-ECGF in psoriatic epidermis. | Potential role for TP in the pathophysiology of psoriasis and a probable target for antiangiogenesis therapy in the treatment of this disease |
| (Giatromanolaki et al. 1998) | Focal expression of TP in non-small-cell lung cancer is associated with marked stromal lymphocytic infiltration in the area of focal TP overexpression. | Direct association of TP/PD-ECGF and angiogenesis, stromal macrophage and fibroblast reactivity. Important in understanding the behaviour of non-small-cell lung cancer |
| (Stevenson et al.1998) | Effects on the rate of microvessel growth by TP and the degradation products, substrate and the various metabolites of the enzyme reaction. | PD-ECGF/TP affects angiogenesis by changing the relative concentrations of pyrimidine based compounds and their metabolites in the interstitial fluid surrounding the ECs. |
| (Leek et al. 1998) | Immunohistochemical studies show that TNF-α upregulates TP expression in tumour cells in vitro. There is also a positive correlation between TNF-α bound to its receptor on tumour cells and metastasis. | Explains the relationship between increased macrophage infiltration and angiogenesis in breast cancer and strengthens the idea that tumour-associated macrophages represent an important anti-angiogenic target. |
| (Balzarini et al. 1998) | 7-deazaxanthine inhibits the enzymatic reaction catalysed by PD-ECGF in a concentration dependent manner. It prevents neovascularisation in CAM. | It is the first purine derivative shown to be a potent inhibitor of purified TP and is shown to have marked inhibitory effect on angiogenesis. |
| (Nakata et al. 1998) | TP activity in gastric cancer cells was found to correlate with venous invasion. | TP plays an important role in tumour angiogenesis as well |
| (Kitazono et al. 1998) | Increased expression of TP prevents apoptosis induced by hypoxia. Among the degradation products of thymidine, 2-deoxy-D-ribose and thymine partially prevent hypoxia induce-apoptosis. | Inhibitors of TP and analogs of the degradation products of thymidine suppress growth of tumours by promoting apoptosis. |
| (Gasparini et al. 1999) | High levels of TP and low levels of VEGF characterise patients with Node Positive Breast Cancer (NPBC). When treated with adjuvant cyclophosphamide methotrexate 5-fluorouracil (CMF) the NPBC patients have the highest likelihood of favourable outcome. | Novel therapeutic strategies of adjuvant therapy based on angiogenesis. |
| (Sivridis et al. 1999) | Immunohistochemical studies reveal that TP is not a major angiogenic factor in endometrial carcinomas. | TP expression cannot be used as a prognostic factor. |
| (Tang et al. 2000) | Immunohistochemical staining of uterine neoplasm suggest that TP expression in stromal cells rather than in tumour cells plays an important role in promoting microvessel growth | Angiogenesis may have an association with tumour cell invasion in cervical squamous cell carcinoma of the cervix. |
| (Hata et al. 2000) | Transvaginal colour, pulsed Doppler ultra-sonography analysis of blood flow velocity have shown that intra-tumoral PSV is a good indicator of ovarian malignancy. | Biological difference between physiological angiogenesis (TIE-2 expression correlates with PSV in normal ovary) and pathological angiogenesis (TP expression correlates with PSV in ovarian cancer). |

those in the adjacent non-neoplastic tissues. TP may perhaps have an important role to play in the proliferation of solid tumours like those of oesophageal, gastric, colorectal and lung carcinomas (Takebayashi et al. 1996).

TP directly influences tumour oxygenation levels via its role in formation of functional vasculature (Griffiths & Stratford 1997). Inhibitors of TP are expected to suppress the growth and metastasis of tumour cells by inhibition of angiogenesis (Hirota et al.1997). The crystal structure of human TP is not yet known. However, the three dimensional structure of Pyrimidine Nucleoside Phosphorylase (PYNP) in close conformation was determined by Pugmire and Ealick (Pugmire & Ealick 1998). Since PYNP (a bacterial enzyme) shares around 40% sequence similarity with human TP, it is believed that they may share significant structural similarity as well. The crystal structure of PYNP has helped us understand the overall function of the human enzyme much better and has given us valuable insights into the rational design of inhibitors based on the crystal structure.

The other area being exploited by researchers is the enzymatic function of TP/PD-ECGF. The enzymatic activity of TP is a major determinant of the toxicity of the drugs used as anti-cancer agents. 5-fluorouracil and its pro-drugs are being extensively studied as potential anti-angiogenic therapeutics (Griffiths & Stratford 1997). A newly synthesised TP inhibitor (TPI), 5-chloro-6-[1-2-iminopyrrolidinyl)methyl]uracil hydrochloride, increases the proportion of apoptotic cells and suppresses tumour growth and inhibits angiogenesis (Matsushita et al. 1999). A new class of inhibitors has been developed which are base analogs that are not converted into nucleoside by TP, and thereby represent pure non-substrate inhibitors of TP (figure 19). Among these are the 6-(phenylalkylamino) uracil derivatives, which at μM levels inhibit both catabolic and anabolic reactions of human TP *in vitro* (Focher et al. 2000).

### *4.2.6. Angiogenin*

Angiogenin is a potent inducer of angiogenesis like the VEGF. It binds to high affinity endothelial cell surface receptors, and with lower affinity to the extracellular matrix. Among the various angiogenic molecules known, angiogenin is an enzyme (Fett et al. 1985). It is a member of the pancreatic ribonuclease (RNase A) superfamily of proteins (33% amino acid sequence identity) that has the ability to stimulate new blood vessel formation from pre-existing vasculature. The weak catalytic activity of angiogenin is believed to be very important for its angiogenic properties (table 5). In addition, the cell-binding region and the nuclear localisation signal are both important for the molecule's angiogenicity (for recent reviews see Riordan 1997, Adams & Subramanian 2000).

The crystal structure of angiogenin(Acharya et al. 1994) is very similar to that of RNase A (figure 20). It was shown that the catalytic residues in the RNase superfamily are well conserved spatially. Also the strucutre pointed out notable structural differences between angiogenin and RNase and thus providing a structural basis to the differences in the activities of the two proteins. The structure is now one of the focal point in the development of

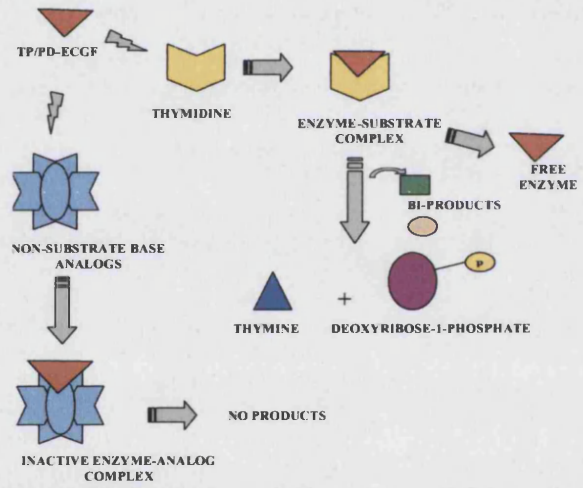


**Figure 19** A schematic representation of the reaction catalysed by TP/PD-ECGF

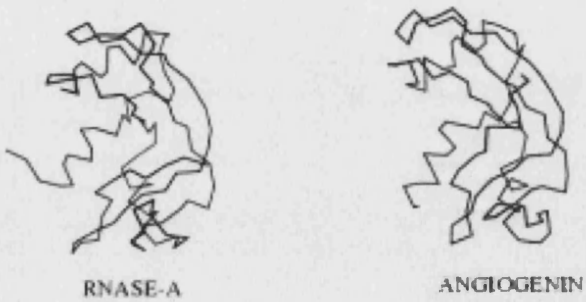


**Figure 20** The Cα traces of RNase-A and Angiogenin showing the structural similarities as well as the differences between the two (Acharya et al. 1994, Wlodawer et al. 1982)

**Table 5** *History of angiogenin*

| References | Organs / molecules / processes affected | Biological significance |
|---|---|---|
| (Koga et al. 2000) | Ovaries | Angiogenin acts as a local angiogenic factor for the development of the ovarian follicle and the ensuing formation of corpus luteum. Up-regulation is by hCG, cAMP or hypoxia. |
| (Etoh et al. 2000) | Macrophage infiltration | Angiogenin, induced by pro-inflammatory cytokines derived from the infiltrating macrophages, advances tumour angiogenesis in colorectal cancer. This results in a very low survival time for patients with high angiogenin expression. |
| (Shimoyama & Kaminishi 2000) | Gastric tissues | Increased angiogenin expression in the serum of patients with gastric cancer correlates with cancer progression. |
| (Eberle et al. 2000) | Brain tissues | Angiogenin causes malignant transformation of the glioma cells |
| (Hatzi et al. 2000) | Aortic smooth muscles | Activates second messenger pathways and inhibits proliferation of the aortic smooth muscle cells. |
| (Hu et al. 2000) | HUVECs | Nuclear translocation of angiogenin suggests a possible role in gene expression by direct binding to DNA. |
| (Hartmann et al. 1999) | Melanocytes | Up-regulation of angiogenin expression in malignant melanoma by hypoxia. The expression is associated with metastatic potential of the melanoma. |
| (Lee et al. 1999) | Ovary | Involved in the morphological changes in the ovary during the endometrial cycle. There is decreased level of angiogenin expression during regression. |
| (Malamitsi-Puchner et al. 1998) | Organs affected by IDDM | Proliferative lesions in various organs. Levels of angiogenin vary with gender and not age. Increased levels are found in women than in men. |
| (Kolben et al. 1997) | Pregnancy | In normal pregnant women, increased levels of angiogenin reflect a persisting placental transformation and remodelling processes. In patients with highly pathological Doppler flow findings, these processes are disturbed and thus the placental function is disrupted. |
| (Ozaki et al. 1996) | Eyes | Elevated levels of angiogenin reflect a breakdown of the blood-ocular barrier in the eyes and causes proliferative diabetic retinopathy. |
| (Hosaka et al. 1995) | Synovial fluid | ELISA and Immunohistochemical studies reveal that the expression levels of angiogenin are not upregulated. Hence, angiogenin possibly has a role to play in the physiology of normal synovium. |
| (Hu et al. 1994) | Endothelial cells | Enhances ECs to digest the ECM components and degrade the basement membrane and positively induces the process of neo-vascularisation. |
| (Soncin et al. 1994) | Metastasis | Angiogenin acts as an effective substrate for tumour cell adhesion during metastasis. |
| (Hu & Riordan 1993) | Cell surface actin | Angiogenin binds and displaces the cell surface actin during cell migration and tumour invasiveness. |
| (Soncin, 1992) | ECs and fibroblasts | Angiogenin acts as an effective substratum for cell adhesion and is critical in the process of angiogensis. |
| (Xiao et al. 1989) | Aortic smooth muscle cells | Depresses aortic smooth muscle cell cAMP by pertussis toxin sensitive mechanism and hence provides evidence for interaction between cellular signalling pathways. |
| (Bicknell & Vallee 1989) | Phospholipase-A2 | Activates phospholipase-A2 and stimulates EC prostacyclin secretion. This suggests mechanisms of agonist-induced intracellular arachidonate mobilisation and relevance to angiogenesis. |

inhibitors, which can be used to modulate the activity of angiogenin *in vivo* (Chen & Shapiro 1997, Papageorgiou et al. 1997, Leonidas et al. 1999).

## 5. Involvement of the heparan sulphate proteoglycans in angiogenesis and metastasis

Proteoglycans are negatively charged polysaccharide chains composed of repeating disaccharide units, attached to a core protein (Lindahl et al. 1994). Proteoglycans are found in almost all cell types. The wide spectrum of their biological functions ranges from being a purely mechanical support molecule to the one with complex cellular functions such as adhesion, proliferation and differentiation. These biological effects are due to the ability of these molecules to act as receptors for a wide range of molecules. One group of proteoglycans known as the Heparan Sulphate Proteoglycans or HSPGs is involved in both physiological as well as pathological angiogenesis.

The biological functions of HSPGs are due to their ability to bind to different kinds of angiogenic molecules including growth factors, extracellular matrix proteins, and protease inhibitors (Lindahl et al. 1994). Some of the angiogenesis related HSPG binding molecules are:

- Fibroblast Growth Factors
- Vascular Endothelial Growth Factor
- Placenta Growth Factor
- Heparin-binding Epidermal Growth Factor
- Hepatocyte Growth Factor
- Transforming Growth Factor-β
- Interferon-γ
- Platelet Factor-4
- Interleukin-8
- Macrophage Inflammatory Protein
- Interferon-γ inducible protein-1
- HIV-Tat transactivating factor
- Platelet-Derived Growth Factor
- Plieotropin

The interaction of HSPGs with the molecules depends on distinct structural features. These interactions depend mainly on the size of the polysaccharide chain and on the degree and distribution of the sulphate groups (table 6, Maccarana 1993). HSPG binding does not depend solely on the sulphate groups. For example Ishihara et al. (1994) showed that the carboxyl group of heparin is important for FGF-2 interaction. The interaction also depends on the biochemical properties of the protein. The HSPG binding regions on most proteins have been identified as a cluster of basic amino acids (Zhang et al. 1991, Zhu et al. 1990, Eriksson et al. 1991).

The binding of these growth factors to the ECM associated HSPGs is in the range of 5-50 nM. These growth factors usually accumulate in the HSPGs of ECM and the endothelial cell surface indicating that the HSPGs act as a physiological sink for these growth factors. Different mechanisms (both enzymatic and non-enzymatic) mobilise the ECM-stored growth factors. Some of the enzymes involved are plasmin (a serine protease), heparitinase, heparinase, heparanase, phospholipase C and the non-enzymatic free glycosaminoglycans.

HSPGs mediate the binding of these growth factors to their respective tyrosine kinase receptors (Mustonen & Alitalo 1995). There exists a controversy about the absolute requirement of HSPGs for the interaction of the growth factors with their receptors. One group reported that heparin deficient CHO cells transfected with the FGFR-1 do not bind to FGF in the absence of heparin. On the other hand, another group showed that FGFR-1 expressed in the CHO cell mutants retain their ability to bind to FGF even when heparin is not present (Yayon et al. 1991, Roghani et al. 1994). Recent solution experiments have shown that

**Table 6** *The different sulphate groups on HSPGs*

| Angiogenic molecules | The important sulphate groups |
|---|---|
| FGF-2 | 2-O- and N-sulphate groups (Macarena, 1993) |
| FGF-1 and FGF-4 | 2-O- and 6-O-sulphate groups (Grimond et al. 1993) |
| HGF | 6-O-sulphate groups of $GlcNSO_3$ (Lyon & Gallagher, 1994) |
| HIV-Tat Protein | 2-O- and 6-O- and N-sulphate groups (Rusnati et al. 1994) |
| Interleukin-8 | 6-O-desulphated heparin, N-desulphated/N-acetylated heparin (Rusnati et al. 1994) |

heparin although is not absolutely necessary for the formation of the receptor-ligand complex, it does enhance the binding of FGFR to FGF-2 (Rusnati et al. 1994). What is common about the ternary interaction among the HSPGs, growth factors and the receptors is the ligand-induced receptor dimerisation for activating the tyrosine kinase receptors that is required to induce a mitogenic response (Ruoslahti & Yamaguchi 1991, Vlodavsky et al. 1991). The storage of the HSPGs in the ECM increases the local concentration of the growth factors and hence a decrease in the radii of diffusion. This favours ligand oligomerisation, receptor interaction and thus signalling (figure 21).

Molecules that bind to an angiogenic growth factor that will interfere with the latter interacting with ECM associated proteoglycans are potential compounds (table 7) with angiostatic activity. These molecules fall into two categories:

- Molecules that compete with the heparin-binding growth factors for HSPG interaction (eg. polycationic compounds).
- Molecules that compete with the HSPGs for growth factor binding interaction (eg. polyanionic compounds).

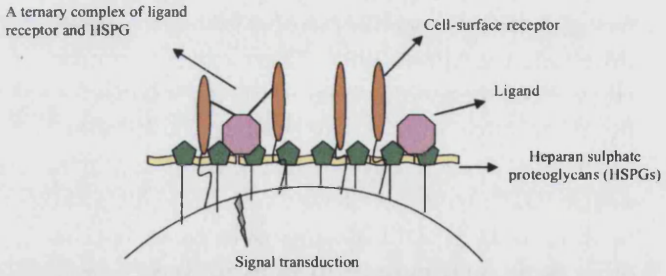


Figure 21 Role of HSPGs in ligand-induced receptor dimerisation and thus cell signalling

## 6. Role of Proteases and Protease Inhibitors in Angiogenesis

Controlled degradation of extracellular matrix (ECM) is essential in many physiological processes including developmental tissue remodelling, angiogenesis, tissue repair, and normal turn over of the ECM. One of the important steps in angiogenesis is the local proteolysis of the basement membrane. Local proteolysis is facilitated by proteases outside the endothelial cell or perhaps bound to the cell surface and/or secreted by the cell itself. Extracellular proteases cooperatively influence matrix degradation and invasion of cells through proteolytic cascades (figure 22). Every individual protease has a distinct role to play in angiogenesis and also in tumour-induced growth, invasion and

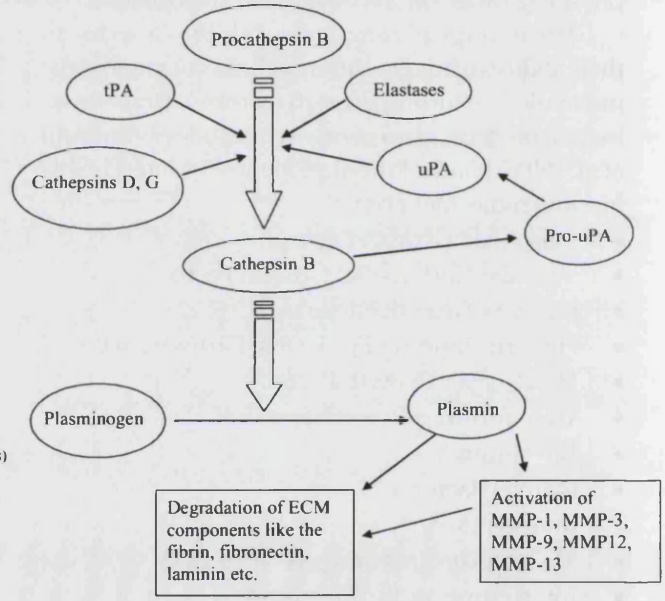


Figure 22 An example of a proteolytic cascade

**Table 7** *Some of the known inhibitors of HSPGs*

| Inhibitors | Mechanism of action |
|---|---|
| Protamine | Compete with the heparin-binding growth factors for HSPG interaction |
| Chemokines | Compete with the heparin-binding growth factors for HSPG interaction |
| Tecogalan sodium | Compete with the heparin-binding growth factors for HSPG interaction |
| Alpha-2 Macroglobulin | Compete with the HSPGs for growth factor binding interaction |
| Suramin and its derivatives | Compete with the HSPGs for growth factor binding interaction |
| Pentosan polysulphate | Compete with the HSPGs for growth factor and receptor binding interaction |

metastasis. Endopeptidases are classified into five major categories:

- Cysteine proteases: cathepsins B, L, K, Q, S; caspases; bleomycin hydrolases.
- Aspartic proteases: cathepsins D and E.
- Serine proteases: plasmin, urokinase-type plasminogen activator.
- Threonine proteases: proteasome.
- Metallo proteases: gelatinases A and B.

Proteases are normally synthesised as latent forms known as zymogens that must be converted to their active, mature forms. This conversion may occur via other enzymes or autocatalytically. It is hypothesised that the proteases interact with each other resulting in the activation of other proteases that then collectively degrade all the ECM components. For example, the activation of Procathepsin B by tPA is thought to initiate a proteolytic cascade whereby the end product of each degradation acts as an enzyme for the conversion of another latent form of a protease into its mature active enzyme. Overexpression and activation of one protease may provide a proteolytically active environment for the onset of local proteolysis of the basement membrane during angiogenesis. Cathepsin B was the first lysosomal protease that was associated with breast carcinoma.

Degradation of the extracellular matrix is also important for tumour cell invasion. Experiments have revealed that most proteases can degrade the ECM components both extracellularly at neutral *p*H under normal physiological conditions and at acidic *p*H under pathological conditions (Griffiths 1991, Montcourrier et al. 1997, Baron 1989). Intracellular degradation of the ECM is also important for tumour cell invasion. Coopman et al. (1996) have shown a correlation between the invasive ability of different cancer cell lines and their ability to phagocytose the ECM.

Proteases play a role in cancer other than degradation of the extracellular matrix. Masson et al. (1998) suggest that MMPs are involved in early alterations leading to tumour formation. Mice deficient in stromelysin-3 exhibit lower tumour incidence and tumour size after carcinogen treatment. Overexpression of some MMPs leads to development of pre-malignant and pre-neoplastic lesions (Sympson et al. 1995), suggesting that MMPs participate in different stages of tumour progression prior to invasion and metastasis (Wilson et al. 1997, Moser et al. 1990, Su et al. 1992). Proteases are believed to increase cell proliferation by activating growth factors or liberating them from the ECM where they are sequestered. Growth factors like bFGF, EGF, IGF, TGF-β and VEGF are bound to ECM and can be released upon proteolysis of the ECM components (Taipale & Keski-Oja 1997, Whitelock et al. 1996). Noel et al. (1993) showed that the ability of fibroblasts to promote tumourigenicity of MCF7 cells requires matrigel containing low-molecular weight factors. MMP inhibitors abolish the tumour promoting effects of the fibroblasts, suggesting that MMPs from the fibroblasts release growth factors from the matrigel. Proteases enhance the growth of the tumours beyond 1-2 mm.

We now know that proteases are important for the process of angiogenesis and their increased expression, increased activity and altered localisation of several proteases are associated with tumour progression. Depending upon the source or the cell type of a cytokine, they can act as both inducers and

**Table 8** *Matrix Metallo Proteinases and their receptors*

| MMPs | Substrates |
|---|---|
| Colagenase -1 (MMP-1), An interstitial collagenase | IGFBP-2, IGFBP-3, IL-1b and TNF-a |
| Gelatinase A (MMP-2), type-IV collagenase | FGFR1, IGFBP-3, IGFBP-5, IL-1b, TNF-a, TGF-b1, and MCP-3 |
| Stromelysin-1 (MMP-3), proteoglycanase | HB-EGF, IGFBP-3, IL-1b and TNF-a |
| Matrilysin (MMP-7) | TNF-a |
| Gelatinase B (MMP-9), type-V collagenase | IL-1b, IL-8, PF-4, TGF-b1 and TNF-a |
| Stromelysin -3 (MMP-11) | IGFBP-1 |
| Metalloelastase (MMP-12), a macrophage elastase | TNF-a |
| MT1-MMP (MMP-14) | TNF-a |
| MT2-MMP (MMP-15) | TNF-a |

as inhibitors of MMP expression (table 8). Both *in vitro* and *in vivo* studies have shown that protease inhibitors reduce tumourigenicity. The wide expanse of knowledge gained so far still leaves several key questions unanswered. Analysis of multiple proteases in a single model system would further enhance the elucidation of the function of proteases both in normal physiological conditions and in tumour environment. This will help in the design of better protease-based drugs and inhibitors to combat malignancy.

## 7. Involvement of environmental factors in the process of angiogenesis

The location and the ability to synthesise various vasoactive mediators make the vascular endothelium a key component in the maintenance of vascular homeostasis. Physiology of angiogenesis is constrained by the need for various environ-mental factors that affect vascular functions by disturbing the vascular endothelium. Some of these factors that contribute to the process of angiogenesis have been discussed below:

### *7.1. Hypoxia*

Endothelial cells have to cope with all changes occurring within the blood as they are the first cell layer in contact with the blood. One of these changes is the variation in the oxygen tension. Hypoxia directly regulates the expression of several genes, activates ECs and initiates a series of reactions. One of the most spectacular effects of hypoxia on endothelial cells is to increase their adhesiveness for neutrophils (Ginis et al. 1993, Milhoan et al. 1992, Arnould et al. 1993). Neutrophils not only adhere both *in vitro* and *in vivo* (Baudry et al. 1998) but are also activated when in contact with hypoxic HUVECs (Arnould et al. 1994). On the other hand, hypoxia has been shown to decrease free radical production and cytokine synthesis by neutrophils in response to various stimuli (Derevianko et al. 1996). The contact of neutrophils with activated endothelial cells delays apoptosis, thereby prolonging their useful life (Ginis & Faller 1997). Hypoxia can also initiate all stages of recruitment and activation of neutrophils in ischemic organs.

ECs display a marked increase in pro-coagulant activity when subjected to hypoxia for more than 24 hours (Gertler et al. 1991). This correlates with a marked decrease of thrombomodulin expression at the surface of endothelial cells (Ogawa et al. 1990). The expression of tissue factor and of some cytokines like interleukin-6 (IL-6), interleukin-1α (IL-1α), interleukin-8 (IL-8) and MCP-1 is also enhanced under hypoxic conditions (Yan et al. 1995, Karakurum et al. 1994). The production of these pro-inflammatory cytokines may perhaps explain the development of an inflammatory response in ischemic tissues.

Both vasorelaxing and vasoconstricting agents release ECs to regulate the vascular tone under normal conditions (Chien et al. 1998). Hypoxia favours vasoconstriction (Kourembanas & Bernfield 1994). In contrast, hypoxia generally enhances the release of endothelin-1 (ET-1) by endothelial cells *in vitro* but this depends on experimental conditions since, in two cases, a decrease has been reported (Gertler & Ocasio 1993, Kourembanas et al. 1991, Markewitz et al. 1995). *In vivo*, an increase in ET-1 circulating level is also observed and this is correlated with an induction of ET-1 gene transcription in lungs (Aversa et al. 1997).

Short-term hypoxic endothelial cell-conditioned medium induces the proliferation of smooth muscle cells (SMCs). In addition, hypoxia increases the release of growth factors by stimulating the production of some ECM proteins, such as TSP-1 in human endothelial cells (Phelan et al. 1998). Kourembanas' laboratory suggest that the proliferative response is regulated by a negative feed back loop mediated through smooth muscle cell-derived CO. Hypoxic SMCs release CO which decreases the transcription of PDGF-BB and ET-1 by increasing the c-GMP content of the ECs. This cascade eventually inhibits smooth muscle cell proliferation initiated by hypoxia (Morita & Kourembanas 1995).

Endothelial cell proliferation itself is also affected by hypoxia. The most potent and specific mitogen for the endothelial cells is VEGF. It is known to initiate angiogenesis *in vivo* (Ferrara & Davis-Smyth 1997). Its expression is upregulated by hypoxia in numerous cell types including endothelial cells (Liu et al. 1995, Mukhopadhyay et al. 1995b). Hypoxia functionally up-regulates the mitogenic response inducing receptor of VEGF,

namely VEGFR-2 or kinase-insert domain receptor (KDR) (Gerber et al. 1997, Waltenberger et al. 1996).

The release of all these cytokines and growth factors by cells like the endothelial cells is generally the result of a complex cascade of processes that ends with the activation of transcription factors. The prototype of all transcription factors regulated by hypoxia is the hypoxia inducible factor-1 (HIF-1). HIF-1 is a heterodimer composed of the two subunits, HIF-1α and HIF-1$^-$ or ARNT (aryl/hydrocarbon receptor nuclear translocator), the first being upregulated by hypoxia (figure 23; Wang & Semenza 1993a, Guillemin & Krasnow 1997, O'Rourke et al. 1997).

A recent observation states the presence of HIF-1 binding sites in the 5' or 3' flanking regions of the genes that encode erythropoietin, VEGF (Liu et al. 1995), VEGF receptor, Flt-1 (Gerber et al. 1997), glucose transporter, Glut-1 (Behrooz & Ismail-Beigi 1997) and several glycolytic enzymes (Semenza et al. 1994). However, the hypoxia-dependent increase of VEGF expression seems to be controlled both at the transcriptional level and at the level of mRNA stability (Ikeda et al.1995, Shima et al.1995). Hypoxia strongly affects the regulatory pathways of ECs as well as of SMCs, resulting in the formation of conditions favourable to vasoconstriction and proliferative activity, participating in the remodelling of the vascular wall.

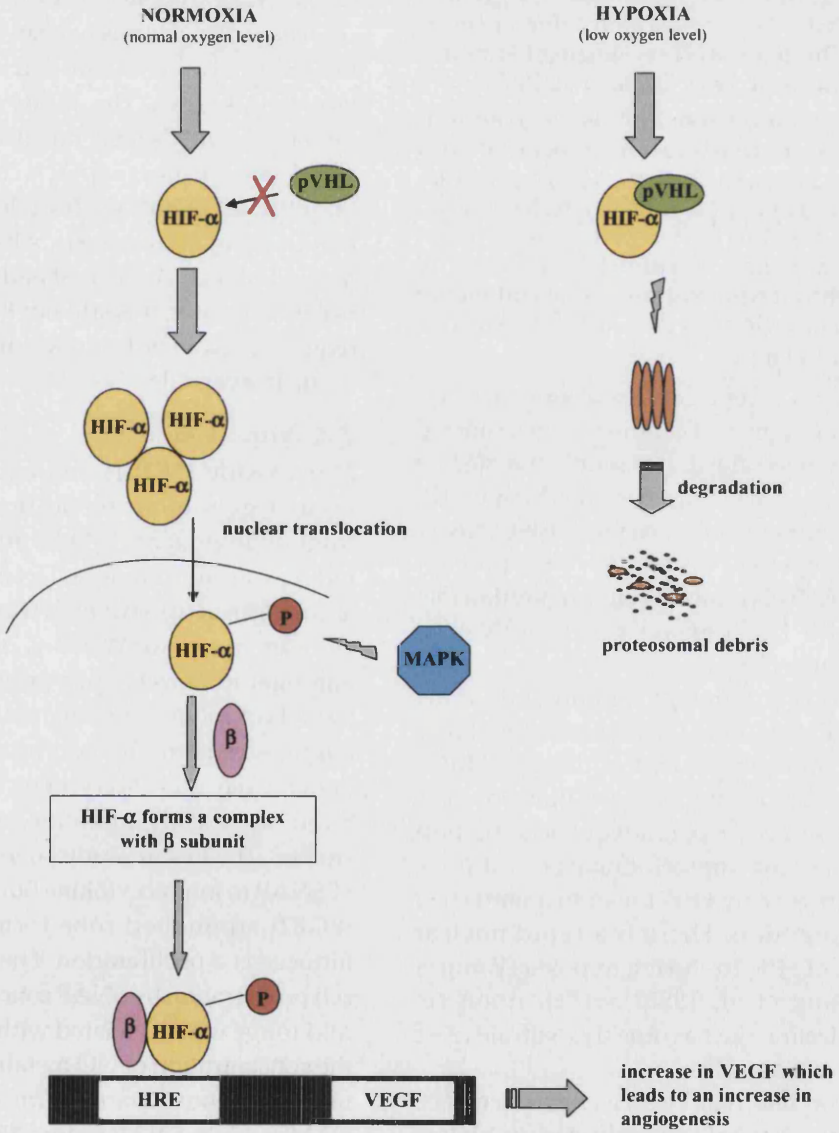


**Figure 23** A schematic representation of HIF-a induced signal transduction under normal and low oxygen levels

When chronic hypoxic conditions persist, the expression of growth factors, cytokines and pro-coagulation molecules is increased which gives rise to several pathological conditions. Some of these conditions are:

- Blood stasis, a situation commonly associated with chronic venous insufficiency (Michiels et al. 1996).
- Second, adaptation to hypoxia represents a key step in tumor progression. On the one hand, these conditions induce the activation of HIF-1 allowing the cells to survive via an adaptive modification of their energetic metabolism. On the other hand, HIF-1 is involved in the establishment of vascular supply. Neoangiogenesis is triggered off by growth factors released by the tumour which itself is becoming hypoxic while enlarging (Semenza 1998a, Zhong et al. 1999, Dachs et al. 1997).
- Pulmonary hypertension is a frequent hemodynamic complication associated with respiratory system disorders. A short exposure of the pulmonary circulation to a reduced level of oxygen tension both *in vitro* and *in vivo* elicits an immediate vasoconstrictive response probably through potassium channel inhibition in smooth muscle cells (Vender 1994, Ward & Robertson 1995).

Analysis of erythropoietin gene regulation uncovered a general mechanism of cellular oxygen sensing and transcriptional control. Transiently transfected reporter genes linked to the erythropoietin enhancer showed similar hypoxia-inducible activity in both the hepatoma cells and a wide variety of non-erythropoietin-producing cells, suggesting that many other HIF-1-responsive genes might exist (Wang & Semenza 1993b).

HIF-1$^-$/ARNT-deficient tumour cells show reduced vascularity, and provide strong evidence linking erythropoietin regulation, through HIF-1, to hypoxia and tumour angiogenesis (Ratcliffe et al. 1998). At cellular level, cooperative effects amongst factors necessary for transcriptional activation of erythropoietin gene by HIF-1 lead to a pattern of inducible expression. There is a rapid nuclear accumulation of HIF-1α during hypoxia (Wang et al. 1995, Huang et al. 1996), which upon re-oxygenation declines just as quickly (half-life of ~5 minutes) in normoxic cells.

Hypoxic conditions induce a well-characterised battery of genes that help the cells cope with low oxygen concentrations (Semenza et al. 1998b, Tacchini et al. 1999, Guillemin & Krasnow 1997). Transcriptional upregulation has been shown to play a major role in the hypoxic induction of these genes, and action mediated by the specific binding of HIF-1 to the hypoxic response element (HRE). HIF-1$\alpha$ and HIF-1$^-$ are both constitutively expressed and do not seem to be significantly modified by hypoxia. However, while the HIF-1$^-$ protein is found in normoxic cells, nearly no HIF-1$\alpha$ can be detected under these conditions. Knockout studies show that tumour cells deficient in HIF-1$\alpha$ as well as in HIF-1$^-$ grow slower than tumours derived from control tumour cells and also show reduced levels of vascularisation (Ryan et al. 1998, Adelman et al. 1999).

The molecular mechanism by which cells sense low-oxygen concentration and induce HIF-1$\alpha$ is still largely unknown. The ability of endothelial cells (EC) to not only sense, but also to adapt to, acute and chronic changes in pO2 is critical to maintaining endothelial metabolic functions and, in turn, to maintaining homeostasis. Although the involvement of hypoxia in several pathological conditions becomes clearer, it is still not known what level of oxygen tension will render the alterations of the tissue irreversible.

### *7.2. Nitric Oxide*

Nitric oxide (NO) is an important molecule in regulating tumour blood flow and stimulating tumour angiogenesis. Inhibition of NO synthase induces an anti-tumour effect by limiting nutrients and oxygen to reach tumour tissue and thus affecting vascular growth (de Wilt et al. 2000). NO is both anti-angiogenic as well a pro-angiogenic in different models of *in vivo* angiogenesis. The role of NO in angiogenesis-mediated processes using the NO donor, S-nitroso N-acetyl penicillamine (SNAP) and S-nitroso N-acetyl glutathione (SNAG) have been studied. The *in vitro* studies demonstrated the ability of SNAP to inhibit cytokine fibroblast growth factor (FGF2)-stimulated tube formation and serum-induced cell proliferation. The inhibitory effect on cell proliferation by SNAP concentrations above the mM range was associated with significant shifts in the concentration of NO metabolites. Furthermore, using the mouse matrigel implant model and the CAM models, SNAP induced 85-95% inhibition of neovascularisation induced by FGF2 in both

*in vivo* models (Powell et al. 2000). Endothelium-derived nitric oxide (EDNO) plays an important role in the regulation of angiogenesis, whereas hypercholesterolemia (HC) impairs EDNO release. Studies in a rat model of unilateral hindlimb ischemia showed that the collateral vessel formation and angiogenesis in response to hindlimb ischemia were significantly attenuated in rats with dietary HC, possibly through decreased NO bioactivity. Augmentation of the tissue NO activity by oral L-arginine supplementation restored the impaired angiogenesis in HC, accompanied by improved NO/cGMP production (Duan et al. 2000). Therefore, a possible therapeutic approach to treat patients with peripheral arterial occlusive diseases would be to augment endogenous NO bioactivity (Duan et al. 2000).

Nitric oxide (NO) is a messenger molecule that regulates many physiological functions like immunity, vascular tone and serves as a neurotransmitter. Experiments on rats with transdermal wounds treated with Sodium Nitroprusside (SNP)/L-arginine showed a decrease in the wound collagen content. On the other hand, application of N-Nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NO synthase, resulted in an increase in the wound collagen content. The results indicate that NO plays an important role in regulating the collagen biosynthesis in skin model of a healing wound (Shukla et al. 1999).

NO has a multi-factorial role to play in tumorigenesis. NO mediates DNA damage in early phases of tumorigenesis and also augments tumour progression via angiogenesis and suppression of the immune response. NO decreases the ability of the hypoxic enhancer to bind to HIF-1 and thus suppresses VEGF by via a cyclic GMP-mediated signal pathway (Liu et al. 1998).

Nitric oxide, unlike most of the other regulators of angiogenesis, has been shown to possess both anti- and pro-angiogenic properties. Most experiments performed so far indicate that the pro-angiogenic effects of NO are due to the NO produced through the constitutive NO synthase (cNOS) and is cyclic GMP dependent. On the other hand the NO produced via the inducible NO synthase (iNOS) is mainly involved in the anti-angiogenic effects. In the CAM, NO donors have been shown to inhibit basal angiogenesis and the NOS inhibitors to promote the process (Pipili-Synetos et al. 1995). Increased iNOS expression, iNOS activity, and NO production are a means of providing an endogenous brake on the process of blood vessel formation.

### *7.3. Copper*

Preclinical and *in vitro* studies have determined that copper is an important cofactor for angiogenesis. Copper stimulates proliferation of human endothelial cells under culture. Incubation of HUVECs for 48 hours with 500 μM $CuSO_4$ in a serum-free medium, in the absence of exogenous growth factors, results in a two fold increase in cell number. This is similar to the cell number increase induced by 20 ng/ml of basic fibroblast growth factor under the same conditions (Hu 1998). Serum copper and zinc concentrations and copper/zinc ratios have been shown to increase in several types of human malignancies. Yoshida et al. (1993) showed that the tissue copper concentrations are significantly higher in metastatic carcinomas and malignant gliomas than control tissues.

Metallothionein (MT) is an intracellular storage molecule for metal ions such as zinc ($Zn^{++}$) and copper ($Cu^{++}$). Cerebral neoplasms sequester copper to modulate angiogenesis. Brem et al. (1990) have demonstrated regional differences in the responsiveness of the endothelium, the distribution of copper, and the activity of cuproenzymes. They showed that the metabolic and pharmacological withdrawal of copper suppresses intracerebral tumour angiogenesis. This finding suggests that pharmacological and metabolic alteration of the cellular microenvironment to inhibit invasiveness represents a novel therapeutic approach, especially for tumours of the brain in which malignancy is a function of regional invasiveness.

Raju et al. (1982) tested the ability of ceruloplasmin, the copper carrier of serum, for its ability to induce new capillary formation in the cornea. The molecules chosen were fragments of the ceruloplasmin molecule with and without copper, heparin and glycyl-L-histidyl-L-lysine (bound or not bound to copper ions). All the three molecules were able to induce angiogenesis provided that they were bound to copper. Fragments of the ceruloplasmin molecule also induced angiogenesis but only when copper was bound to the peptides. It was interpreted that mobilisation of copper ions is involved in the

sequence of events leading to angiogenesis and that the carrier molecules may be of quite a different nature.

### *7.4. Hypoglycaemia*

Hypoglycaemia is yet another factor that modulates the process of blood vessel formation. It usually comes into play along with hypoxia. It reduces proliferation and increases apoptosis in wild-type (HIF-1$\alpha^{+/+}$) embryonic stem (ES) cells. However, it is ineffective in ES cells with the genotype (HIF-1$\alpha^{-/-}$). Since HIF-1$\alpha$ forms a complex with aryl-hydrocarbon-receptor nuclear translocator (ARNT), the latter is crucial in the response to hypoglycaemia. A decrease in the bio-availability of ARNT reduces the intensity of neovascularisation and consequently leads to defective angiogenesis in ARNT deficient embryos (Maltepe et al. 1997). In embryos with the normal genotype (HIF-1$\alpha^{+/+}$), there occurs a situation when cells undergo nutrient deprivation during increase in tissue mass (organogenesis). This leads to an increase in the expression of VEGF that promotes vascularisation of the developing embryo. Favard et al. (1996) demonstrated that vascular endothelial growth factor expression is enhanced *in vitro* by hypoxia and hypoglycemia; and its immunoreactivity is increased in some although some experiments later showed that VEGF expression does not increase in all diabetic patients. A decrease in its bio-availability reduces the intensity of neovascularisation.

### *7.5. pH*

Among the various environmental factors affecting angiogenesis, *p*H is another independent variable that regulates the angiogenic process. Immuno-histochemical studies reveal that in the regions surrounding the necrotic tissues, cells are exposed to acidic *p*H. Shi et al. (1999) showed that acidosis along with hypoxia leads to overexpression of the cytokine IL-8 that in turn contributes to the aggressive biology of the human pancreatic cancer. Experimental studies by Hayashi et al. (2000) reveal that *p*H levels regulate the growth and metastasis of tumours. Macrophages secrete substances that stimulate angiogenesis and mitogenesis at wound sites. High lactate concentration and low *p*H regulate the secretion of angiogenesis factor or factors and change the expression of macrophage mitogens (Jensen et al. 1986). One of the most potent stimuli for increased VEGF production is hypoxia. This teleologically sound response is conserved across a wide variety of cell and tumour lines, including osteoblasts. The increased VEGF production in response to hypoxia arises from a combination of *p*H and lactate expression. Effects of elevated hydrogen ion or lactate concentration on VEGF protein production are additive.

## 8. The Physiological Importance of Endogenous Modulators in Angiogenesis

Identification of the molecular pathways of the angiogenic response has been a major focus of interest in academia and industry. Although the full identification of players and their cross-talk is still at its infancy, it appears that partial blockade of one of the steps in the angiogenesis cascade, is sufficient to affect capillary morphogenesis. In addition to the generation of antibodies or chemical mimetics to interfere with particular steps during vascular organisation, several endogenous (or physiological) molecules have also been identified. The list of endogenous modulators of angiogenesis is growing and can offer additional and important tool for the generation of therapies to restrain tumour vascularisation. This section of the review will focus on the major endogenous molecules that modulate the process of blood vessel formation.

### *8.1. Integrins*

Integrins are a family of heterodimeric cell surface receptors, which mediate adhesion of cells to the ECM proteins and sometimes to other cells. Endothelial cells can express at least 5 or 6 different types of integrins (Luscinskas & Lawler 1994). Cell surface expression of integrins can be controlled by various growth factors, including, notably VEGF. They are regulated by the growth factors that are known to control the process. Integrins, especially the av integrins mediate those biological processes that are needed to organise a vasculature like adhesion, proliferation, migration and are expressed by cells that are involved in the process of vessel formation (Varner et al. 1995, Stromblad & Cheresh 1996a, Hynes & Bader 1997)

The av integrins represent a sub-family of integrins that share a common av subunit in combination with one of the five ß subunits (ß1, ß3, ß5, ß6 and ß8). Endothelial cells can express at least avß3 and avß5 and perhaps avß1 as well since they do

express ß1. Resting ECs express little or no avß3. However, this gets markedly upregulated on vessels undergoing angiogenesis (Varner et al. 1995, Stromblad et al. 1996b, Brooks et al. 1995, Friedlander et al. 1996). Integrin avß3 has been implicated in the pathophysiology of malignant tumours. It is suggested that the interaction of the circulating tumour cells with platelets represent a potential mechanism for tumour cell arrest within the vasculature (figure 24). During blood flow, dynamic forces physically oppose cell attachment. Therefore, integrins are essential to support cell arrest. For example, intravascular attachment of leukocytes and platelets during inflammation and thrombus formation is highly regulated and dependent upon integrin activation (Diamond & Springer 1994, Altieri 1999).

Characterisation of angiogenic factors has revealed that remodelling of the ECM occurs during angiogenesis, mediated by integrins that are found on the endothelial cell surface membrane. Integrins function in cell-to-cell and cell-to-extracellular matrix (ECM) adhesive interactions and transduce signals from the ECM to the cell interior and vice versa. Since these properties implicate integrin involvement in cell migration, invasion, intra- and extravasation, and platelet interaction. Tumour progression leading to metastasis appears to involve equipping cancer cells with the appropriate adhesive (integrin) phenotype for interaction with the ECM.

Integrin subunits can heterodimerise in over 20 combinations. Different integrin combinations may recognise a single ECM ligand, while others bind several different ECM proteins. Of the wide spectrum of integrin subunit combinations that are expressed on the surface of cells, avß3 has been identified as having an especially interesting expression pattern among vascular cells during angiogenesis and vascular remodelling. The expression of avß3 on activated endothelial cells suggests that this integrin may have an important function during angiogenesis and perhaps developmental neovascularisation. In tumour models, inhibition of blood vessels by av integrin antagonists, not only blocked tumour-associated angiogenesis but in some cases caused tumour regression as well (Kerr et al. 1999). The ability of some organs to support neovascularisation, even in the absence of av integrins, may provide a clue to some of the alternate integrins that may mediate vessel formation. Two

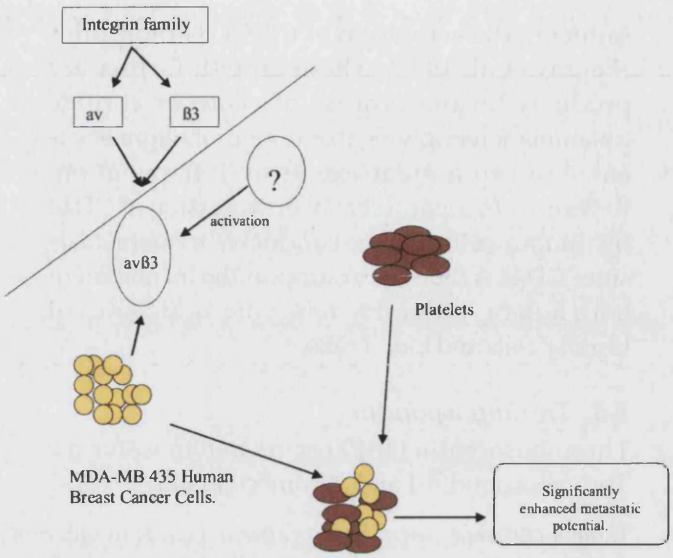


**Figure 24** Control of metastatic potential in human breast cancer via integrin activation

angiogenic pathways are characterised by distinct av integrins, avß3 and avß5. The biological relevance of these distinct angiogenic pathways remains a challenge because the temporal and spatial coordination between the various molecular players still needs to be addressed.

### *8.2. CD44*

CD44 is a multi-functional adhesion molecule involved in cell-to-cell and cell to extracellular matrix interactions, regulation of cell traffic and trapping of growth factors and cytokines. CD44 functions as a growth factor presenting molecule. The growth factors and cytokines modify the expression of CD44 in such a way that it results in changes in the biological properties of the cells. These serve as both suppressors and promoters in carcinogenesis and the subsequent progression of malignancy. Hyaluronic acid is the principal ligand of CD44 (Underhill 1992, Iozzo & Muller-Glauser 1985). It has been reported that a matrix rich in this compound supports tumour growth and invasion. The modification of CD44 in both host and tumour cells is believed to play an important role in tumour progression. These serve as both promoters and suppressors in carcinogenesis and their progression to malignancy. Growth factors modify CD44 expression and function (table 9) by either increasing or decreasing their expression of the different isoforms of CD44 or affecting the glycosylation pathways of CD44 (Kincade et al. 1997) or by

inducing the activation of the CD44 molecules (Legras et al. 1997). These growth factors are produced in the process of acute or chronic inflammation implying that tumour malignancy is enhanced to a great extent by inflammation. Experiments suggest that overexpression of CD44 by tumour cells may be conducive to metastasis since CD44 is thought to support the formation of both homotypic and heterotypic aggregates of tumour cells and blood cells.

### *8.3. Thrompospondin*

Thrombospondin (TSP) occurs in two isoforms: Thrombospondin-1 and Thrombospondin-2. TSP-1 is a homotrimeric, matricellular, adhesive, 450 kDa glycoprotein. Thrombospondins regulate cellular phenotype during tissue genesis and repair. It facilitates the bringing together of the ECM components, cytokines, growth factors, membrane receptors and extracellular proteases (Chen et al. 2000). TSP facilitates smooth muscle cell proliferation and also suppresses capillary growth in angiogenesis assays (figure *25*). TSP-1 is known to reduce tumour size in transgenic overexpressors (Iruela-Arispe et al. 1999). TSP-1 binds to several cell-surface receptors. The binding sites for these receptors on TSP-1 are dispersed throughout the molecule and most domains are known to bind

**Table 9** *Different growth factors affecting CD44 in different types of target cells.*

| Growth factors | Endothelial cells | Tumour cells | Other cell types | Fibroblasts | References |
|---|---|---|---|---|---|
| VEGF | HUVECs | | | | (Griffioen et al. 1997) |
| FGF-2 | HUVECs | | | Human lung fibroblast | (Romaris et al. 1995) (Griffioen et al. 1997) |
| IFN-α (interferon-alpha) | | Renal cell carcinoma | | | (Hathorn et al. 1994) |
| PDGF | | Human neuroblastoma | | | (Fichter et al. 1997) |
| TGF-β | | Rat mammary carcinoma | | Human lung fibroblast | (Hamada et al. 1998) (Romaris et al. 1995) |
| IFN-χ (interferon-gamma) | | | Myelomonocytic cell | | (Mackay et al. 1994) |
| TNF-α | | | Myelomonocytic cell | | (Mackay et al. 1994) |
| IL-2 | | Human renal cell carcinoma | | | (Hathorn et al. 1994) |
| NGF (nerve growth factor) | | Human neuroblastoma | | | (Fichter et al. 1997) |
| IGF-1 (insulin growth factor-1) | | Human neuroblastoma | | | (Fichter et al. 1997) |
| HGF | Human Vascular Endothelial Cells | | | | (Hiscox & Jiang, 1997) |
| EGF | | | | Mouse fibroblast | (Zhang et al. 1997) |
| GM-CSF (Granulocyte, Macrophage-Colony Stimulating Factor) | | | CD34+ human hematopoietic progenitor cell. | | (Legras et al. 1997) |
| IL-3 | | | CD34+ human hematopoietic progenitor cell. | | (Legras et al. 1997) |
| SCF (Stem Cell Factor) | | | CD34+ human hematopoietic progenitor cell. | | (Legras et al. 1997) |

more than one receptor. The anti-angiogenic domain of TSP-1 has been mapped to the type-I (properdin) repeat (Iruela-Arispe et al1999). A naturally occurring inhibitor of TSP-1 is the Histidine-rich glycoprotein (HRGP), which co-localises with TSP-1 in such a way that the anti-angiogenic epitope of the molecule gets masked (Simantov et al. 2001).

Experiments done by Taraboletti et al. (2000) demonstrated that TSP-1 specifically promotes angiogenesis *in vivo* but neutralises neo-vascularisation induced by fibroblast growth factor-2 (FGF-2). The molecule has dual behaviour due to the presence of domains with opposite functions that also differ in the ability to activate the degradative and invasive behaviour of the vascular endothelium. The pro-angiogenic effect of TSP-1 is on account of the 25kDa heparin-binding fragment that is conversely counteracted by a 140kDa fragment. According the experiments conducted by this group this dual role of TSP-1 in angiogenesis most probably relies on the environmental set up whereby one or the other proteolytic fragment becomes available or functional and thus has different end-results.

Disruption studies with thrombospondin-2 gene have shown that the TSP-2 null mice have a very complex phenotype characterised by a variety of connective tissue abnormalities and increased microvessel density in skin and sub-cutaneous tissues (Bornstein et al. 2000). It is believed that this phenotype is probably due to the non-clearance of MMP-2 in the extracellular matrix (ECM). Experiments have shown that the TSP-2/MMP-2 complexes are endocytosed by Low-density Lipoprotein-receptor Related Protein (LRP). This helps in regulating the MMP-2 levels in the ECM (Yang et al. 2001).

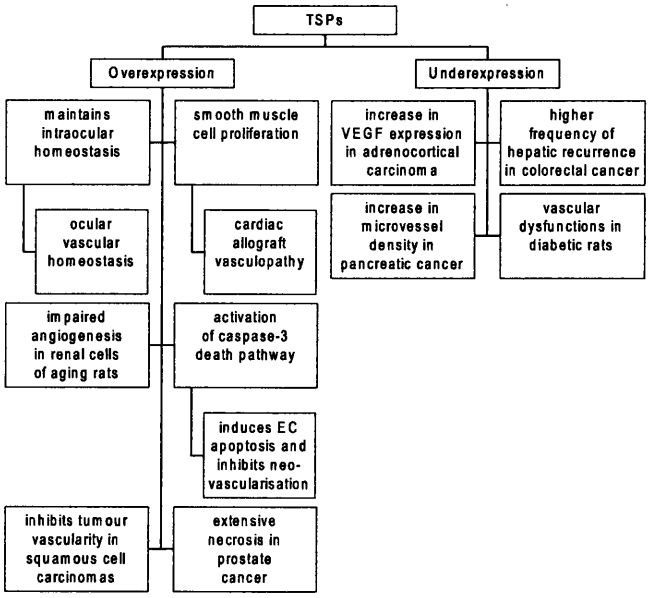


**Figure 25** An insight into the role of thrombospondins when overexpressed and underexpressed in the cells

Neovascularisation is a common feature of human cancers and associated with this phenotype is the decreased expression of TSP-1. The gene for TSP-1 (THBS-1) is regulated by p53, anoxia (Tenan et al. 2000) and Rb. Methylation of the 5' CpG island of the THSB-1 gene leads to down-regulation of its expression (Li et al. 1999). The unique *cysteine-serine-valine-threonine-cysteine-glycine* (CSVTCG) binding domain of TSP-1 also has an important role to play. Immunohistochemistry and computer image analysis have revealed that TSP-1 and its CSVTCG receptor have a decreased expression during foetal repair as cells migrate to the epithelial surface thereby suggesting that the receptor finds a role in keratinocytic maturation, differentiation and epithelial surface formation (Roth et al. 1999). Primary tumour cells sometimes produce high levels of TSP-1 in order to prevent the progressive growth of smaller tumours. Volpert et al. (1998) verified this by showing that human fibrosarcoma line HT1080 induce concomitant resistance and held the experimental B16/F10 melanoma metastases in the lung of nude mouse in check by producing high levels of TSP-1.

TSP-1 interacts specifically with several cell-surface receptors, growth factors, ECM components and heparan sulphate proteoglycans. This ability of TSP-1 to bind to several molecules and its ability to inhibit proteases suggests that exposure to TSP-1 probably initiates many intracellular signals and influences specific responses from different cell types. Integration of these molecular signals and interactions would lead to novel therapeutic strategies for controlling cancer progression and metastases (Roberts 1996). However, one likely drawback is that the therapeutic application of TSPs might potentially be associated with impaired wound vascularisation and tissue repair (Streit et al. 2000).

### *8.4. Angiopoietins*

Angiopoietins are VEGF's most important partners in vascular formation. The angiopoietins were

discovered as ligands for the TIEs, a family of tyrosine kinase receptors. These receptors, TIE-1 and TIE-2, are expressed within the vascular endothelium as well as some other cells such as those involved in haematopoiesis. The angiopoietin family consists of four members of which only two have been well characterised, namely Ang-1 and Ang-2 (figure 26). All these represent the spliced counterparts of the same gene (Maisonpierre et al. 1997, Valenzuela et al. 1999). The primary receptor for these ligands is the TIE-2. It still remains unclear if TIE-1 has independent ligands or if these current angiopoietins bind to TIE-1 as a second component of a heterodimer complex under certain conditions. The angiopoietins influence a variety of pathophysiological conditions (figure 27)

### *8.4.1. Angiopoietin-1*

Experiments with mice engineered to lack Ang-1 have shown that embryos lacking in either or both of Ang-1 and TIE-2 develop a normal vasculature. However, this vasculature does not undergo normal remodelling (Suri et al. 1996, Dumont et al. 1994). The heart seems to be the most affected with failures in association between the endocardium and the myocardium. The endothelial cells (ECs) in many vascular beds do not integrate properly with the underlying support cells that provide Ang-1 to its receptor. As a result the vascular beds cannot remodel into small and large vessels (Suri et al. 1996). Ang-1 optimises the way in which the ECs associate with the support cells so that they can receive other important signals from the environment for vessel formation (Suri et al. 1996, figure 28).

Overexpression of Ang-1 in transgenic mice results in marked increase in vessel size unlike VEGF that increases the vessel number. Ang-1 stabilises the vessel wall. They lead to vessels that are resistant to leak. These properties of Ang-1 suggest that Ang-1 counters the effect of VEGF on vascular function and integrity (Larcher et al. 1998, Thurston et al. 1999). Diseases characterised by damaged and leaky blood vessels like diabetic retinopathy, oedema would benefit enormously from the Ang-1 therapeutic treatment. This belief is supported by experiments involving adenoviral administration of Ang-1to adult animals (Thurston et al. 2000).

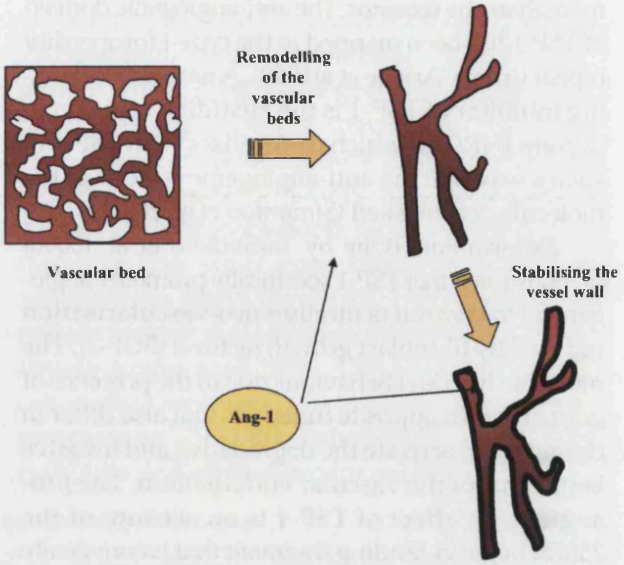


**Figure 26** Schematic representation of the role of Angiopoietin-1 during vessel formation

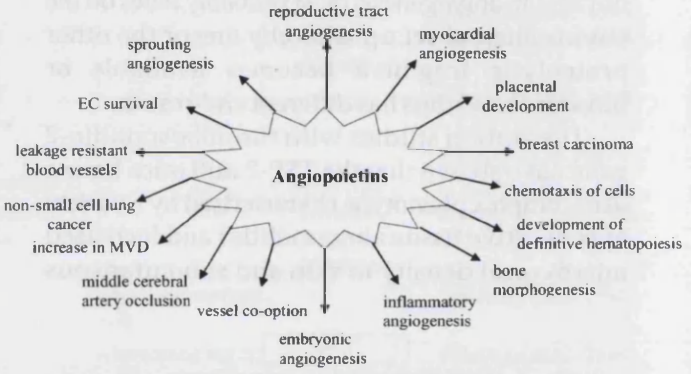


**Figure 27** Angiopoietin induced biological effects in different cells and tissues

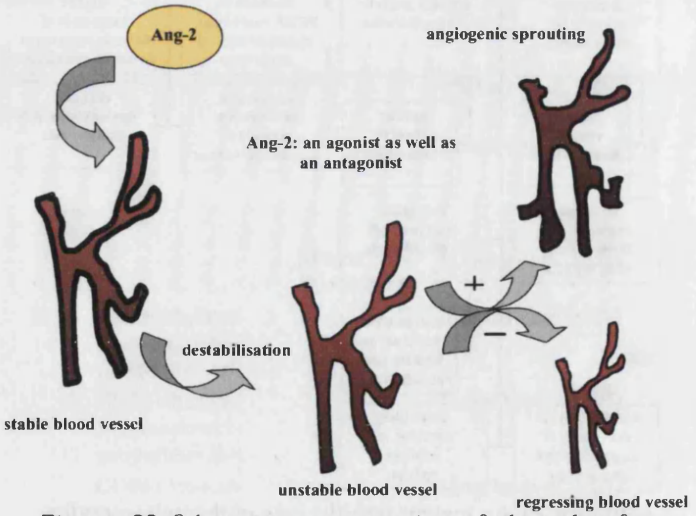


**Figure 28** Schematic representation of the role of Angiopoietin-2 during vessel formation

### *8.4.2. Angiopoietin-2*

Ang-2 is homologous to Ang-1 and can bind to TIE-2 with similar affinity as Ang-1. However, this Ang-1 homolog differs from its isofrom in its ability to either activate or inhibit/antagonise TIE-2 depending upon the cell or source of the receptor. It is proposed that Ang-2 acts as a destabilising signal that reverts the endothelial cells to a more plastic and tenuous state (Maisonpierre et al.1997, figure 29).Ang-2 is one of the earliest markers of tumours that develop by co-opting with the existing host vessels. Ang-2 is a marker for the co-opted vessels themselves and not the tumour cells. Hence, Ang-2 can perhaps be used to detect presence of tumours even when the tumour itself cannot be detected. It is thought that Ang-2 blocks interaction of Ang-1 with the TIE-2 and hence the leaky and haemorrhagic vessels are formed by the tumour- derived VEGF (Holash et al.1999, Zagzag et al. 1999).

### *8.5. Angiotensins*

The components of the Renin-Angiotensin System (figure 29) function as growth factors besides controlling blood volume and homeostasis. It is an important factor in pathogenesis of cardiovascular diseases including hypertension and congestive heart failure.

The angiotensins act in opposition (table 10) both as growth regulators and in the control of some cardiovascular functions. These opposing action suggest that the components of RAS (figure 30) may modulate the endogenous healing process and/or the persistence of chronic inflammation and proliferative pathology.

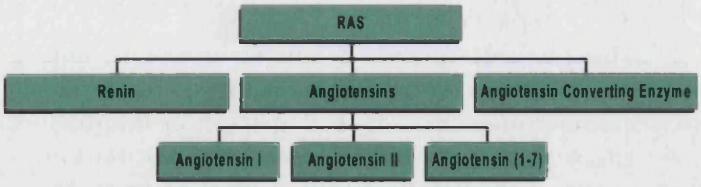


**Figure 29** The components of the Renin-Angiotensin System (RAS)

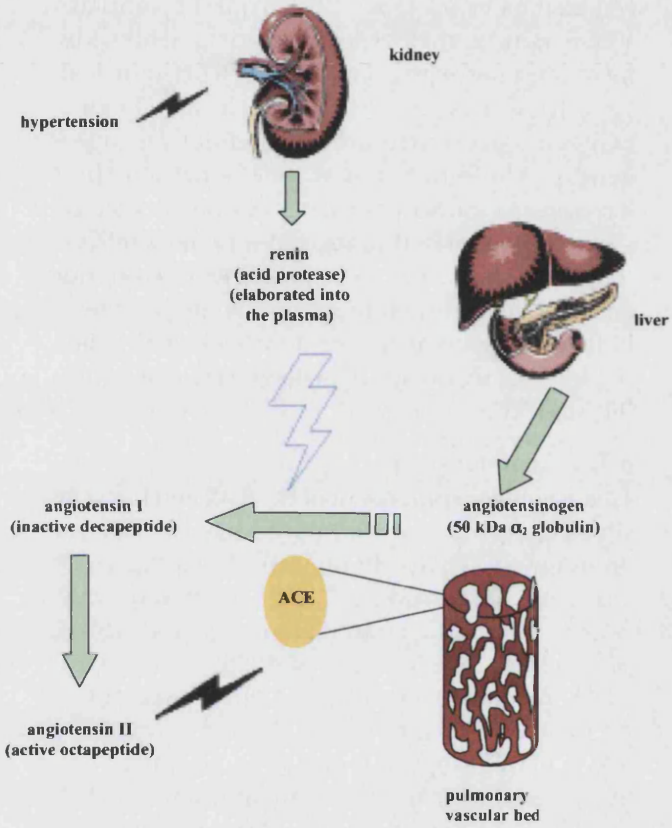


**Figure 30** A schematic overview of the Renin-Angiotensin System

**Table 10** *The differences between the two receptor types of the angiotensins*

| AT1 | AT2 |
|---|---|
| Responsible for all known physiologic actions of angiotensin II | The role of AT2 receptor has not been as well characterised as the AT1 receptor |
| mediates cell growth or cell proliferation | activation of the receptor induces inhibition of cell proliferation and possibly mediates cell differentiation |
| facilitates angiogenesis | inhibits angiogenesis |
| involved in vasoconstriction | involved in vasodilation |
| increases accumulation of angiotensin II mRNA via activation of PKC and release of $Ca^{2+}$ ions | inhibits accumulation of angiotensin II mRNA via activation of protein tyrosine phosphatases. |
| Angiotensin II induces the expression of Angiopoietin-2 via AT1. | Angiotensin II induces the expression of Angiopoietin-2 via AT2. |

*8.5.1. Angiotensin II*

This molecule plays an important role in the remodelling of the heart and the vessels after myocardial infarction via cell growth-promoting effects(Baker etal.1992).It has been shown to induce angiogenesis in the rabbit cornea, embryonic chorioallantoic membrane and rat cremaster muscle (Fernandez et al. 1985, Le Noble et al. 1993, Munzenmaier & Greene 1996). Ang II potentiates VEGF-induced angiogenesis in the retinal MECs by increasing the expression of VEGFR2 (Otani et al. 1998). It also transactivates EGFR via heparin-binding EGF and thus contributes to modulating angiogenesis(Abramovitch et al.1998).Angiotensin II increases the transcriptional rate of Angioppoietin-2 gene without affecting the stability of its mRNA (Fujiyama et al. 2001). It is a potent vasoconstrictor and has been shown to stimulate angiogenesis induced by sponge in mice (Machado et al. 2000). Angiotensin II mediates its actions via two receptors: AT1 and AT2.

*8.5.2. Angiotensin (1-7)*

This is another component of the RAS and has only seven peptides as compared to the ten of angiotensin II. This heptapeptide mediates its biological functions via different ways including the release of vassopressin (Schiavone et al. 1988), prostaglandins (Santos et al. 1996; Jaiswal et al. 1992) and nitric oxide (Li et al. 1997). The receptors AT1 and AT2 do not reverse the anti-angiogenic effects of this exogenous angiotensin peptide. This indicates that there exists an angiotensin (1-7) specific receptor that is different from the AT1 and AT2 receptors. This receptor is responsible for the anti-angiogenic effects of the heptapeptide (Benter et al. 1993, Fontes et al. 1994, Jaiswal et al. 1992).

*8.5.3. Angiotensin Converting Enzyme*

ACE as it is commonly known as, Angiotensin Converting Enzyme is a $Zn^{2+}$ metallopeptidase. It is so called because it cleaves the C-terminal dipeptide from the oligopeptide, Angiotensin I to produce the potent vasopressor, Angiotensin II. ACE is abundantly found in the lungs where is gets localised on the luminal surface of the plasma membranes of the endothelial cells in the pulmonary vascular bed (Ryan et al. 1975; Caldwell et al. 1976). The lungs are the most important site for the generation of the circulating AngII. An insight into the expression and regulation of ACE would open up possible therapeutic strategies for inhibition of angiotensin-mediated angiogenesis.

***8.6. Endothelin***

Endothelins (ET) are peptides expressed in many tumours. These may stimulate angiogenesis and desmoplasia (Alanen et al. 2000). Endothelins occur as big ET-1 (1-38), a precursor that is cleaved into smaller peptides ET-1 (1-32), ET-3 by Endothelin Converting Enzyme-1 (ECE-1). ECE-1 occurs in four different isoforms: ECE-1a, ECE-1b, ECE-1c and ECE-1d. Vessel tone is determined by the balance of various vasodilatory and vasoconstrictor factors. ET-1 is one such vasoconstrictor. It participates in angiogenesis and vascular remodelling (Akimoto et al. 2000). ET-1 is an important factor in gastric healing along with VEGF and NO. The active and the healing stage of gastric ulcer is characterised by ET-1 and iNOS positive ECs in the vascular wall (Akimoto et al. 2000). Endothelins, ET-1 and ET-3, mediate angiogenic effects via the ET-A receptor. The angiogenic effect on the ECs is not mediated by leukocytes and is unaffected by ET-B receptor-antagonist (Bek & McMillen 2000). ET-1 induces angiogenic phenotype in cultured ECs and stimulates neovascularisation in vivo. ET-1 can exert its angiogenic effects both as an autocrine and as a paracrine factor. For instance ovarian carcinomas express ET-1 as an autocrine factor via the ET-A receptor (Salani et al. 2000) while angiogenesis and stromal growth in lung cancer is induced by a paracrine ET-1 (Ahmed et al. 2000). ET-1 negatively regulates hCG secretion by the trophoblast of the receptive phase endometrium (Sunder & Lenton 2000). ET-1 modulates tumour angiogenesis either directly or in part via VEGF.

***8.7. Prolactin***

Prolactin is a multifunctional protein hormone that exists in several molecular forms (figure 31) due to post-translational modification of the predominant 23kDa form (Nicoll 1980, Russell 1989). Spatial molecular heterogeneity of prolactin suggests that the protein functions as a pro-hormone precursor that can be processed to biologically active forms. These different molecular forms of the hormone occur in the anterior hypophysis, the immune system and the brain. The 23kDa form is

proteolytically cleaved into different N-terminal fragments that have some specific properties that are not share with the parent form, although they do retain some of the original bioactivities.

The 16kDa PRL has anti-angiogenic properties that are mediated by a unique receptor (Clapp et al. 1993). [3H]-Thymidine incorporation experiments were carried out by Clapp et al. (1993). They showed that 16K PRL antiserum blocks the inhibitory effects of the hormone and the rat neurohypophyseal conditioned medium on the b-FGF stimulated bovine brain capillary endothelial cells. The 16K PRL also stimulates natriuresis and diuresis in anesthetised rats. This form of the hormone is located throughout the anterior hypophysis. D' Angelo et al. (1995) showed that the human 16K PRL is also anti-angiogenic. The protein inhibits the phosphorylation and subsequent activation of the MAP kinases by both VEGF and b-FGF. Another set

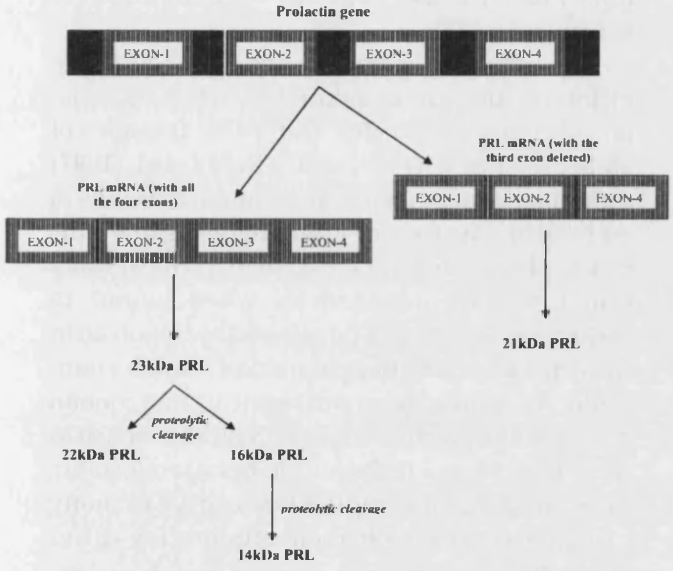


**Figure 31** *The different splice-variants of Prolactin (PRL)*

of experiments carried out by Clapp et al. (1998) showed that the endothelial cells themselves produced prolactin and prolactin-like proteins. The results of the experiments were consistent with the hypothesis that the EC proliferation is supported by autocrine regulation within the tissue micro-environment via these PRL-related proteins. The angiogenic activity is believed to be a general property of the members of the PRL family. The intact molecules are angiogenic whereas the proteolytic N-terminal fragments of the protein are anti-angiogenic (table 11). It has been suggested that the ratio of the intact protein to that of the cleaved fragments is probably the angiogenic switch regulated by the expression level of the protein and the proteases that digest the whole-length prolactin (Struman et al. 1999).

The opposite actions of these proteins are mediated by receptors specific to each protein. They do not compete for binding to the same receptor. They act by interfering with the MAP-kinase signalling pathway. The proteases that digest the full-length protein reside within the same compartment as the hormone itself. For instance, Cathepsin D that cleaves PRL is found at the deciduoplacental interface along with PRL and perhaps regulates the vascularisation of the human placenta.

### *8.8. Angiostatin and Endostatin*

Perhaps two of the most exciting and promising discoveries in the field of angiogenesis and cancer research are Endostatin and Angiostatin. Their discoveries came in the wake of the realisation that primary tumours release angiogenic inhibitors in order to suppress metastatic growth.

Angiostatin is a 38 kDa proteolytic fragment of plasminogen that was first isolated from mouse urine and serum by O'Reilly et al.(1994). Angiostatin

**Table 11** *The in vivo and in vitro properties of Prolactin (PRL)*

| *in vivo* | | *in vitro* | |
|---|---|---|---|
| 1) Growing capillaries | | 1) Bovine Brain Capillary Endothelial Cells | |
| *16kDa Prolactin fragment:* | *anti-angiogenic* | *16kDa Prolactin fragment:* | *anti-angiogenic* |
| *full-length prolactin* | *no effect* | *full-length prolactin* | *no effect* |
| 2) Queiscent vasculature | | 2) Pulmonary fibroblasts | |
| *16kDa Prolactin fragment:* | *no effect* | *16kDa Prolactin fragment:* | *stimulates NO production* |
| *full-length prolactin* | *angiogenic* | *full-length prolactin* | *no effect* |

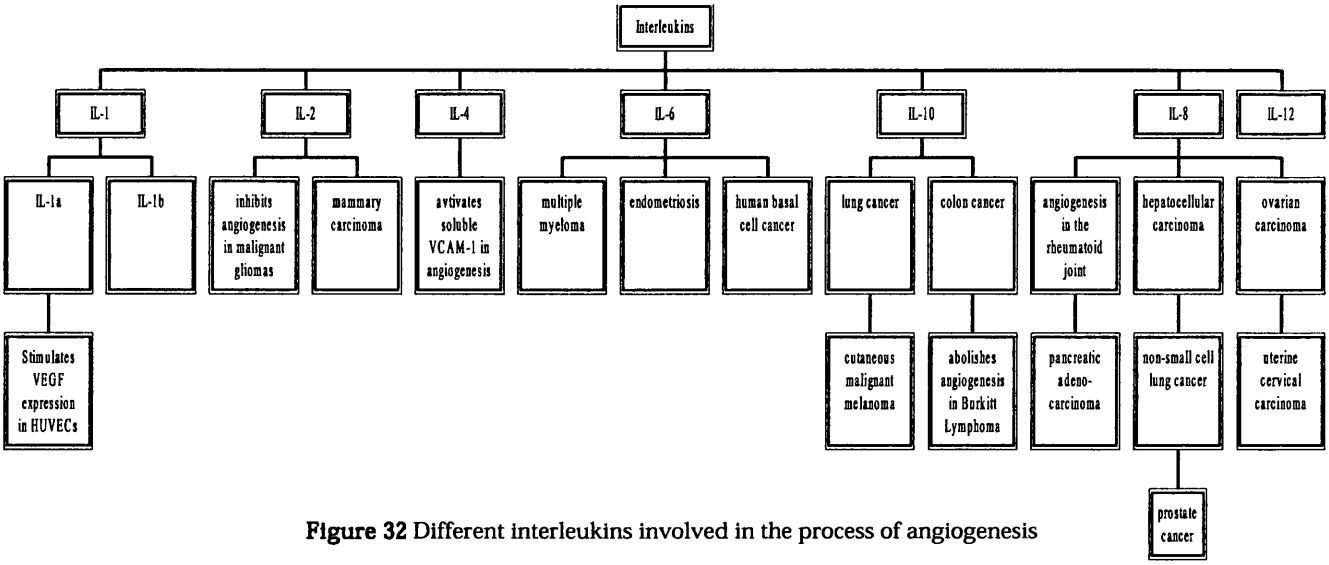


**Figure 32** Different interleukins involved in the process of angiogenesis

can almost completely suppress metastatic cell growth and is able to specifically inhibit proliferation of ECs of different origin. It is believed that angiostatin perhaps inhibits angiogenesis by inducing apoptosis, which consequently kills the endothelial cells (Lucas et al.1998). Systemic administration of angiostatin has shown nearly complete inhibition of angiogenesis and for the first time there wasn't any trace of detectable toxicity or resistance (O'Reilly et al. 1996, Sim et al. 1997). Adenovirus mediated gene transfer experiments have shown that targeted antiangiogenesis is a more promising concept than the delivery of the anti-angiogenic factors as their bolus injections (Griscelli et al. 1998).

Endostatin is a 20 kDa C-terminal fragment of collagen XVIII. Generation of endostatin from collagen XVIII is a two-step process. The first step is dependent on a metal and the latter step is elastase activity dependent (Wen et al. 1999). It specifically inhibits EC proliferation, angiogenesis and tumour growth (O'Reilly et al. 1997). Endostatin is also derived from collagen XV but this form differs from the collagen XVIII-derived endostatin both in structural and binding properties. It also differs in its tissue distribution and anti-angiogenic activity (Sasaki et al. 2000). Like angiostatin, endostatin also does not cause the onset of any resistance to the treatment (Taddei et al.1999). According to Standker et al. (1997), human endostatin is in circulation. It has been shown to have a high affinity for heparin (Hohenester et al. 1998) although the binding sites for endostatin and that for FGF-2 are discrete (Chang et al. 1999).

Endostatin binds zinc ions and it was found that metal chelating agents induce N-terminal degradation of endostatin (Boehm et al. 1998). Thoughts of Hohenester et al. (1998) and O'Reilly et al. (1997) differ in the functional importance of zinc ions in endosatin. The former group believes that zinc rather plays a structural role than a critical functional role in angiogenesis when bound to endostatin. A variety of proteinases are involved in the generation and degradation of human endostatin. All of them generate fragments that contain the N-terminal within the same 15-residue stretch as those which occur in the physiological endostatin, indicating that this region is sensitive to many proteinases although their efficiencies differ markedly.

Both angiostatin and endostatin are specific, cleaved fragments of larger, ubiquitous proteins. This may be a general mechanism by which the body promotes or inhibits angiogenesis. Use of these fragments in therapetics requires the knowledge of the extent of these mechanisms in physiological as well as pathological settings.

The crystal structure of endostatin revealed that this endogenous inhibitor of angiogenesis closely resembles the carbohydrate recognition domain of the mammalian C-type lectins (Hohenester et al. 1998). Despite a high degree of structural similarity,

endostatin does not use calcium to bind to oligosaccharides like the other c-type lectins. It is hypothesised that endostatin inhibits angiogenesis by interfering with the heparin-binding requirement of FGF-2 signalling. The crystal structure of endostatin reveals a highly basic region on the surface of the molecule that comprises of eleven arginine residues of the total fifteen for endostatin. This basic patch is hypothesised to be the putative heparin-binding site for endostatin. The three dimensional structure of endostatin is currently being used as a reference to delineate the heparin-binding site using site-directed mutagenesis as well as to define the mode of inhibition of angiogenesis by endostatin. The structural insights are proving helpful in the development of new anti-angiogenesis therapeutic approaches.

## 9. Cytokines in Angiogenesis

Cytokines are a class of small peptide messenger molecules. These are secreted by the immune system cells and transported in the blood (table 12). Their main function is to facilitate a number of immune system responses. These molecules play an important role in both normal and pathologic cellular activities. Cytokines act mainly as intracellular messengers especially in response to infection, wounds etc. They cause the proliferation and differentiation of immune cells like the T-cells (Tasaki et al. 2000), B-cells, and the macrophages. Lymphocytes in culture can be stimulated to produce active cytokines that perform a very important function of cellular communication. As mentioned before growth factors, interferons, interleukins (figure 32) can all be categorised as cytokines. This confusion in the naming system stems from the fact that many of these were initially named as growth or stimulating factors before they were all traced to originate from various immune system cells.

Cytokines act by binding to their receptors that are present on the surfaces of many cell types throughout the body. The binding of the biochemical messengers to their receptor results in transduction of an intracellular message across the cell membrane. This signal activates the genes responsible for different cellular activities like growth, differentiation and proliferation. This signal also stimulates the production of other kinds of cytokines. The cytokines often complement each other. These molecules are extremely potent which is evident from their active concentration range lying between picomolar and femtomolar values. Even at such a small range the effects exerted by the cytokines are far-reaching and very rapid. Cytokines are known to interact with and orchestrate several cellular activities that are central to the process of inflammation and healing.

## 10. The Tumour Necrosis Factor-a and Angiogenesis

Tumour necrosis factor-alpha (TNF-α) is multifunctional and has effects in inflammation, sepsis, lipid and protein metabolism, haematopoiesis, angiogenesis and host resistance to parasites and malignancy (Gordon & Galli 1990). TNF-α is a pro-inflammatory cytokine that can directly modulate the inflammatory response by altering the integrity of the endothelial barrier, resulting in an increase in endothelial permeability. TNF-α is an important mediator during the inflammatory phase of wound healing. It stimulates the secretion of active MMP-2, a type IV collagenase (figure 33).

The role of TNF-α in angiogenesis has been controversial (figure 34). *In vitro* TNF inhibits proliferation of endothelial cells (EC) whereas in the cornea it appears to stimulate vessel growth. The disc angiogenesis system developed by Fajardo et al. (1992) indicate bimodal, dose-dependent opposing effects and explain some of the in vitro versus in vivo paradoxical results. TNF (native or exogenous) may have opposing effects on microvessels of neoplasms and inflammatory reactions, depending on its local tissue concentrations (Fajardo et al. 1992). TNF-α was first described in activated macrophages. IgE-dependent activation of cultured or peritoneal mast cells induces extracellular release of TNF-α and augments levels of TNF-α messenger RNA and bioactivity (Gordon & Galli 1990). These findings suggested that the mouse mast cells are a home to both the existing and the immunologically inducible TNF-α (Gordon & Galli 1990). TNF-α is secreted by activated macrophages that are believed to mediate tumour cytotoxicity.

TNF-α can also inhibit the proliferation of ECs derived from the brain or the adrenal cortex. This suggested that the molecule can also act as a

**Table 12** *The different cytokines produced by different cell types.*

| Cell Types | Cytokines |
|---|---|
| Platelets | Angiopoietins, EGF, FGFs, IGF-1, IL-1β, PDGF, TGF-α, TGF-βs, VEGF |
| Endothelial cells | Angiopoietins, EGF, ET, FGFs, IL-1β, IL-6, IL-8, MCP-1, PDGF, TNF-α, TGF-βs, VEGF |
| Mast cells | ET, FGFs, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-18, MCP-1, MIP-1α, MIP-2, PDGF, TGF-α, TGF-βs, TNF-α, VEGF |
| Macrophages | Angiopoietins, EGF, ET, FGFs, IFN-α/β, IGF-1, IL-1β, IL-6, IL-8, IL-10, IL-18, MCP-1, MIP-1α, MIP-2, PDGF, SLP1, TGF-α, TGF-βs, TNF-α, VEGF |
| Fibroblasts | Angiopoietins, EGF, KGF, FGFs, IFN-α/β, IGF-1, IL-1β, IL-6, IL-8, MCP-1, MIP-1α, MIP-2, PDGF, TGF-α, TGF-βs, TNF-α, VEGF |
| Keratinocytes | Angiopoietins, EGF, ET, FGFs, KGF, IL-1β, IL-6, IL-8, IL-18, SLP1, TGF-α, TGF-βs, TNF-α, VEGF |

negative regulator of angiogenesis *in vivo* and can also induce selective cytotoxicity of capillary ECs. This explains perhaps why TNF-α induces haemorrhagic necrosis of certain solid tumours (Schweigerer et al. 1987). Many genes are down-regulated by the TNF-α including thrombomodulin, protein C, eNOS, the Fas ligand, and the adhesion molecule, ICAM-2.

## 11. Macrophages in Angiogenesis

Macrophages are specialised cells that induce immune response through release of specific cytokines, prostanoids and other mediators of host defenses. Although antigen processing and presentation are important functions of macrophages, Evidence states that progressively growing solid tumours reprogramme the cellular machinery of the activated macrophages resulting in immune suppression of host defenses and hence a disordered response. These macro-phages are known as the Tumour-associated Macrophages (TAMs). TAMs are an important component of many tumours, cancers. But it has also been seen that TAMs can influence tumour regression as well (figure 35). The mechanisms by which TAMs enhance metastatic spread are:

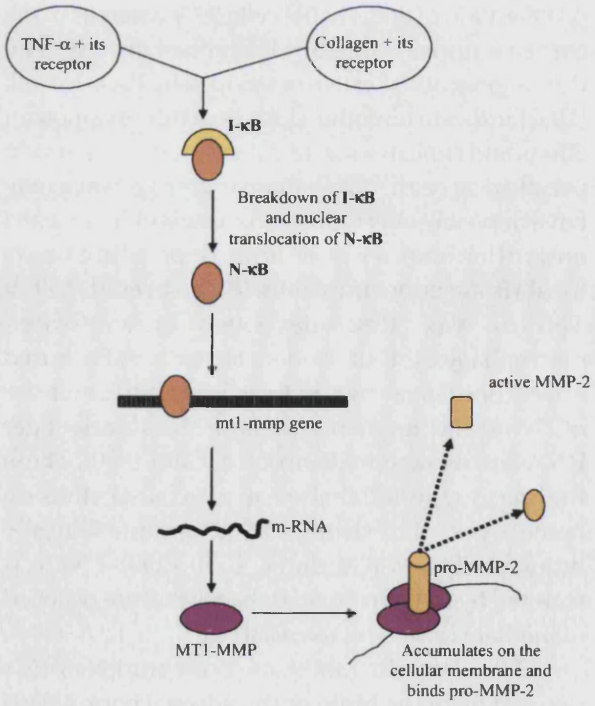


**Figure 33** A schematic representation of the various steps involved in the active secretion of MMP-2 by TNF-α

- Enhanced growth mediated by growth factors like the EGF, PDGF, TGF-β, cytokines like the IL-6, IL-1 and TNF-α.
- Enhanced angiogenesis through the production of various cytokines like IL-1, IL-6, IL-8, TGF-α, TGF-β, and prostanoids.
- Invasion and dissemination via proteolytic enzymes and prostanoids.
- Immunosuppression mediated by cytokines (IL-10) and TGF-β.

Tumour regression by TAMs is brought about by direct cellular cytotoxicity, macrophage induced apoptosis, antibody-dependent cytotoxicity and by secreting cytotoxic and cytostatic products like eicosanoids, NO, TNF-α. The outcome of these complex interactions between macrophages, neoplastic cells and other types of immune cells is enhanced tumour growth both in terms of progression and regression.

## 12. Role of Cyclooxygenases in Angiogenesis

Cyclooxygenases are the key rate-limiting enzymes involved in the synthesis of prostaglandins and

other eicosanoids from arachidonic acid (Lysz & Needleman 1982). The metabolites of the enzyme, known as the eicosanoids or the prostanoids mediate signals to the adjacent cells for the delicate regulation of cellular functions (Katori & Majima 2000). These metabolites play an important role in both physiological as well as pathological processes, especially in the gastrointestinal tract. The eicosanoids are responsible for maintenance mucosal integrity, mucus stimulation, secretion of electrolytes and inflammatory processes (Adaikan & Karim 1976, Isselbacher 1987, Wallace 2001). The enzyme occurs in two isoforms, the constitutively expressed cyclooxygenase-1 or COX-1 and the mitogen-inducible cyclooxygenase-2 or COX-2 (Kujubu et al. 1993, Wen et al. 1993). COX-1, also known as the housekeeping enzyme, is cytoprotective and is proposed to regulate physiological functions. The inducible isoform of the enzyme is of maximum interest in the field of angiogenesis because COX-2 overexpression is linked to a variety of tumours and cancers (Fosslien 2001). COX-2 overexpression can be induced by a variety of mitogens including cytokines, growth factors, tumour promoting genes (Gately 2000) COX-2 can contibute to the development of tumours and cancers in a variety of different ways. These include inducing cell survival (Seed et al. 1997), increase in angiogenesis and invasiveness (Rozic et al. 2001) and sometimes by regulating immuno-suppression. The increase in angiogenesis is brought about by increase in the expression of angiogenic factors like VEGF, EGF, FGF-2 and MMPs (Majima et al. 1997, Majima et al. 2000, Ottino & Bazan 2001).

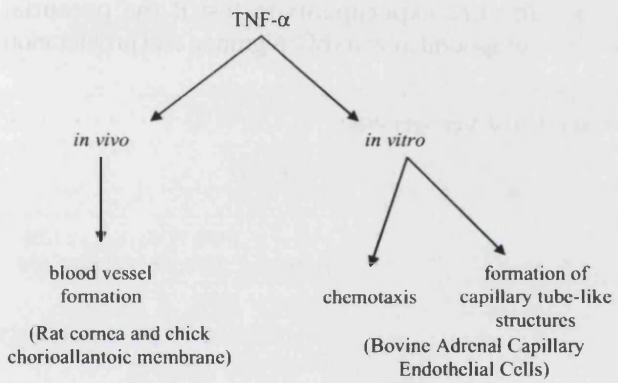


**Figure 34** The in vivo and in vitro effects of TNF-α on different models of angiogenesis

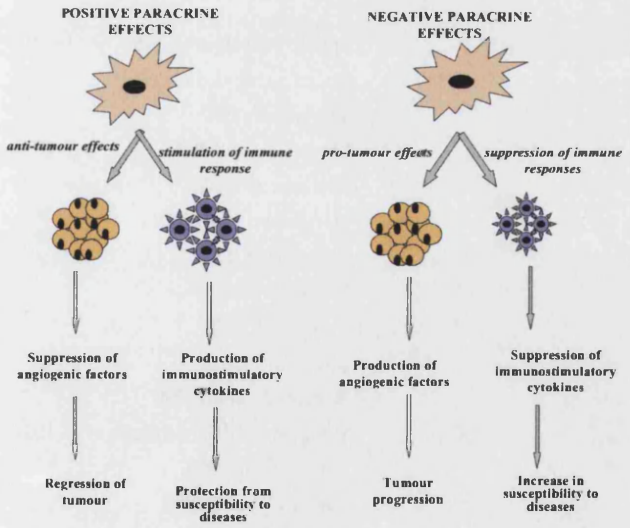


**Figure 35** Positive and negative paracrine interactions of macrophages with tumour cells and immune system

There are two classes of cyclooxygenase inhibitors, the Non-steroidal anti-inflammatory drugs (NSAIDs) and the selective COX-2 inhibitors. As the names suggest, the NSAIDs are non-specific COX inhibitors that are effective against both COX-1 and COX-2 whereas the COX-2 inhibitors (modified NSAIDs) are specific for the COX-2 enzyme (Capriotti 2000). Selective inhibition of COX-2 is preferred over nonselective inhibition because it inhibits cancer cell proliferation, reduces cancer cell survival and restores apoptosis. Selectivity against COX-2 prevents inhibition of the cytoprotective activity of COX-1 in the gastrointestinal tract. However, recently experiments have raised concerns that the COX-2 inhibitors might cause thrombosis in the infarcted heart by reducing the production of vascular prostacyclin ($PGI_2$). This disrupts the balance between thromboxane and $PGI_2$. This has made it imperative that the efficacy of COX-2 inhibitors as a potential therapeutic utility in several types of cancers be re-evaluated especially in view of their effects in cardiovascular events (Bing & Lomnicka 2002, Mukherjee 2002, Gryglewski et al. 2001).

## 13. Transcriptional Regulation of Vascular Development

Transcription factors can regulate the expression of other genes in a tissue-specific and quantitative

manner and are thus major regulators of embryonic developmental processes. Several transcription factors that regulate specific genes involved in angiogenesis have been described. Some of them have been described in table 13.

## 14. From Basic Research to Clinical Trials

The key to the clinical control of cancer and other angiogenesis dependent diseases is to find a solution to one of the great scientific query of this era: What regulates blood vessel formation? The hypothesis introduced by Judah Folkman at Harvard (USA) almost three decades ago has evloved into a paradigm. The rapid pace of discoveries coming from laboratories, research centers and industries worldwide has brought the search for an effective, non-toxic, anti-angiogenesis therapy a dream within reach.

There are several mechanisms to attack cancer cells by cutting off their blood supply. These are:

- Inhibit angiogenesis promoting factors
- Inhibit the activated endothelial cells
- Inhibit the proteolytic enzymes that digest the extra cellular components
- Inhibit the EC specific adhesion molecules
- Use of chelators of copper
- Gene Therapy
- Endocrine Therapy

The basic pathway followed to translate a potential compound from the laboratory to the clinic has five major steps (Brem 1999):

- Identification of a molecular target
- Testing the functional activity of putative inhibitor eg. receptor binding, mRNA inactivation etc
- *In vitro* experiments to test if the potential compound inhibits EC migration and proliferation

**Table 13** *Transcriptional regulation of angiogenesis*

| Transcription factor | Process regulated | References |
|---|---|---|
| Ets factors | Transactivation of Tie-1 and Tie-2 genes | Iljin et al. 1999; Wakiya et al. 1996; Dube et al. 1999; Kappel et al. 2000 |
| AP-2 | | Iljin et al. 1999 |
| SCL/tal factor | Yolk sac erythropoiesis and angiogenesis | Kappel et al. 2000; Visvader et al. 1998; Shivdasani et al. 1995 |
| GATA factors | | Kappel et al. 2000 |
| AP-1 | Extra-embryonic vascularisation | Schreiber et al. 2000 |
| LKLF | Smooth muscle architecture of tunica media, ECM deposition, decrease in no of ECs and pericytes | Kuo et al. 1997 |
| Tfeb | Placental vascularisation | Steingrimsson et al. 1998 |
| Hox $D_3$ | $\alpha_v\beta_3$ expression, EC response to angiogenic factors, | Boudreau et al. 1997 |
| Tel | Extra-embryonic angiogenesis | Wang et al. 1997 |
| Fra1 | Endothelial differentiation | Schreiber et al. 2000 |
| Vezf1 | Endothelial differentiation | Xiong et al. 1999 |
| HESR1 | Endothelial tube formation and development of arteries | Henderson et al. 2001 |
| Hox $B_3$ | Capillary morphogenesis | Myers et al. 2000 |
| PPAR-$\gamma$ | Endothelial tube formation | Xin et al. 1999 |
| SMAD-5 | Muscle development | Yang et al. 1999 |
| MEF2C | Muscle development | Lin et al. 1998 |
| dHAND | Vascularisation of the yolk sac | Yamagishi et al. 2000 |
| ARNT/HIF-$\alpha$ | Yolk sac angiogenesis, stimulation of production of angiogenic factors | Wang et al. 1995; Maltepe et al. 1997 |
| COUP-TFII | Regulates angiopoietin-1 levels | Pereira et al. 1999 |
| AML-1 | Regulates angiopoietin-1 levels | Takakura et al. 2000 |
| Fli-1 | Tie-2 gene regulation | Hart et al. 2000 |
| Lmo 2 | Bridging molecule between the GATA factors and the E-box proteins | Wadman et al. 1997 |

- Further studies are done with the chick chorioallantoic membrane and the rabbit cornea
- Finally the ultimate rigorous evaluation of the compound as an angiogenesis inhibitor is done in a vascularised organ such as the brain. Once the compound being tested has passed the different rigorous *in vitro and in vivo* tests in animals, it is administered to human volunteers in different phase trials (table 14).

Apart from inhibition of angiogenesis, stimulation of the process is also pursued for treating diseases that arise from lack or deficiency of blood supply like the ischemic heart disease. Therapeutic stimulation of angiogenesis is done by one of the following mechanisms (Thompson et al. 1999Thompson et al., 1999):

- topical application of angiogenic growth factors
- gene transfer vascularisation
- laser-induced vascularisation (Miller et al. 1990, Yi et al. 1997, Kovacs et al. 1974, Edelman et al. 2000)

There are several agents (table 15) that are currently being studied for their ability to block the blood supply to the tumours. The first stage of clinical trials have given several feedbacks and ideas pertaining to what needs to be dealt with the next line of therapeutic modalities (Thompson et al. 1999).

### *14.1 Structure Based Drug Design*

The three-dimensional structures of target macromolecules provide starting point for drug design once the lead compounds have been screened and identified. The three-dimensional structures define the interface of the ligands and their receptors or other macromolecular targets. Study of the key residues at the active site and/or protein-protein interface gives strong insights into the function of the target molecule. These help in the design of drugs that would enhance the desired interactions with the target macromolecule and at the same time reduce the unwanted ones. Design is the combination of docking and linking of available compounds. Structure dictates what and where structural modifications are required to improve the pharmacodynamic properties of the lead compound.

The availability of structures has helped in the design and identification of many drugs that selectively target the macromolecule. Recently, three-dimensional structures have impacted heavily on the drug design approaches. One recent example of such a designed drug is that of Anginex (Griffioen et al. 2001). Anginex is a βpep-peptide designed by using the three dimensional structures of several anti-angiogenic proteins. These proteins have a structural commonality among them comprising of anti-parallel β-sheet structure. The structural and compositional characteristics of these anti-angiogenic proteins have been combined to design Anginex. Anginex is a potent inhibitor of EC adhesion and migration and functions by inducung inducing apoptosis. Successful clinical trials are required before Anginex can be used to treat arthritis, tumour growth, retinopathy and restenosis.

### *14.2. Anti-angiogenic Gene Therapy for Cancer*

Relative instability, economic constraints posed by mass manufacturing and high chronic dosage

**Table 14** *A brief summary of the aim of the different clinical trail phases (Hagedorn 2000)*

| Trial stages | Duration/ no of subjects | Definition of the stage of clinical trial |
|---|---|---|
| Pre-clinical | at least 2-3 months | Evaluation of pharmacological effects of the compound through *in vivo* and *in vitro* animal (rodent and non-rodent) experiments. The animals are tested on the basis of the genotoxicity of the drug, the metabolism of the drug and the excretion pathways. |
| Phase I | 20-80 subjects | The new drug is administered to healthy or diseased volunteers for the first time under closely monitored conditions and the subjects are observed for drug metabolism, side effects relative to dosage |
| Phase II | several hundred patients | The main aim is to obtain preliminary data on the effectiveness of the new drug. This again is carried out under close monitoring |
| Phase III | several hundred or thousand patients | Expanded controlled and uncontrolled trial studies to determine the benefit-risk relationship of the drug |

**Table 15** *The various inhibitors that have reached the clinical trial stage*

| Drug | Inhibitor type | Endogenous / synthetic | Trial Status | Diseases |
|---|---|---|---|---|
| Endostatin | EC inhibitor | Endogenous | Phase II | Solid tumours |
| Suramin | Growth factor inhibitor | Synthetic | Phase II | Glioblastoma |
| Vitaxin | Adhesion molecule inhibitor | Synthetic | Phase II | Leiomyosarcoma |
| Thalidomide | Growth factor inhibitor | Synthetic | Phase II | |
| Penicillamine | Protease inhibitor, EC inhibitor | Synthetic | Phase II | Glioblastoma |
| Farnesyl Transferase Inhibitor | EC inhibitor | Endogenous | Phase I | Soild tumours and glioblastoma |
| Marimastat | Protease inhibitor | Synthetic | Phase III | Breast cancer, malignant glioma, non-small cell lung carcinoma, pancreatic carcinoma |
| Tetrathiomolybdate | Copper chelator | Synthetic | Phase I/II | Advanced metastatic cancer and multiple tumour types |
| EMD121974 | Adhesion molecule inhibitor | Synthetic | Phase I/II | Kaposi's sarcoma, brain tumours |
| PTK787/ZK22584 | Growth factor inhibitor | Synthetic | Phase I/II | Kaposi's sarcoma, glioblastoma, von Hippel-Lindau disease |
| Captopril | Copper chelator | Synthetic | Phase I/II | - |
| Interpheron-alpha | Growth factor inhibitor | Endogenous | Phase II/III | - |
| Interleukin-12 | - | Endogenous | Phase I/II | Ovarian, renal cell, gastrointestinal cancers, soild tumours and Kaposi's sarcoma |
| SU5416 | Growth factor inhibitor | Synthetic | Phase I/II | Kaposi's sarcoma, colorectal carcinoma |
| Bay 12-9566 | Protease inhibitor | Synthetic | Phase III | Lung cancer, pancreatic cancer and ovarian cancer |
| Squalamine | EC inhibitor | Synthetic | Phase III | Prostate cancer, non-small cell lung carcinoma, pancreatic carcinoma |
| IM862 | - | Synthetic | Phase III | AIDS-related Kaposi's sarcoma |
| AG3340 | Protease inhibitor | Synthetic | Phase III | Prostate cancer, non-small cell lung carcinoma, pancreatic carcinoma |
| R115777 | EC inhibitor | Synthetic | - | - |
| CAI | - | Endogenous | Phase II/III | Renal cell, non-small-cell lug and ovarian cancers |
| CGS 27023A | Protease inhibitor | Synthetic | Phase I/II | - |
| COL-3 | Protease inhibitor | Synthetic | Phase I | - |
| Combretastatin | EC inhibitor | | | - |
| TNP-470 | EC inhibitor | Synthetic | Phase II | Lymphomas and acute leukemias |
| ABT-627 | ET-1 receptor agonist | Synthetic | Phase I/II | Prostate cancer, glioblastoma |
| -L-778,123 | EC inhibitor | Synthetic | - | - |
| -SCH66336 | EC inhibitor | Synthetic | - | - |
| Neovastat | Protease inhibitor | Endogenous | Phase III | Colon cancer, non-small cell lung carcinoma, pancreatic carcinoma |
| PNU-145156E | | Synthetic | Phase I/II | Solid tumours |
| Anti-VEGF Antibody | VEGF inhibitor | | Phase II/III | Lung cancer, renal cancer, prostate cancer, breast cancer and colorectal cancer |
| SU6668 | Receptor inhibitor | Synthetic | Phase I | Advanced cancer |
| CM101/ZDO101 | EC inhibitor | Synthetic | Phase I | - |
| BMS-275291 | Protease inhibitor | Synthetic | Phase I | - |
| Purlyin (SnET2) | ET-1 receptor inhibitor | Synthetic- | Phases I/II/III | Cutaneous skin carcinoma, breast carcinoma |
| Suradista | Blocks binding of growth factors | Synthetic | Phases I/II/III | Brain cancers, glioblastoma, prostate cancer, ovarian cancer |

requirements for recombinant endogenous inhibitors make the anti-angiogenic gene therapy a very attractive treatment. Two gene strategies have been proposed for this therapeutic treatment:

**(a) Tumour Directed Gene Therapy**

- Facilitates paracrine activity
- Avoids systemic toxicity of anti-angiogenic gene product
- Requires an established tumour blood supply to deliver the vector
- Less efficient against micrometastases
- Difficulty in producing tumour-specific gene-delivery systems

**(b) Systemic Gene Therapy**

- Facilitates the use of normal host tissues for the production of elevated levels of the anti-angiogenic products.

Gene delivery systems can be classified as:

- **Viral vectors**: These are more efficient in entering eukaryotic cells and at inducing gene expression. But they are also toxic being natural pathogens and thus are susceptible to being eradicated by the human immune systems.
- **Non-viral vectors**: These are less efficient in entering host cells and at inducing gene expression. But they are not toxic as opposed to the viral vectors.

Several *in vitro* and *in vivo* anti-angiogenic gene therapy approaches have been used to evaluate a variety of endogenous inhibitors. Table 16 lists some of gene transfer methods for a few important inhibitory molecules.

### *14.3. The Endostatin Paradox*

About 5 years ago, Judah Folkman's endostatin produced dramatic results in laboratory experiments. This led to clinical trials using several gene transfer approaches in order to establish its magical properties in shrinking tumour in mice virtually to nothing. However, this result was not obtained all the research groups that tried their hand with this wonder molecule. Several questions were raised regarding the properties of endostatin as projected by the Harvard group. The reason for paradoxical behaviour of this molecule lies in the method of delivery to the body. It seems that gene transfer approach is not as effective as when the protein is injected into the body. But then again according to Folkman this behaviour itself is not consistent as some gene therapy experiments have given promising results and not just his laboratory. According to him, the inefficiency of the gene transfer method may be because of endostatin rendered inactive at high concentrations produced by the gene therapy. Although the perplexity of this paradox is still not resolved, Folkman and his group believe that whilst one continues to investigate into the discrepancies of the varied results, one should not stop the clinical trials with endostatin. Further research in this field would enhance our understanding of the anti-angiogenic properties of this molecule.

## 15. Future prospects

The aim of angiogenesis research is to characterise the endothelial cells that originate from tumour tissues. Today, one of the major future directions for angiogenesis research is to continue to identify the biochemical targets and elucidate the three-dimensional structures of all the macromolecules involved in the process of angiogenesis. It is important to model and quantify the structure-functional relationship of these macromolecules in

**Table 16** *Gene transfer methods evaluated for some important inhibitory molecules*

| Genes | References |
|---|---|
| Angiostatin | *In vitro* transfection (Cao et al. 1998); cationic liposome:DNA complex (Liu et al. 1999); Adenovirus (Griscelli et al. 1998) |
| Endostatin | *In vitro* transfection (Yoon et al. 1999); polymerised plasmid DNA (Blezinger et al. 1999); cationic liposome:DNA complex (Chen et al. 1999) |
| TIMPs | Adenovirus (Fernandez et al. 1999); *In vitro* transfection (Valente et al. 1998) |
| ILs | *In vitro* transfection (Stearns et al. 1999); sf-virus (Asselin-Paturel et al. 1998) |
| IFNs | Retro-viral transduction (Dong et al. 1999) |
| TSPs | Cationic liposome:DNA complex (Xu et al. 1998) |

parametric terms and correlate the dynamics of the process with these parameters. This will help in incorporating the molecular properties of these targets in a cellular context, which will unravel the complex pathways that are integrated into the process of blood vessel formation. Also, it is necessary to develop new experimental models for angiogenesis so that the authenticity of the molecule being tested can be verified to a larger extent than now. For this we also need to evaluate angiogenesis in human tumours as opposed to animal tumours as a prognostic tool. It is necessary to design small molecule antagonists taking into account the macromolecular target in terms of its biological endpoint. The therapeutic applications that need to be developed should promote tumour regression by death of tumour cells and not normal, healthy cells. Also, the need of the hour is to develop drugs or vaccines that are easy to deliver, free of delivery risk and with low dosage requirements to combat poor immunogenicity, which is one of the major obstacles to the development of successful antagonists. Furthermore, it is necessary to explore the induction of immunological memory to reduce the number of dosages and prevent disease relapses.

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