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A mechanism for glu-plasminogen binding: an important aspect of the plasminogen activation cascade

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A mechanism for glu-plasminogen binding: an important aspect of the plasminogen activation cascade.

A thesis submitted in fulfillment of the requirements for the award of the degree

Doctor of Philosophy

From

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By

Nicholas Matthew Andronicos

Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilmant of the degree of Doctor of Philosopy. It does not include any material previously pubished by another person except where due reference is made in the text. The experimental work described in this thesis is original work and has not been submitted for a degree in any University or Institution.

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Table of Contents

Table of Figures

Table of Tables

Table of Abbreviations

2-PGA	2-phospho-D-glycerate
AH	Amino-Hexyl
APUD	Amine-precursor uptake and decarboxylation
Arg	Arginine
Asp	Aspartate
Cys	Cystine
DFP	Diisopropylfluorophosphate
DNP	Dinitrophenyl
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERM	Enolase related molecule
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate (isomer 1)
G	Acceleration due to gravity (9.8 ms^{-2})
GAM-HRP	Goat anti-mouse horse radish peroxidase conjugate
GAR-HRP	Goat anti-rabbit horse radish peroxidase conjugate
Glu	Glutamate
HGF	Hepatocyte growth factor
HGFL	Hepatocyte growth factor-like
HRG	Histidine-rich glycoprotein
K1	Kringle 1
K2	Kringle 2
K3	Kringle 3
K4	Kringle 4
K5	Kringle 5
k_a	Association rate constant
K_a	Association equilibrium constant
k_{cat}	Catalytic rate constant
K_d	Dissociation equilibrium constant
k_d	Dissociation rate constant
KDa	Kilodaltons
K_m	Michalis-Menton kinetic constant
LBS	Lysine binding site
Lp(a)	Lipoprotein a
Lys	Lysine
MAb	Monoclonal antibody
MSF	Macrophage stimulating factor
NHS	N-hydroxysuccinimide-N-ethyl-N'-(diethylaminopropyl)carbodiimide
NTA	Nitrilotriacetic acid
NTP	N-terminal peptide
Pab	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-type 1
PAI-2	Plasminogen activator inhibitor-type 2
PD	Protease domain
PEP	Phosphoenolpyruvate
PI	Propidium iodide
Plg	Plasminogen
PMSF	Phenylmethylsulfonylfluoride
Pn	Plasmin
RCL	Reactive centre loop
r-α-enolase	Recombinant human alpha-enolase
Sc	Single chain
Ser	Serine
SERPIN	Serine protease inhibitor
SF	Scatter factor

TA	Tranexamic acid
tc	Twin chain
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
tPA	Tissue plasminogen activator
TSD	Thermostable domain
Trp	Tryptophan
Tyr	Tyrosine
uPA	Urokinase-type plasminogen activator
Val	Valine
ΔH	Change in enthalpy
ΔS	Change in entropy
α2-AP	Alpha2-antiplasmin
ε-ACA	Epsilon-amino caproic acid

List of Publications

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Ranson, M., **Andronicos, N.M.**, O'Mullane, M.J. and Baker, M.S. (1998) Increased plasminogen binding is associated with metastatic breast cancer cells: differential expression of plasminogen binding proteins. *Br. J. Cancer. 77, 1586-1597.*

Ranson M., **Andronicos N.M.**, Saunders D., & Baker M.S. Rapid autocatalytic processing of Glu-plasminogen occurs on malignant breast cancer cell surfaces. *Fibrinolysis & Proteolysis.* 1998, 12 (Supp. 1): 14. (Abstract).

Ranson, M., **Andronicos, N.M.**, and Baker, M.S. Do plasminogen receptors play a role in breast cancer malignancy?. *Fibrinolysis and Proteolysis.* 1997, 11 (Supp. 3):56. (Abstract).

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PAPERS IN PREPARATION

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CONFERENCE PRESENTATIONS

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Ranson, M., **Andronicos, N.M.**, Lackmann, M., and Baker, M.S. The biochemistry of plasminogen binding to receptors - relevance to breast cancer metastasis. *Australian Society for Medical Research, 36th National Scientific Conference. Adelaide, Australia. November, 1997.*

Ranson, M., **Andronicos, N.M.**, and Baker, M.S. Do plasminogen receptors play a role in breast cancer malignancy? *9th Lorne Cancer Conference, Special joint conference with the American Association for Cancer Research. Lorne, Vic., Australia. February, 1997.*

Andronicos, N.M., Ranson, M., Bognacki, J., and Baker, M.S. Characterization of recombinant α-enolase, an important cancer cell plasminogen receptor. *8th Lorne Cancer Conference. Lorne, Vic., Australia. February, 1996.*

Andronicos, N.M., Ranson, M., Bognacki, J., and Baker, M.S. Expression, purification and characterization of α-enolase, an important cancer cell plasminogen receptor. *The Paulo Foundation Symposium. 5th International Workshop on Molecular and Cellular Biology of Plasminogen Activation. Hameenlinna, Finland. August, 1995.*

Andronicos, N.M., Baker, M.S., and Ranson, M. Generation, purification and characterization of α-enolase, an important cancer cell plasminogen receptor. *7th Lorne Cancer Conference. Lorne, Vic., Australia. February, 1995.*

Summary

The lysine-dependent, activation-resistant, conformation of glu-plasminogen is converted to an activation susceptible conformation after binding to cell-surface lysine residues. These lysine-dependent interactions of glu-plasminogen are mediated by its lysine binding sites (LBS's). Thus, it is hypothesised that lysine residues from the cell surface plasminogen receptors compete with lysine residues from glu-plasminogen for occupation of its LBS's, thereby inducing a conformational change to glu-plasminogen. Previously, an α-enolase-related molecule was identified as a cell surface plasminogen receptor. Hence, the plasminogen binding characteristics of recombinant human α-enolase (r-α-enolase) were assessed.

R-α-enolase bound glu-plasminogen in a lysine-dependent manner with an apparent K_d of 1.9 μM. This interaction [1] was dependent on the C-terminal lysine residue of r-α-enolase, [2] enhanced the activation rate of glu-plasminogen and [3] blocked α2-antiplasmin from binding glu-plasminogen.

BIACORE kinetic analysis of the interaction suggested that the dissociation of glu-plasminogen from r-α-enolase was mediated by at least two components with apparent dissociation rate constants of $k_{d1}=4.7\times10^{-2}s^{-1}$ and $k_{d2}=1.6\times10^{-3}s^{-1}$. Global analysis of the interaction suggested that it was a two-state conformational change reaction, mediated by a concentration-dependent increase in the initial association rate. Intrinsic fluorescence spectroscopy confirmed that r-α-enolase induced a more open conformation of glu-plasminogen. Thus, the gene product of human ENO1 encoded an authentic plasminogen binding protein and the binding of glu-plasminogen to α-enolase is mediated by an initial lysine-dependent competition reaction that results in a conformational change to the zymogen.

The binding of glu-plasminogen to the metastatic breast cancer cell line MDA-MB-231 in this study was: [1] lysine-dependent [2] low affinity (K_d = 1.8 μM), but high capacity (5.0×10^7 sites/cells) and [3] dependent on the viability status of the cells. Multiple plasminogen binding proteins may be responsible for localising glu-plasminogen to the cell surface. The MDA-MB-231 cells were capable of generating large amounts of plasmin compared to the non-metastatic breast cancer cell lines which did not have a high plasminogen binding capacity.

Therefore, glu-plasminogen binds to the cell surface by a lysine-competitive, two-step binding event that results in a more open, activation-susceptible conformation. This competitive-lysine reaction mechanism of glu-plasminogen explains the relationship between binding, conformation and activation of glu-plasminogen.

Chapter 1

Plasminogen binding: a fundamental event, essential to the plasminogen activation cascade

The plasminogen activation system is a serine protease mediated cascade that results in the formation of plasmin, a broad spectrum, trypsin-like, serine-protease. This system is responsible for mediating several important physiological (e.g. fibrinolysis, cell migration) and pathological (e.g. inflammation, local tumour invasion, and metastasis) processes. Physiologically, the control of plasminogen activation is multi-fascitated and involves both spatial and temporal regulatory mechanisms. Since glu-plasminogen is the central constituent of the plasminogen activation system (figure 1.1) detailed knowledge of its structure is essential for understanding the physiology and pathology of the plasminogen activation cascade.

An important biological process that must be considered in the activation of plasminogen is the relationship between the conformation of glu-plasminogen and its activation rate. Glu-plasminogen changes from an activation-resistant to an activation-susceptible conformation after binding small lysine analogues. This is referred to as a lysine-dependent conformational change. This same type of conformational change is also hypothesised to occur when glu-plasminogen binds in a lysine-dependent manner to the plasminogen binding proteins such as fibrin and α-enolase. Lysine-dependent binding of glu-plasminogen to these proteins also targets the proteolytic activity of plasmin to specific sites within the body, thereby adding a

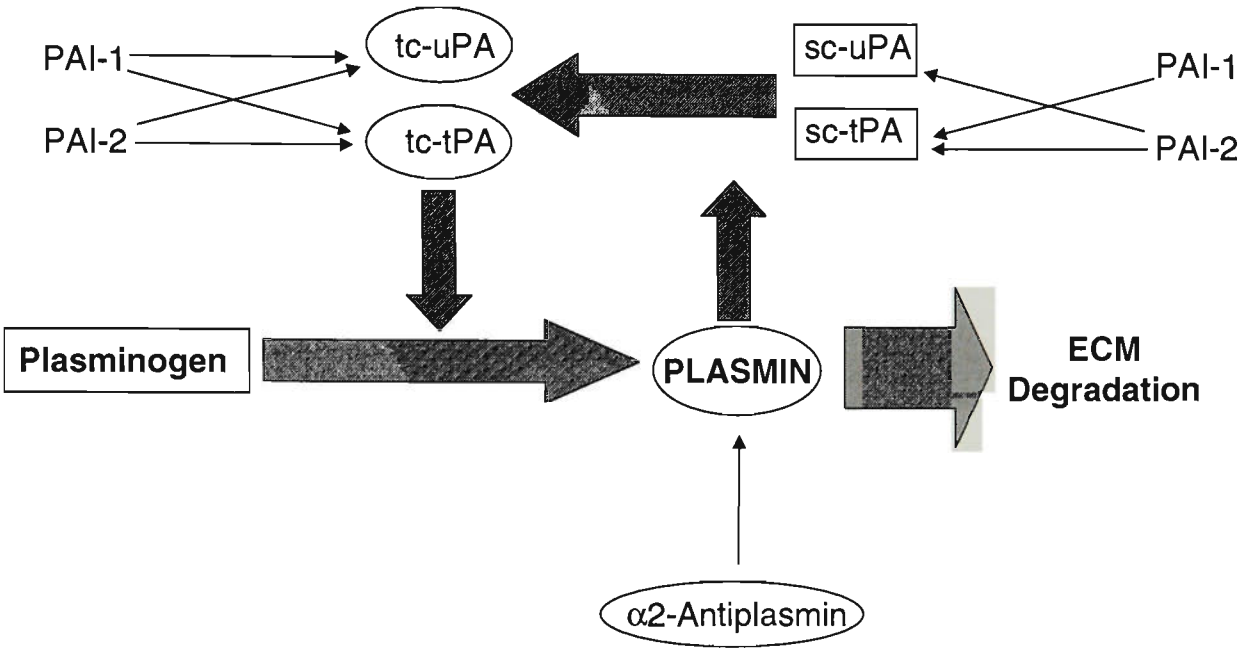

Figure 1.1: Solution-phase plasminogen activation:

Plasminogen is activated by either single-chain or twin-chain urokinase plasminogen activator (sc-uPA, tc-uPA) and by either single-chain or twin-chain tissue plasminogen activator (sc-tPA, tc-tPA). Once formed the serine protease plasmin can degrade fibrin clots, and extracellular matrix (ECM) either directly or indirectly via the activation of metalloproteases. The serpins PAI-1 and PAI-2 are physiological inhibitors of the plasminogen activators. Solution phase plasmin is inhibited by the serpin α2-antiplasmin (α2-AP) in a lysine-dependent manner. An activation event is represented by ➡, whereas → represents an inhibition event.

further dimension of regulation to the cascade. Hence, the lysine-dependent binding of glu-plasminogen to fibrin and protein receptors is hypothesised to induce conformational changes in glu-plasminogen promoting activation, as well as targeting plasmin activity to a specific tissue. If plasminogen binding proteins induce a lysine-dependent conformational change to glu-plasminogen upon binding, then this may be considered fundamental for the correct physiological outcomes of the plasminogen activation cascade. Furthermore, aberrations of the lysine-dependent plasminogen binding capacity of a tissue may result in, or contribute to pathologies associated with plasmin-induced proteolytic damage.

This chapter will provide an overview of the plasminogen activation cascade, focussing on the relationship between the lysine-dependent conformation of glu-plasminogen and its activation. Secondly, the consequences of lysine-dependent glu-plasminogen binding on the rate of fibrinolysis will be discussed and compared to

glu-plasminogen binding and activation at the cell surface. Finally, aberrations of the plasminogen activation cascade associated with breast cancer cell invasion and metastasis will be reviewed, highlighting the lack of research into the expression of plasminogen binding potential of both benign and metastatic breast cancer cells.

1.1 Plasminogen Activation Cascade Components

1.1.1 Glu-Plasminogen.

Plasminogen (EC 3.4.21.7), a broad spectrum serine endopeptidase zymogen, is a single chain 790 amino acid glycoprotein that has a molecular weight of 92 kDa (Wiman, 1977; Sottrup-Jensen *et. al.,* 1978). It is a β-globulin (Miyashita *et. al.,* 1988) consisting of seven compact co-operative structural domains (Novokhatny *et. al.,* 1984). Plasminogen is composed of both a heavy chain (approximately 65 kDa), containing an N-terminal acidic domain with a basic hinge region (NTP; amino acids 1-77) (Forsgren *et. al.,* 1987), as well as five triple-disulfide-bonded kringle (K) structures on which the lysine binding site (LBS) motifs are located, and a light chain (25 kDa) or protease domain chain (Sottrup-Jensen *et. al.,* 1978) (figure 1.2). The chromosomal location of the plasminogen gene is 6q26 of the human genome (Murry *et. al.,* 1987). The liver is the primary plasminogen producing site of the body (Raum *et. al.,*1980), however, other tissues such as microglia cells of the brain (Nakajima *et. al.,* 1992a,b). When synthesised, plasminogen has 809 amino acids. The 19 amino acid signal peptide of plasminogen (figure 1.2) is removed during the secretion process resulting in mature, glu-plasminogen (790 amino acids). The concentration of glu-plasminogen in the blood is approximately 2 μM (Rabiner *et. al.,* 1969; Raum *et.*

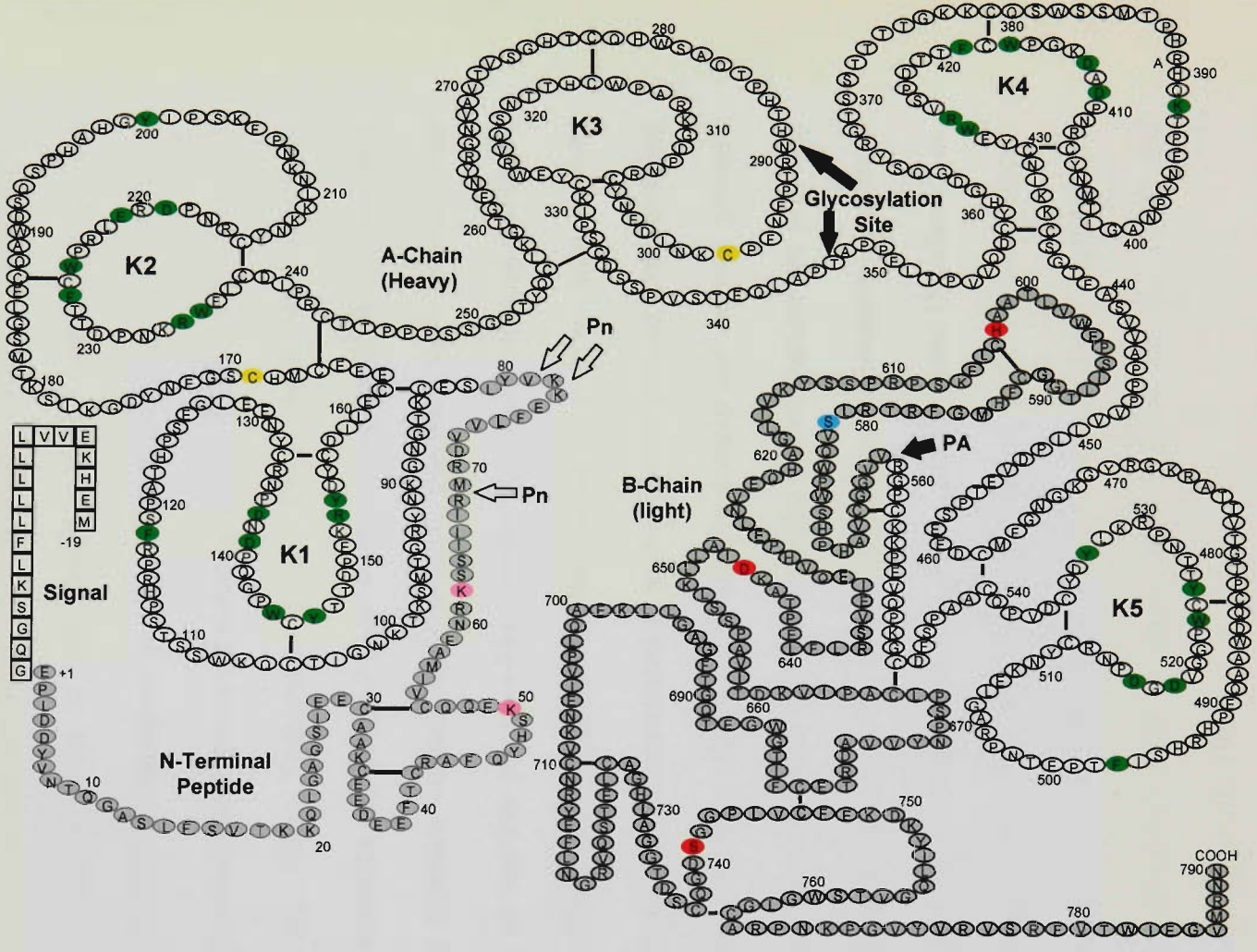

Figure 1.2: The amino acid sequence and domains of glu-plasminogen.

Human glu-plasminogen is a 92 kDa glycoprotein composed of 790 amino acids. The amino acids of the different types of domains of glu-plasminogen are represented by distinct symbols (i.e. the 19 amino acid secretion signal peptide □, the amino acids of the NTP ●, the heavy chain amino acids of the mature protein (O) and the protease domain light chain amino acids ● The constituents of the LBS motifs ●. The amino acids of the catalytic triad of plasmin is defined by ●. The glycosylation sites as well as the plasminogen activation and the plasmin cut sites are identified by arrows. The lysine residues (Lys_{50} and Lys_{62}) implicated as conformational determinants in the NTP identified by ●. The phosphorylation site of glu-plasminogen (Ser_{578}) is denoted by ●. The cystine residues that form the interkringle disulfide bond linking K2 and K3 into a "super kringle" are represented by ●. Adapted from Pollanen *et. al.*, (1991).

1980).

1.1.1.1 Plasminogen-like growth factor family of proteins

Plasminogen is a member of the family of proteins known as the plasminogen-like growth factors. Other proteins of this family include: hepatocyte growth factor/scatter factor (HGF/SF) (Nakamura *et. al.,* 1989; Weidner *et. al.,* 1991) and hepatocyte growth factor-like/macrophage stimulating factor (HGFL/MSF) (Degen *et. al.,* 1991; Han *et. al.,* 1991; Yoshimura *et. al.,* 1993), both of which have approximately 50% sequence similarity to human plasminogen (BLAST database). These growth factors typically contain an N-terminal peptide (NTP) domain, four kringle (K) domains, an inactive protease-like domain and a plasminogen activation site. Donate *et. al.,* (1994) has suggested that this family of proteins have evolved from a common ancestral gene that consisted of an N-terminal domain corresponding to the NTP of plasminogen, three copies of the kringle domain and a serine protease domain. The precursor forms of these plasminogen-like growth factors are probably substrates for plasminogen activators. For example, urokinase plasminogen activator (uPA) activates pro-HGF by cleavage of its Arg_{494}-Val_{495} peptide bond (Naldini *et. al.,* 1992), which is homologous to the Arg_{560}-Val_{561} activation bond of plasminogen. These growth factors do not have proteolytic activity but have been implicated in various physiological processes such as angiogenesis (Grant *et. al.,* 1993) and organogenesis (Sonnenberg *et. al.,* 1993).

1.1.1.2 Glu- and lys-plasminogen- secondary and tertiary structures.

Three distinct types of domains exist within secreted plasminogen (figure 1.2). Firstly, the NTP domain, which is responsible for maintaining the conformation of the molecule. The second type of domain within plasminogen are the kringle domains which contain the LBS motifs of the protein that are responsible for regulating the conformation and tissue specific interactions of plasminogen. The final domain is the

trypsin-like protease domain located at the C-terminal end of plasminogen. As discussed in detail below, the combination of these domains confers unique properties on plasminogen that facilitates its biological activity (i.e. activation of plasminogen and the tissue specificity of plasmin).

There are two *in vivo* species of plasminogen namely glu-plasminogen and lys-plasminogen. The apparent half lives of iodinated glu- and lys-plasminogen in the circulation are 2.2 and 0.8 days, respectively (Collen and Verstraete, 1975). Glu-plasminogen, (native plasminogen) designated as such because the N-terminal amino acid of the NTP is glutamate (Wiman and Wallen, 1975). The crystal structure of glu-plasminogen has not been solved. However, a combination of low-resolution physical techniques as well as biochemical studies have advanced the hypothesis that glu-plasminogen exists in a closed right-handed spiral conformation. Electron microscopy of glu-plasminogen demonstrates that it has a right-handed spiral conformation with a diameter of 9-11 nm (Tranqui, *et. al.,* 1979; Weisel *et. al.,* 1994). Small angle, electron scattering experiments also suggest that the structure of glu-plasminogen in solution is compact. Glu-plasminogen has a radius of gyration of 3.05 nm and a maximum intramolecular distance of 9.1 nm (Ponting, *et. al.,* 1992), which is comparable to the electron microscopy data of Tranqui, *et. al.,* (1979) and Weisel *et. al.,* (1994). However, the precise dimensions of the zymogen remain controversial since Mangel *et. al.,* (1990), using neutron scattering techniques, describes glu-plasminogen as a prolate ellipsoid with radius of gyration and the maximum intramolecular distance of 3.39 and 15 nm respectively. Chemical cross-linking analysis of glu-plasminogen demonstrates that Lys_{204} of K2 and Tyr_{672} of the C-terminal protease domain are approximately 3 Å from each other in the protein (Banyai and Patthy, 1984, 1985). Taken together these studies suggest that glu-plasminogen exists in a tight right hand spiral, known as the closed conformation.

Lys-plasminogen (Lys_{78}-Asn_{790}), in which the NTP (Glu_1-Lys_{77}) is absent, has a more open conformation than glu-plasminogen. Electron microscopy of lys-plasminogen demonstrates that it exists in a U-shaped conformation with approximate dimensions of 14 nm × 7 nm (Wiesel *et. al,* 1994). This open conformation has also been described as a Debye random coil with a radius of gyration of 56 Å (Ramakrishnan *et. al.,* 1991). The N-terminal fragment of lys-plasminogen (K1-3) is described equally well by either a Debye random coil or an elongated, prolate ellipsoid, shapes with a radius of gyration of 29 Å (Ramakrishnan *et. al.,* 1991). This more open conformation of lys-plasminogen is also associated with an increase in the intrinsic fluorescence of the protein (Markus *et. al.,* 1979). The secondary structure of both glu- and lys-plasminogen are very similar (Sjoholm *et. al.,* 1973; Misselwitz *et. al.,* 1994), suggesting that a negligible change in secondary structure is associated with a large tertiary conformational change. Thus, the spatial relationship of the individual domains with each other is different between the closed to the open conformations of glu-plasminogen, whilst the structures of the domains themselves remains constant. Taken together, these studies suggest that the NTP domain may act as a conformational determinant of glu-plasminogen.

The NTP domain of glu-plasminogen contains two disulfide bonds, Cys_{30}-Cys_{54} and Cys_{34}-Cys_{42} and is predicted to have a loop-in-loop topology (Donate *et. al.,* 1994). The solution phase tertiary structure for the Glu_1-Ser_{57} fragment of the NTP, determined by ^{1}H-NMR, demonstrates that this fragment has very low secondary structure content (about 10% α-helix) (An *et. al,* 1998a). Similarly, the tertiary structure of the hairpin loop containing domain of HGF (Gly_{31}-Asn_{127}), which has approximately 27% sequence similarity to the NTP domain of glu-plasminogen, has been solved by ^{1}H-NMR and also contains approximately 10% α-helix secondary structure (Zhou *et. al.,* 1998).

Site-directed mutagenesis of the basic amino acid hinge region of the NTP domain (Cys_{54}-Val_{79}) demonstrates that this plasmin susceptible region is directly involved in maintaining the conformation of glu-plasminogen (Horrevoets *et. al.*, 1995; figure 1.2). Plasmin catalysed proteolysis of glu-plasminogen at Arg_{68}-Met_{69}, Lys_{77}-Lys_{78} or Lys_{78}-Val_{79} (figure 1.2) results in the autocatalytic removal of the 8 kDa NTP of the protein and the formation of lys-plasminogen (Wiman, 1973; Summaria *et. al.*, 1973; Gonzalez-Gronow *et. al.*, 1977). The conformational change from glu- to lys-plasminogen, mediated by the removal of the NTP domain, is reversible since addition of the isolated NTP back to lys-plasminogen restores the native lysine-dependent conformation of glu-plasminogen (Sjoholm *et. al.*, 1973). This suggests that the conformation of glu-plasminogen is regulated by non-covalent, lysine-dependent interactions between the NTP of glu-plasminogen and the LBS motifs of the kringle domains of the protein (Wiman and Wallen, 1975; Violand *et. al.*, 1975). This further implicates the NTP domain of glu-plasminogen as a lysine-dependent conformational determinant of the protein.

Lys-plasminogen is more readily activated by the plasminogen activators (Claeys and Vermylen, 1974), and has a higher specific activity when converted to plasmin (Lucas *et. al.*, 1983) than glu-plasminogen. Thus, the lysine-dependent interactions between the kringle domains and NTP of glu-plasminogen may modulate the biological role of glu-plasminogen by regulating the conformation of the protein. Therefore, detailed structural knowledge of the NTP and the kringle domains of plasminogen is required to understand the conformational regulation of glu-plasminogen and hence, biological activity of the protein.

1.1.1.3 The kringle domains of plasminogen.

The LBS motifs of glu-plasminogen are contained within the five kringle domains (figures 1.2 and 1.3) of the heavy chain of the zymogen. These motifs

mediate the lysine-dependent binding of glu-plasminogen to various proteins such as fibrin (Sottrup-Jensen *et. al.,* 1978). The kringles contain approximately 80 amino acids each (figures 1.2 and 1.3A) and are homologous to kringle domains of several other proteins, including: tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), prothrombin, factor XII (reviewed by Patthy, 1985) and apolipoprotein(a) (McClean *et. al.,* 1987; Tomlinson *et. al.,* 1989). Structurally, kringles are triple disulfide bonded domains characterised by three short perpendicular antiparallel β-strands (figure 1.3B).

All of the plasminogen kringle domains have been isolated and individually characterised with respect to their affinities for the lysine analogues that structurally resemble either the N-terminal, C-terminal or internal lysine residues of proteins. The binding of lysine analogues, such as ε-amino caproic acid (ε-ACA), to K1-3 or isolated K1, K4 and K5 thermodynamically stabilise the native structures of these kringles (Menhart *et. al.,* 1991; Menhart *et. al.,* 1993; Castellino *et. al.,* 1981). Furthermore, both the enthalpy (ΔH) and entropy (ΔS) of the interactions are the driving forces for the binding of ε-ACA and other analogues to kringles such as K4 (Sehl and Castellino, 1990). Thus, the binding of lysine analogues to isolated, functional kringle domains of plasminogen is energetically favourable and results in the stabilisation of these domains.

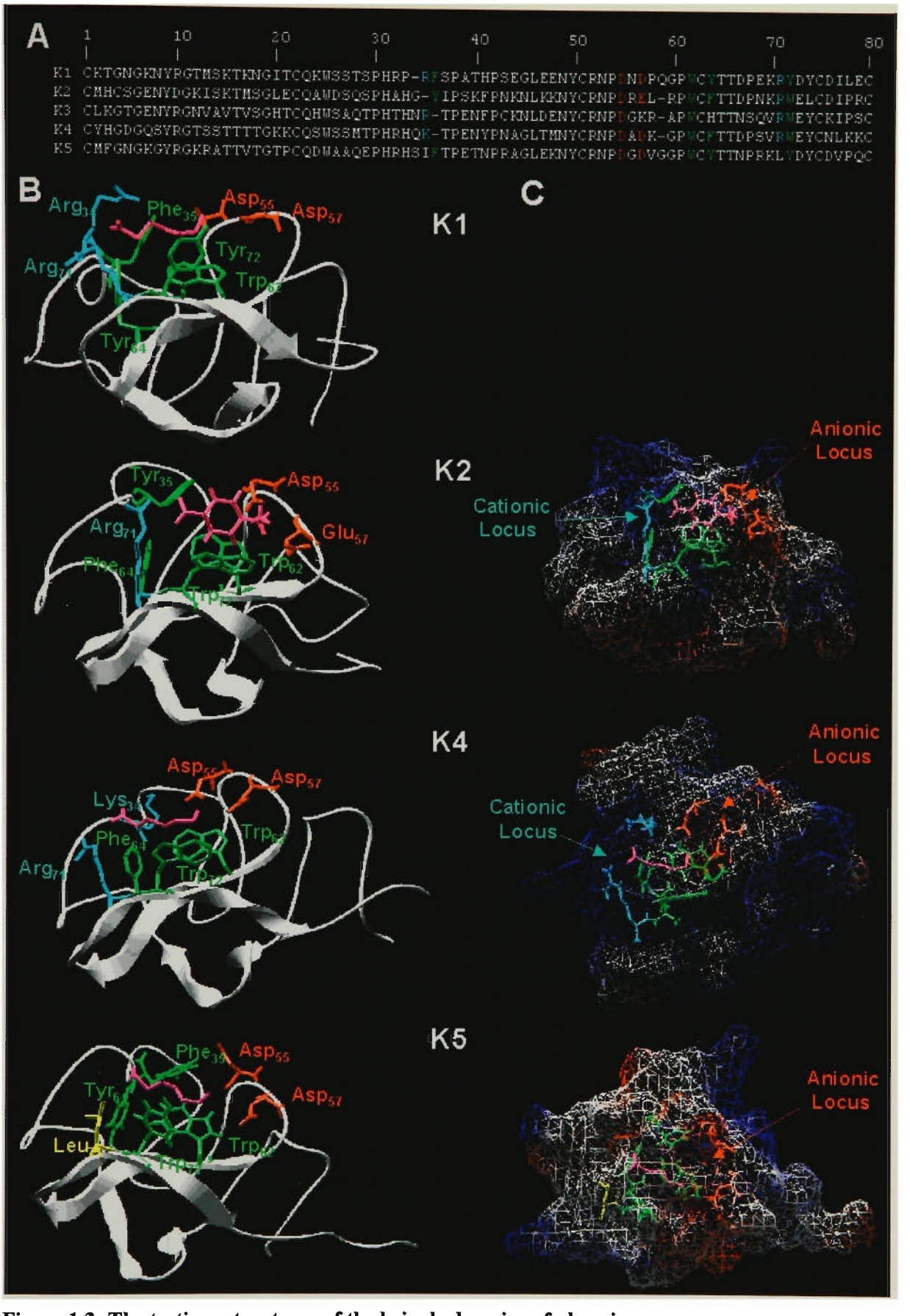

Figure 1.3: The tertiary structures of the kringle domains of plasminogen.

(A) Sequence alignment of the kringle domains of glu-plasminogen. Amino acids of the anionic, cationic loci and the hydrophobic clefts (red, blue and green respectively). (B) The tertiary structures of K1 (Matthews *et. al.*,1996), K2 (Marti *et. al.*,1999), K4 (Wu *et. al.*,1991) and K5 (Chang *et. al.*,1998) of glu-plasminogen complexed with either ε-ACA or TA (pink). Note, K2 tertiary structure was resolved using ^{1}H-NMR, whereas K1, K4 and K5 tertiary structures were derived using x-ray crystallography. (C) The anionic loci (-1.80; red), cationic loci (+1.80; blue) separated by the hydrophobic clefts (green) of the kringle domains.

K1 contains the high affinity LBS for ω-amino carboxylic acid ligands such as ε-ACA and some cyclic analogues such as *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (t-AMCHA; table 1.1). The LBS motif of isolated K1 displays a preference for C-terminal lysine analogues as opposed to internal lysine residue analogues (table 1.1). Furthermore, the LBS motif of K1 binds in a lysine-dependent manner to the plasmin inhibitory serpin α2-antiplasmin (Wiman *et. al.,* 1979) and the plasma plasminogen binding protein, histidine rich glycoprotein (Lijenen *et. al.,* 1980).

The lysine binding characteristics of K2 and K3 of human plasminogen are not as well characterised as K1, K4 and K5 possibly due to the presence of an inter-kringle disulfide bond between Cys_{169}-Cys_{297} (figure 1.2) (Cys_4 of K2 to Cys_{43} of K3, kringle numbering convention). This disulfide bridge permits the generation of a K2-K3 "supermodule". Recombinant K2 (r-K2) and r-K3 were engineered to remove the inter-kringle disulfide bond so the individual lysine binding characteristics could be studied. The LBS motif of K2 is designated as the lowest affinity LBS within plasminogen for all the classes of lysine analogues examined (table 1.1). Furthermore, it exhibits weak binding to lysine-Bio-Gel (Marti *et, al.,* 1994). Isolated K2 binds to the C-terminal lysine analogue *N*α-acetyl-L-lysine with an affinity approximately 40-fold lower than the LBS motifs of K1 and K4 (table 1.1; Marti *et. al.*, 1997). Despite its very low affinity for small lysine analogues, r-K2 as opposed to r-K1 and r-K3, binds to an internal sequence of the streptococcal surface plasminogen binding protein PAM with an apparent K_a of 4.5×10^7 M^{-1} (Wistedt *et. al.,* 1998), which

Table 1.1 The ligand affinities of isolated human plasminogen kringles and the ligand concentration required to induce a half maximal conformational change to glu-plasminogen.

Ligand	Ligand-type	Equilibrium association constant (K_a) (mM^{-1})[a]					[Ligand] that induces a ½ maximal glu-plg conformation change (mM)
		Glu-Plg[o]	K1	K2[a]	K4	K5	
4-aminobutyric acid	ω-aminocarboxylic acid	Nd	5.1 (2.6[k])	0.22	11	0.2	20.7[j]
5-aminopentanoic acid	ω-aminocarboxylic acid	Nd	44.6(36[k])	3.4	16[c]	0.72	4.9[j]
6-aminohexanoic acid (ε-ACA)	ω-aminocarboxylic acid	0.37[m]	74.2(77[k])	2.3	21[c](37[k])	10.6[h](7.1[k])	2.4[j]
7-aminoheptanoic acid	ω-aminocarboxylic acid	Nd	13.8 (7[k])	0.45	6.6[c]	2.1	17.4[j]
L-lysine	Free amino acid	14.7[n]	2.7[b]	0.68	24.4[c]	0.1[h]	25.1[j]
N^{α}-acetyl-L-lysine	C-terminal lysine	0.09[m]	41[b]	0.96	37[c]	0.18	Nd
L-lysine methyl ester	N-terminal lysine	Nd	0.16[b]	0.1	1.5[f]	0.17	Nd
N^{α}-acetyl-L-lysine methyl ester	Internal lysine	0.0018[m]	0.16[b]	0.1	0.2[f]	0.28	Nd
L-arginine	Free amino acid	Nd	Nb[b]	0.31	Nb[c] (0.08[m])	Nb[h](53[m])	Nd
N^{α}-acetyl-L-arginine	C-terminal arginine	Nd	0.72	0.55	0.32[c]	Nb[h]	Nd
L-arginine methyl ester	N-terminal arginine	Nd	Nb	0.03	Nb[c]	0.17	Nd
N^{α}-acetyl-L-arginine methyl ester	Internal arginine	Nd	0.09	0.07	0.08[c]	Nb[h]	Nd
γ-guanidinobutyric acid	Arginine R-group	Nd	2.8[b]	0.28	2.6	0.37	Nd
t-AMCHA[l]	Cyclic ligands	1.25[m]	>300[b](>300[k])	7.3	159[e](204[k])	44.2 (46[k])	0.5[j]
BASA	Cyclic ligands	Nd	82.6[b]	4.0	74[g]	2.2[h]	Nd
Benzylamine	Cyclic ligands	Nd	0.16	0.04	0.18[c]	6.3[h]	Nd
Benzamidine	Cyclic ligands	Nd	0.08	0.03	Nb[c]	3.4[h]	Nd
CB-Pre-activation domain	Glu_1-Met_{57} of PAD	N/A	4.6[l]	3.3[l]	6.2[i]	2.3[i]	Nd

Nb: non-specific or very weak binding (i.e. K_a<0.03 mM^{-1}) to isolated kringle domains; Nd: not determined. N/A: Not applicable

[a] Unless specified all equilibrium association constants for the isolated kringles were obtained from Marti *et. al.*, 1997 using ^{1}H-NMR spectroscopy; Other equilibrium association constants have been derived by [b]Rejante, 1992 (as cited in Marti *et. al*, 1997); [c]Rejante *et. al.*, 1991; [d]Ramesh *et. al.*, 1987; [e]De Marco *et. al.*, 1987; [f]Petros *et. al.*, 1989; [g]Hochschwender *et. al.*, 1983; [h]Thewes *et. al.*, 1987; [i]An *et. al.*, 1998; [j] The concentration of ligand required to induce a half maximal conformation change to glu-plasminogen evaluated by titration of the change in $S^{o}_{20,\omega}$of glu-plasminogen 2 with these ligands: Violand *et. al.*, 1978. Equilibrium association constants for isolated kringles[k] (K1 from Menhart *et. al.*, 1991; K4 and 5 from Menhart *et. al.*, 1993); [m]Novokohatny *et. al.*, 1989 or intact glu-plasminogen[m] (glu-plg) (Christensen and Molgaard, 1992) were determined using intrinsic fluorescence studies. [l] *trans*-4-(Aminomethyl)cyclohexanecarboxylic acid. [n] Brockway and Castellino, 1972

suggests an important biological function. In contrast, r-K3 is devoid of a binding affinity for ω- aminocarboxylic acids (Marti *et, al.,* 1994). The binding of ε-ACA to K2 is not inhibited by the presence of K3 in the isolated kringle supermodule conformation (Sohndel *et. al.,* 1996).

Several studies have determined that isolated K4 of plasminogen has slightly lower affinity for ε-ACA than K1 (table 1.1; deMarco *et. al.,* 1987; Lerch, *et. al.,* 1980; Sehl and Castellino, 1987). The surface availability of the LBS motif of K4 within glu-plg may be limited due to the positioning of the K1-3 domains in the parent molecule (Vali and Patthy, 1982). K4 also binds to the plasminogen-K4 binding protein tetranectin (Clemmensen *et. al.,* 1986; Kluft *et. al.,* 1989b).

The LBS motif of K5 displays low affinity for all classes of lysine analogues (table 1.1). It has also been termed the amino-hexyl (AH) site of plasminogen since it preferentially interacts with the AH but not the carboxylate function of lysine residues (table 1.1; Christensen, 1984). The preference of the K5 LBS motif for the AH side chain of lysine residues is further demonstrated by its higher affinites for *N*α-acetyl-L-lysine methoxyester, a lysine analogue that resembles an internal lysine residue of proteins (table 1.1). However, K5 also binds N- and C-terminal lysine residue analogues with a similar affinity (table 1.1). It is predicted that due to this low affinity but broad binding specificity, that LBS motif of K5 may bind internal lysine residues of a protein (Ponting *et. al.,* 1992), such as Lys_{50} or Lys_{62} of the NTP of glu-plasminogen (Cockrell *et. al.,* 1998). In contrast to the other plasminogen kringle LBS motifs, K5 displays selective affinity for both benzamidine and benzylamine (table 1.1; Thewes *et. al.,* 1987; Varadi and Patthy, 1981). Hence, these small cyclic molecules may be used to discern the contribution of K5 to the physiological functions of glu-plasminogen.

Within plasminogen there are four classes of LBS motifs with respect to their affinities for the ω-aminocarboxylic acid analogues: K1>K4>K5>K2. Furthermore, K1 and K4 preferentially bind C-terminal analogues whilst K5 displays a preference for internal lysine analogues. Therefore, each of the kringle LBS motifs may play a slightly different role due to: [1] their differential affinities for the three classes of lysine residues within proteins, and [2] their position in the A–chain of glu-plasminogen. The differential affinities of the kringle LBS motifs for lysine analogues that mimic N-terminal, C-terminal and internal lysine residues of proteins may be due to slight structural differences of the individual LBS motifs of the kringle domains.

1.1.1.4 The Architecture of the LBS motif

The structural characteristics of the LBS motifs located within the isolated kringle domains of plasminogen have been determined using chemical modification studies (Vali and Patthy, 1984; Trexler *et. al.,* 1982; Trexler *et. al.,* 1985), site-directed mutagenesis (Nielsen *et. al.,* 1993; M^{c}Cance and Castellino, 1995), NMR spectroscopy (Hochschwender *et. al.,* 1983; Llinas *et. al.,* 1983; Trexler *et. al.,* 1983; Ramesh *et. al.,* 1987; DeMarco *et. al.,* 1987; DeMarco *et. al.,* 1985; Motta *et. al.,* 1986; Motta *et. al.,* 1987; Thewes *et. al.,* 1987; Petros *et. al.,* 1989; Rejante and Llinas, 1994) and x-ray crystallography (Matthews *et. al.,* 1996; Wu *et. al.,* 1991; Chang *et. al.,* 1998). The crystal structures of K1 (Matthews *et. al.,* 1996), K4 (Wu *et. al.,* 1991; Mulichak *et. al.,* 1991) and K5 (Chang *et. al.,* 1998) demonstrate that the kringle LBS motifs are: (1) preformed and localised to the surfaces of these domains (figure 1.3B, C), and (2) that the kringle domains do not change conformation upon ligand binding (Wu *et. al.,* 1991; Mulichak *et. al.,* 1991; Matthews *et. al.,* 1996). Water probably occupies the apo (unligated) LBS motifs of the kringle domains of glu-plasminogen (Chang *et. al.,* 1998). ^{1}H-NMR of isolated K1 (DeMarco *et. al.,* 1982), K4 (Hochschwender *et. al.,* 1983; Llinas *et. al.,*1983; Ramesh *et. al.,* 1987;

Trexler *et. al.*,1983) and K5 (Thewes *et. al.*, 1987) suggest that these domains exist as compact globular structures in solution.

Each of the plasminogen kringle LBS motifs has a slightly different architecture that may account for the different affinities observed for individual lysine analogues (table 1.1). The basic structural elements required for a functional, high affinity, LBS are anionic and cationic loci separated by a hydrophobic cleft, optimised for the specific electrostatic, hydrophobic and steric requirements of lysine residues (figure 1.3).

The ε-amino group of a bound lysine ligand is stabilised by an ionic interaction with the carboxylate functions of the aspartate residues of the anionic locus (figure 1.3). The aspartate anionic locus is present in all functional lysine binding kringle domains of plasminogen, for example, Asp_{55} and Asp_{57} are rigorously conserved in K1, K4 and K5 of plasminogen (figure 1.3A). This is not unexpected, since the ε-amino group is part of the R group of L-lysine and therefore is common to all classes of lysine residues within proteins. K2 of plasminogen, which has very low affinity for lysine analogues (table 1.1), has a glutamate residue at position 57 (figure 1.3). This may reduce the electrostatic attraction between the anionic locus of K2 and the ε-amino group of the lysine ligand. Thus, the apparent low affinity of K2 for all classes of lysine analogues (table 1.1) may be partially due to this aspartate/glutamate substitution. K3 of plasminogen, which does not bind lysine analogues, has a lysine residue (opposite charge) at position 57 (figure 1.3A). This would sufficiently perturb the charge of the anionic locus of kringle 3 thereby destroying its ability to bind the ε-amino group of a lysine ligand. Substitution of Asp_{57} with alanine or asparagine eliminates the lysine binding capacities of K1, K4 and K5 (Nielsen *et. al.*, 1993; M^{c}Cance and Castellino, 1995). Thus, the anionic locus of a kringle is fundamental to its lysine binding ability.

The amino acids of the cationic locus, which stabilise the carboxyl group of a lysine analogue, are more ambiguous to define than those of the anionic locus. Positively charged amino acids such as either lysine and arginine at positions 34 or 35 and arginine at position 71 define the cationic locus of the kringle LBS (figure 1.3). The x-ray crystal structure for human plasminogen K1 suggests that both Arg_{34} and Arg_{71} may contribute to the cationic locus (figure 1.3; Matthews *et. al.,* 1996). In contrast to human plasminogen K1, mouse plasminogen K1 does not contain a basic residue at a position homologous to Arg_{34} of human plasminogen K1 (Degan *et. al.,*1990). Nevertheless, mouse plasminogen K1 has an affinity for ε-ACA comparable to human plasminogen K1 (Castellino, 1995). Furthermore, the orientation of Arg_{34} the cationic loci in the solution structure of K1 varies significantly from the crystal structure of K1 (figure 1.4; Rejante and Llinas, 1994; Matthews *et. al,* 1996). In contrast, the orientation of the anionic loci of the solution and crystal structures of K1 are comparable (figure 1.4; Rejante and Llinas, 1994; Matthews *et. al,* 1996). Therefore, Arg_{34} may not be a member of the cationic locus of K1 of soluble plasminogen.

Chemical modification of isolated K4 suggests that Arg_{71} is critical for lysine-dependent binding (figure 1.3). In contrast, Lys_{35} may not be an important member of the cationic locus in solution phase K4 (Nielsen *et. al.,* 1993). Thus, Arg_{71} may be the sole constituent of the cationic locus of soluble K1 and K4. In K5 of plasminogen Arg_{71} is replaced by Leu_{71} (neutral R-group) (figure 1.3). Replacement of Leu_{71} with an arginine residue by site-directed mutagenesis increases the affinity of mutant K5 for ε-ACA to levels comparable to K1 and K4 (Chang *et. al.,* 1998). Thus, a single

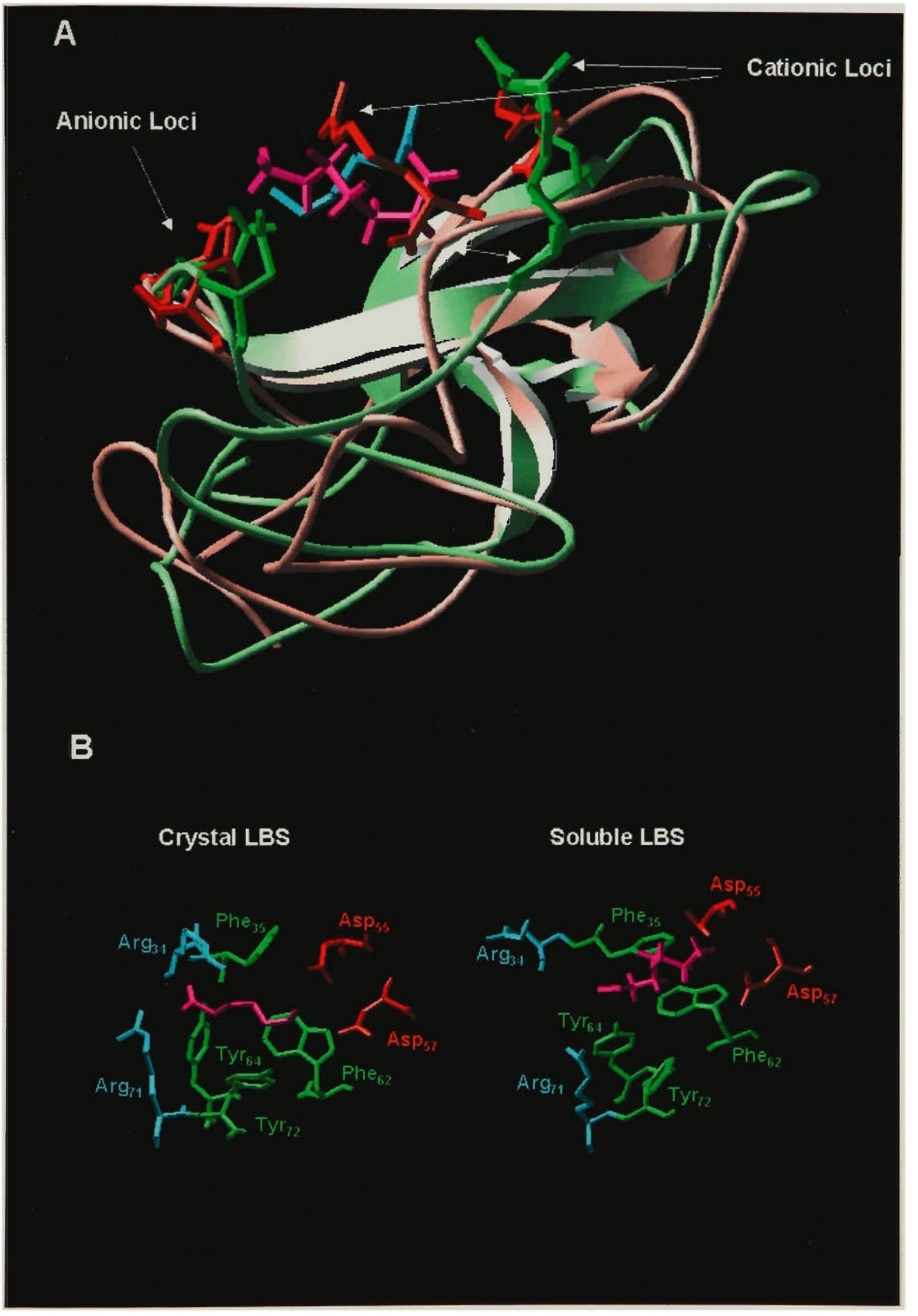

Figure 1.4:Comparison of the tertiary structure of solution-phase and crystallised plasminogen K1.

(A) Alignment of solution-phase (green; Rejante and Llinas, 1994) and crystallised (pink; Matthews *et. al.*, 1996) of plasminogen K1. The relative positions of the anionic and cationic loci of both the solution-phase (green) and crystal-phase (red) are shown. (B) Comparison of the anionic (red), cationic (light blue) and hydrophobic (green) components of the LBS of the solution-phase and crystal-phase complexed to ε-ACA (pink).

basic amino acid at position 71 may be sufficient for the stablisation of the ligand carboxyl group by the cationic locus.

The anionic and cationic loci of the kringle LBS motifs are separated by a series of aromatic amino acids, namely Phe_{36}/Tyr_{36}, Trp_{62}, Phe_{64}/Tyr_{64} and Tyr_{72}/Trp_{72}, organised into a hydrophobic cleft approximately 7.33 Å in length (Marti *et. al.*,1997). The hydrophobic cleft of all plasminogen kringles contains Trp_{62} and Tyr_{72}/Trp_{72} residues. However, only functional, lysine-binding kringles of plasminogen contain Phe_{64}/Tyr_{64} residues, in contrast to the non-functional K3, which has a histidine at position 64 (figure 1.3A). Furthermore, of the lysine binding functional kringles, only K4 does not have a phenylalanine or tyrosine at position 36 (figure 1.3A). The binding of ε-ACA to the hydrophobic clefts of K1, K4 and K5 results in a negative change in the standard heat of capacity (ΔC°) for this interaction of -81, -37 and -40 cal $K^{-1}mol^{-1}$ respectively (Menhart and Castellino, 1995). The absence of Phe_{36}/Tyr_{36} in K4 may contribute to its reduced ligand affinity when compared to ligand affinity of K1, since K4 had the highest ΔC° for ε-ACA binding. This suggests that the aromatic amino acid side chains of the hydrophobic clefts are important for stabilising the interaction of ε-ACA with these kringles (Menhart and Castellino, 1995).

The LBS motifs of K1 and K4 of plasminogen preferentially bind C-terminal lysine residue analogues (table 1.1). Physiologically, the carboxylate function of lysine is only present on free lysine and C-terminal lysine residues of proteins. Thus, the presence of a cationic locus within the LBS motifs of K1 and K4 may confer C-terminal lysine residue specificity to these kringles, as opposed to the internal lysine residue specificity of the K5 LBS, which does not contain a cationic locus (figure 1.3). Hence, the ubiquitous presence of the anionic locus in all functional

plasminogen kringle LBS motifs, facilitates lysine-dependent binding, whilst the cationic locus confers lysine residue-type specificity.

1.1.1.5 Lysine-dependent conformational changes of glu-plasminogen

The lysine-dependent conformations of glu-plasminogen have been defined using small lysine analogues. For example, incubation of glu-plasminogen with L-lysine, ε-ACA, or tranexamic acid induces the fully open, but reversible change in the conformation of glu-plasminogen (table 1.1; Violand *et. al.,* 1978; Brockway and Castellino, 1972; Castellino *et. al.,* 1973; Markus *et. al.,* 1978; Markus *et. al.,* 1979) from a tight right hand spiral to a U-shaped conformation (Weisel *et. al.,* 1994) thereby shifting the conformational equilibrium of the protein (figure 1.5; table 1.1).

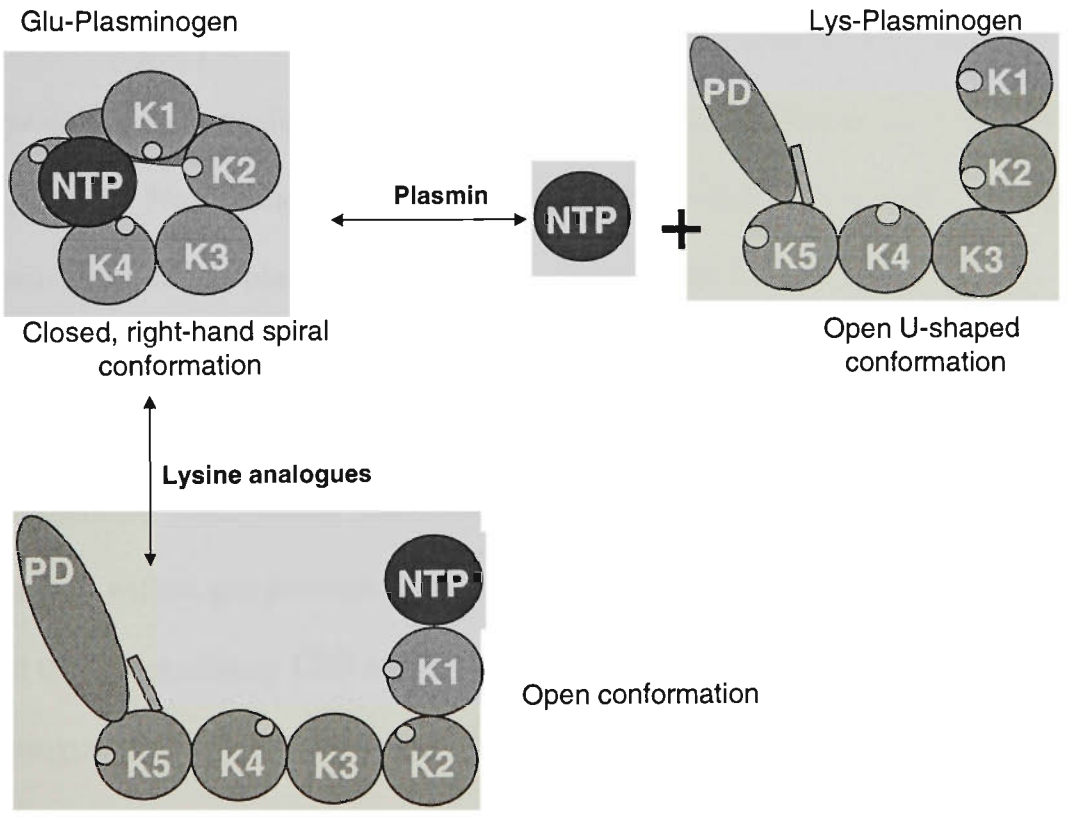

Figure 1.5: Schematic representations of the conformations of glu- and lys-plasminogen.
Glu-plasminogen exists a closed right hand spiral conformation. Plasmin mediated cleavage of the NTP (●) from the A-chain of glu-plasminogen results in the formation of lys-plasminogen (in an open U-shaped conformation). The functional LBS motifs are represented by within the kringle domains ●.

In an manner similar to lys-plasminogen, this lysine-dependent conversion of glu-plasminogen from the closed to the open isomer is not accompanied by a change

in the secondary structure of the molecule (Sjoholm *et. al.,* 1973; Vuk-Pavlovic and Gafni, 1979; Ramakrishnan *et. al.,*1991). Hence, the closed conformation is a product of the interactions between the different domains of glu-plasminogen. Moreover, under acidic conditions the kringle domain and protease domain of miniplasminogen (K5-protease domain) have significant structural and motional independence (Teuten *et. al.,* 1991). Similarly, glu-plasminogen under the same conditions also exhibits domain independence (Teuten *et. al.,* 1991) thereby re-enforcing the notion that the interdomain linker sequences of glu-plasminogen are flexible. Although glu-plasminogen exists predominantly in the closed conformation, regions of the molecule may oscillate ("breath") between the closed and open conformations of the protein. For example the LBS motif(s) responsible for the lysine-induced conformational changes of glu-plasminogen, are estimated to spend between 20% and 40% of their time in the open conformation (Markus *et. al.,* 1978; Markus *et. al.,* 1979; Weisel *et. al.,* 1994). Nevertheless, in the absence of exogenous lysine, the conformational equilibrium of glu-plasminogen exists in favour of the closed isomer.

The LBS motifs of the kringle domains maintain the closed conformation of glu-plasminogen by binding to internal lysine residues of plasminogen. Site-directed mutagenesis experiments that eliminated the lysine binding function of individual kringles within glu-plasminogen, demonstrated that both the high affinity K1 LBS and the lower affinity LBS of K4 (both of which preferentially bind C-terminal lysine analogues (table 1.1)), are responsible for mediating large conformational changes to glu-plasminogen (M^{c}Cance and Castellino, 1995). In contrast, K5 is responsible for minor (or partial) conformational changes of glu-plasminogen (M^{c}Cance and Castellino, 1995). Thus, the closed conformation of glu-plasminogen is maintained by the lysine-dependent interactions of K1, K4 and K5 with lysine residues from other regions of glu-plasminogen. The presence of this intramolecular lysine-binding

network suggests that the LBS motifs of glu-plasminogen are occupied by the internal lysine residues of the protein.

The NTP domain contributes to the closed, lysine-dependent conformation of glu-plasminogen by providing some of the internal lysine residues that bind to the kringle domains. Christensen (1984) first suggested that the closed conformation of glu-plasminogen might be mediated by a lysine-dependent interaction between the Lys_{50} of the NTP and the LBS motif of kringle 5. Incubation of glu-plasminogen with an exogenous peptide containing the Ala_{44}-Lys_{50} residues of the NTP domain induces a conformational change in the molecule (Takada *et. al.,* 1993). Furthermore, isolated K4 binds to this peptide thereby supporting the notion that LBS motifs can bind to the NTP of glu-plasminogen (Ramesh *et. al.,* 1995). However, within this peptide Lys_{50} has a C-terminal configuration and hence does not accurately reflect the chemical properties of Lys_{50} in the NTP of glu-plasminogen. Site-directed mutagenesis studies that eliminated the lysine-binding activity of K5, as well as substituting Lys_{50} or Lys_{62} of the NTP with alanine residues, have provided direct evidence that this kringle does interact with Lys_{50} or Lys_{62} of the NTP to maintain the closed conformation of the protein (Cockrell *et. al.,* 1998). Furthermore, isolated K1, K2, K4 and K5 bind, in a lysine-dependent manner to the Glu_1-Met_{57} fragment of the NTP of glu-plasminogen (which contains Lys_{50} as an internal residue), with affinities ranging from 6.2 mM^{-1} down to 2.3 mM^{-1} with K4 having the highest affinity for Lys_{50} of the NTP (An *et. al.,*1998a). The Ala_{44}-Lys_{50} NTP fragment binds specifically to isolated K4. Thus, Lys_{50} of the NTP of glu-plasminogen has also been suggested as a possible ligand for K4 (An *et. al.,* 1998b).

The amino acid sequence containing Lys_{50} in the NTP is highly flexible (An *et. al.,* 1998a). This flexibility may be important for the interaction of Lys_{50} with either

the K4 or K5 LBS. ^{1}H-NMR and molecular modeling studies have suggested that the Glu_{39}-Lys_{50} stretch in the NTP of glu-plasminogen generates an area that complements both topologically and electrostatically the solvent-exposed K4 LBS surface (An *et. al.,* 1998b). Homology modeling of the NTP of glu-plasminogen using the NTP of HGF (designated as 2hgf in the Brookhaven database; figure 1.6) suggests that both Lys_{50} and Lys_{62} are located on opposite sides of the hairpin loop of the NTP of glu-plasminogen (approximately 23 Å apart). If this model (figure 1.6) were correct, there would be enough space for a kringle domain to bind to each of these lysine residues simultaneously (figure 1.6). Therefore, Lys_{50} and Lys_{62} of the NTP are implicated as the conformational determinants of glu-plasminogen. However, other lysine residues, acting a conformational determinants may exist since K1 has been shown by mutagenesis to be an important conformational determinant of glu-plasminogen (M^{c}Cance and Castellino, 1995).

Exogenous lysine analogues, such as ε-ACA (table 1.1) induce the open conformation of glu-plasminogen by disrupting the intramolecular lysine-binding network of protein. Tranexamic acid (Markus *et. al.,* 1979) and ε-ACA (Markus *et. al.,* 1978) bind to approximately five sites within glu-plasminogen (one high affinity and four low affinity sites). Marshall *et. al.,* 1994, using different lysine analogues, has suggested that glu-plasminogen can actually exist in three distinct conformations: α or closed conformation (in the absence of exogenous lysine analogues); β or partially open conformation (resembling the U-shaped conformation of lys-plasminogen); and γ (fully open, exogenous lysine saturated conformation).

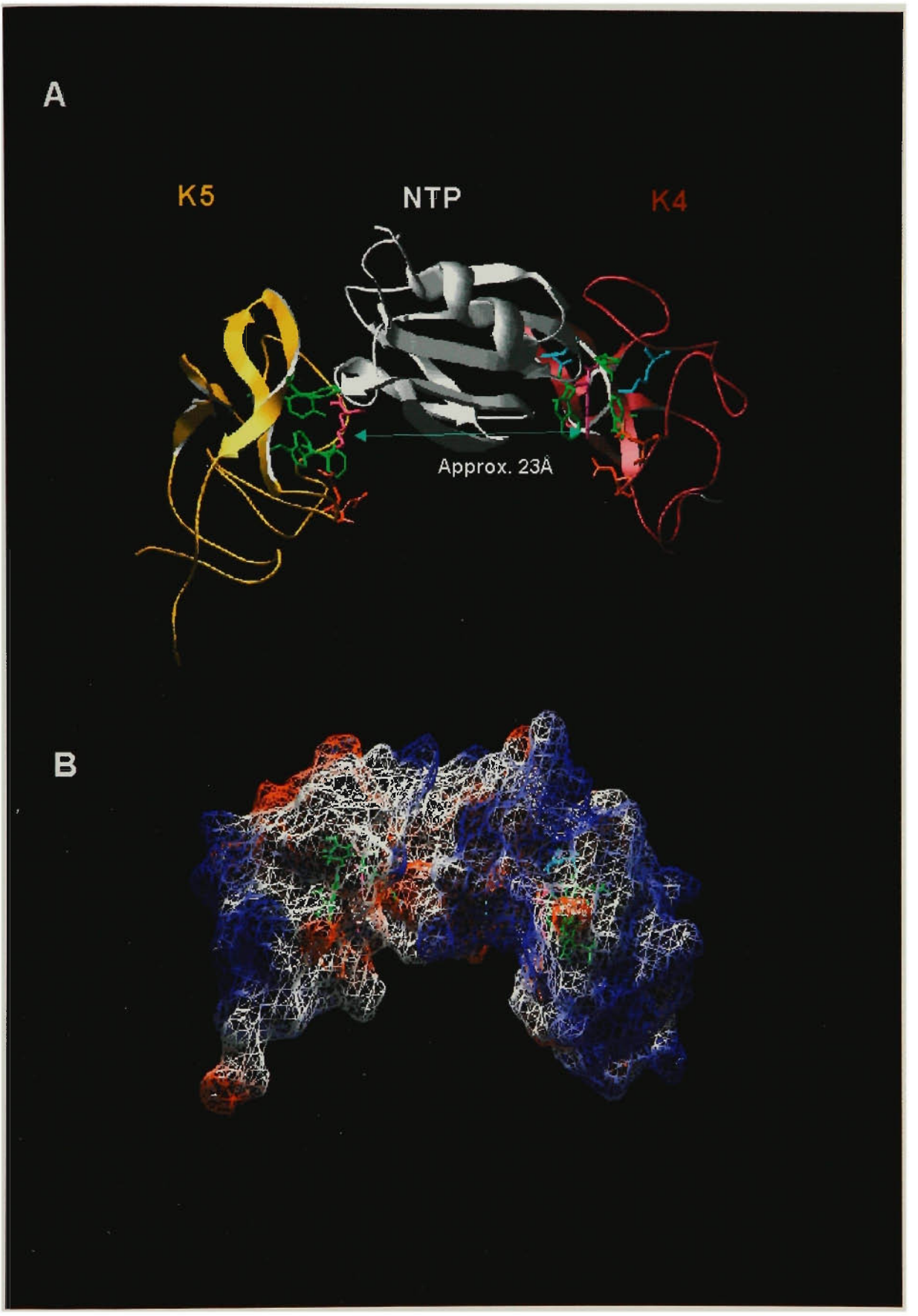

Figure 1.6: A hypothetical model of the NTP of glu-plasminogen and its possible interactions.

A hypothetical model of the NTP of glu-plasminogen modeled on the structure of the NTP of HGF and generated using Swiss Pdbviewer (Peitsch and Guex 1997). (A) Ribbon model of K4 (pink) and K5 (orange) binding to lys_{50} and lys_{62} of the NTP (white). (B) An electrostatic surface model showing the charge (red: –1.80; blue: +1.80) distribution of the K5-NTP-K4 complex.

Incubation of glu-plasminogen α with the K5 specific ligand, benzamidine (table 1.1; Thewes *et. al.,* 1990), induces the formation of glu-plasminogen β (figure 1.7; Marshall *et. al,* 1994). Lys-plasminogen and glu-plasminogen β have a similar conformation, thus disruption of the lysine-dependent binding of K5 to a lysine residue in the NTP for both is responsible for a partial conformational change of glu-plasminogen. Subsequent treatment of glu-plasminogen β or lys-plasminogen with ε-ACA induces the fully open (γ) conformation of these proteins. This suggests that multiple kringle lysine-dependent interactions are responsible for inducing the fully open conformation of glu-plg (Marshall *et. al.,* 1994). Thus, the conformational status of glu-plasminogen is the result of a series of competition reactions between the internal lysine residues of glu-plasminogen and exogenous lysine analogues that disrupt the intramolecular lysine-binding network of glu-plasminogen.

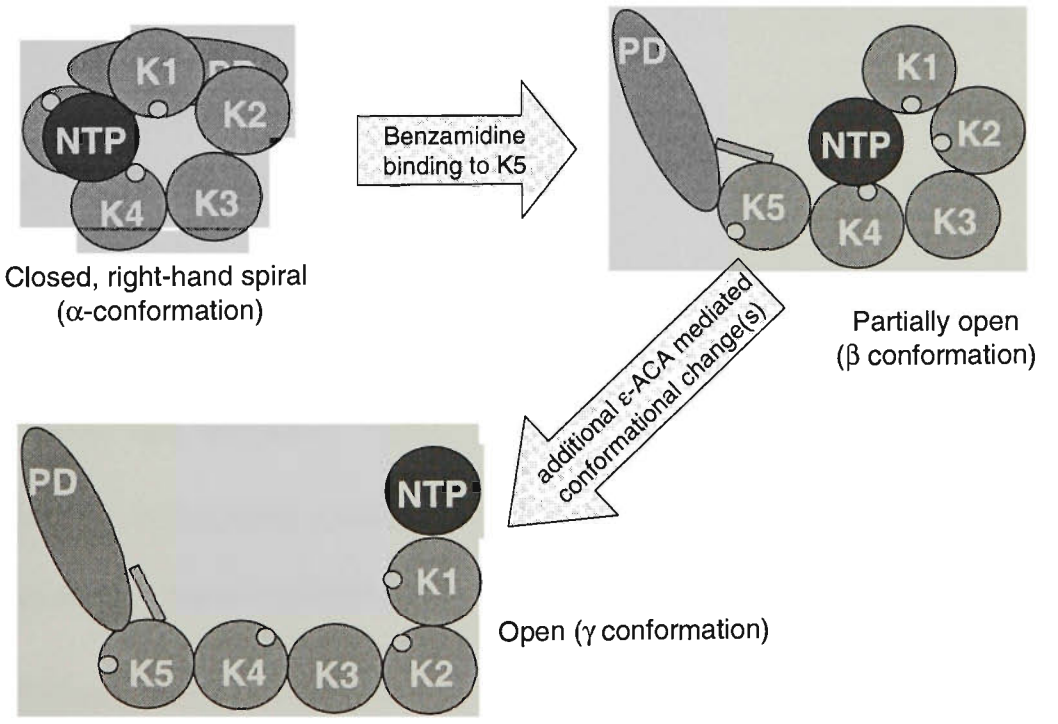

Figure 1.7: Schematic representation of the lysine-dependent conformational changes of glu-plasminogen.

The interaction of multiple LBS motifs with exogenous lysine residues is hypothesised to be required for the complete conformational change of glu-plasminogen to the open, activation-susceptible conformation. Kringle domains (●), NTP (●), protease domain (●) and the LBS motifs ( ).

The interaction of multiple LBS motifs with exogenous lysine analogues is responsible for disrupting the closed conformation of glu-plasminogen (table 1.1). The total lysine-dependent conformational change of glu-plasminogen (i.e. $\alpha \rightarrow \gamma$) involves both the high affinity and multiple, low affinity, kringle LBS motifs over a wide range of lysine analogue concentrations (Markus *et. al.,* 1978; Markus *et. al.,* 1979). Strong binding of the first lysine analogue to glu-plasminogen causes little change in the conformation of the protein. In contrast, saturation of the weak LBS motifs of glu-plasminogen with tranexamic acid or ε-ACA induces most of the lysine-dependent conformational change of the protein (Markus *et. al.,* 1978; Markus *et. al.,* 1979). Similarly, Christensen and Molgaard, 1991 demonstrated that a rapid conformational change of glu-plasminogen ($t_{1/2}$ = 0.01 s) is mediated by the cooperative binding of the LBS of K5 and K4 to ε-ACA (Christensen and Molgaard, 1992). However, the interaction of K1 with ε-ACA does not mediate a conformational change to glu-plasminogen (Christensen and Molgaard, 1992). Both C-terminal and internal lysine analogues are capable of inducing this conformational change to glu-plasminogen (Christensen and Molgaard, 1992). The apparent ε-ACA-induced conformational change rate constants for glu-plasminogen are k_2 = 69 $s^{-1}$and k_{-2} = 3 s^{-1} (Christensen and Molgaard, 1991), suggesting that exogenous ε-ACA locks glu-plasminogen in the open conformation. Thus, the lysine-dependent conformational changes of glu-plasminogen are mediated by its multiple kringle domains.

1.1.1.6 Plasminogen activation and conformation

The lysine-dependent conformation of glu-plasminogen regulates its activation rate. Both the conformations (Weisel *et. al.,* 1994; Sjoholm *et. al.,* 1973) and activation rates (table 1.2; Markus *et. al.,* 1978; Violand *et. al.,* 1978; Peltz *et. al.,* 1982; Lijnen *et. al.,* 1984) of glu-plasminogen in the presence of lysine analogues,

Table 1.2: Activation kinetics of glu-plasminogen by different plasminogen activators in the presence or (absence) of various ligands or cells.

Plg Species (substrate)	Ligand/Cell type	Plg Activator	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Fold increase k_{cat}/K_m and parameter responsible for increase	Reference
Glu-Plg	ε-ACA	tc-uPA	0.8 (0.06)	2.5 (25)	3.2 (0.02)	160; k_{cat}	Lucas *et. al.*, 1983
Glu-Plg	ε-ACA	tc-uPA	3.8 (3.8)	18 (138)	0.21 (0.028)	7.5; K_m	Banyai & Patthy, 1985
Glu-Plg[1]	ε-ACA	tc-uPA	0.8 (1.1)	238 (215)	$3x10^{-3}$($5x10^{-3}$)	0.6	Banyai & Patthy, 1985
Glu-Plg	-	tc-uPA	5.6	11.7	0.5	N/A	Ellis *et. al.*, 1987
Glu-Plg	-	sc-uPA	2.9	47.7	0.06	N/A	Ellis *et. al.*, 1987
Glu-Plg	-	tc-uPA-Lys		3.7	11	N/A	Lenich *et. al.*, 1991
Glu-Plg	-	tc-uPA-Phe		5.9	9.2	N/A	Lenich *et. al.*, 1991
Glu-Plg	Fibrinogen	tc-tPA	0.3 (0.06)	28 (65)	0.01 ($9.2x10^{-4}$)	11; k_{cat}	Hoylaerts *et. al.*, 1982
Glu-Plg	Fibrinogen	tc-tPA	0.76 (0.15)	0.11 (0.13)	6.91 (1.15)	6; k_{cat}	Stack *et. al.*, 1990
Glu-Plg	CNBr Fibrinogen Frag	tc-uPA	1.98 (0.15)	0.17 (0.13)	11.65 (1.15)	10.1; k_{cat}	Stack *et. al.*, 1990
Glu-Plg	Fibrin	tc-tPA	0.1 (0.06)	0.16 (65)	0.625 ($9.2x10^{-4}$)	680; K_m	Hoylaerts *et. al.*, 1982
Glu-Plg	Fibrin	tc-tPA	0.12 ($7.8x10^{-3}$)	0.18 (7.6)	0.67 ($1.4x10^{-4}$)	4786; K_m	Ranby, 1982
Glu-Plg	Fibrin	tc-tPA	0.1 (0.09)	0.017 (6.7)	6.5 (0.014)	464; K_m	Tachias *et. al.* 1996
Glu-Plg	Fibrin	sc-tPA	0.083 ($1.3x10^{-3}$)	0.46 (4.9)	0.18 ($2.7x10^{-4}$)	666.6; k_{cat}	Ranby, 1982
Glu-Plg	HRGP	tc-tPA	0.008 (0.037)	0.123 (2.3)	0.07 (0.02)	3.5; K_m	Borza & Morgan, 1997
Glu-plg	Annexin II	tc-tPA	0.201 (0.02)	0.066 (0.596)	3.05 (0.048)	63.5; K_m	Cesarman *et. al.*, 1994
Glu-Plg	U937 (monocytoid)	-	0.067 (0.6)	0.9 (65)	0.072 ($9x10^{-3}$)	7.8; K_m	Felez *et. al.*, 1996
Glu-Plg	Non-activated platelets	tc-tPA	$9x10^{-3}$ ($7x10^{-3}$)	2.6 (10)	$3x10^{-3}$ ($7x10^{-4}$)	4.3; K_m	Gao *et. al.*, 1990
Glu-Plg	C6 glioma	tc-tPA	3.7 (1.6)	0.49 (9.6)	7.6 (0.17)	44; K_m	Pernod *et. al.*, 1998
Glu-Plg	HUVEC	tc-tPA	$1.2x10^{-4}$($3x10^{-4}$)	0.24 (1.86)	$5x10^{-4}$($1.6x10^{-4}$)	3.2; K_m	Hajjar *et. al.*, 1987

[1] Glu-Plg was crosslinked with 1,5-difluoro-2,4-dinitrobenzene which "freezes" it in the closed conformation.

Table 1.3: Activation kinetics of lys-plasminogen by different plasminogen activators in the presence or (absence) of various ligands or cells.

Plg Species (substrate)	Ligand/Cell type	Plg Activator	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	fold increase k_{cat}/K_m and parameter responsible for increase	Reference
Lys-Plg	-	tc-uPA	1.97	9.0	13.1	N/A	Ellis *et. al.*, 1987
Lys-Plg	-	sc-uPA	0.09	11.7	0.5	N/A	Ellis *et. al.*, 1987
Lys-Plg	-	tc-uPA-Lys	62.1	5.4	11.5	N/A	Lenich *et. al.*, 1991
Lys-Plg	-	tc-uPA-Phe	77	15.2	5.1	N/A	Lenich *et. al.*, 1991
Lys-Plg	Fibrinogen	tc-tPA	0.52 (0.08)	0.5 (0.06)	1.04 (1.33)	7.8; k_{cat}	Stack *et. al.*, 1990
Lys-Plg	CNBr Fibrinogen frags	tc-tPA	0.58 (0.08)	0.04 (0.06)	14.5 (1.33)	10.9; k_{cat}	Stack *et .al.* 1990
Lys-Plg	Fibrin	tc-tPA	0.3 (0.2)	0.02 (19)	15 (0.01)	1500; K_m	Hoylaerts *et. al.*, 1982
Lys-Plg	Fibrin	tc-tPA	0.22 (0.25)	0.04 (0.3)	5.5 (0.8)	6.9; K_m	Ranby, 1982
Lys-Plg	Fibrin	sc-tPA	0.185 ($3x10^{-3}$)	0.04(0.17)	4.6 (0.02)	230; k_{cat}	Ranby, 1982
Lys-Plg	Annexin II	tc-tPA	0.72 (2.1)	0.045 (8.065)	16 (0.26)	61.5; K_m	Cesarman *et. al.*, 1994

and lys-plasminogen are comparable. This suggests that the lysine-dependent, open conformation of glu-plasminogen increases the catalytic efficiency (k_{cat}/K_m) of the activation reaction (table 1.2). This lysine-dependent increase in the activation rate is due to both a concomitant decrease and increase in the K_m and k_{cat} of the reaction, respectively (table 1.2).

The lysine-dependent increase in the activation rate of glu-plasminogen is partially driven by a decrease in the K_m of the reaction. Crosslinking of Lys_{203} of K2 to Tyr_{671} of the protease domain prevents an increase in activation rate of glu-plasminogen, induced by ε-ACA, by impeding the lysine-dependent conformational change of the protein (Banyai and Patthy, 1985; table 1.2). The heavy chain of glu-plasminogen, when the protein is in its lysine-dependent, closed conformation may hinder the access of the plasminogen activators to the Arg_{560}-Val_{561} activation bond of glu-plasminogen. The K_m for unbound, soluble, glu-plasminogen is approximately 10 fold above the physiological plasma concentration of glu-plasminogen. Thus, the plasminogen activators require approximately 10-fold more unbound, soluble, glu-plasminogen than is physiologically available for efficient activation of glu-plasminogen. In contrast, the K_m of glu-plasminogen in the open conformation is less than or equal to the physiological concentration of glu-plasminogen suggesting that when present in this conformation the efficient activation of glu-plasminogen is facilitated under physiological conditions.

The rate of this lysine-induced glu-plasminogen conformational change (70 s^{-1}) (Christensen and Molgaard, 1991) is approximately 20-fold greater than the catalytic activation rate constant (k_{cat}) for the hydrolysis of the plasminogen activation bond of glu-plasminogen (table 1.2; Christensen, 1977; Hoylaerts *et. al.,* 1982; Peltz *et. al.,* 1982; Ranby, 1982; Banyai and Patthy, 1985). This indicates that the time taken for a plasminogen activator to hydrolyse the activation bond of glu-plasminogen

(table 1.2) is greater than the time taken for glu-plasminogen to undergo the lysine-dependent conformational changes. Thus, the open conformation of glu-plasminogen, as opposed to the time taken for the either the conformational change or the hydrolysis of the activation bond, is the rate determining factor in activation of glu-plasminogen. This suggests that the lysine-dependent binding of glu-plasminogen to physiologically relevant sites may modulate its conformation and presents glu-plasminogen as a better substrate to the activator that rapidly converts it into plasmin. The net result would be a decrease in the concentration of lysine-bound glu-plasminogen required for efficient activation.

Chloride ions, in contrast to lysine molecules, act as negative allosteric regulators that impede the activation of glu-plasminogen. Chloride ions act by binding to glu-plasminogen with a K_i of 9 mM (Urano *et. al.,* 1988) and may help to maintain the protein in its closed, activation resistant, conformation in solution (Urano *et. al.,* 1988; Urano *et. al.,* 1987a). Both lysine and chloride ions bind glu-plasminogen simultaneously, suggesting that their binding sites are separate (Urano *et. al.,* 1987a). However, the inhibitory effect of chloride ions is negated in the presence of ε-ACA (Urano *et. al.,* 1987b). For example the K_m of urokinase for the activation of the glu-plasminogen:Cl^- complex is 25 μM compared to 2.2 μM for uncomplexed glu-plasminogen (Urano *et. al.,* 1987a). In contrast, ε-ACA facilitates activation of glu-plasminogen by inducing a favourable conformational change in the molecule for activation by urokinase as indicated by a K_m of 1.8 μM (Urano *et. al.,* 1987a). The physiological concentrations of chloride ions and L-lysine in plasma ranges between 96-106 mM (Murry *et. al.,* 1990) and 105-207 μM (Diem and Lentner,1970), respectively. Since the concentration of L-lysine required to produce a half maximal conformational change in glu-plasminogen is approximately 26 mM (table 1.1;

Violand *et. al.,* 1978) it is predicted that the majority of soluble plasma glu-plasminogen would be in the closed, activation resistant conformation.

1.1.1.7 Post-translational modifications of plasminogen.

Two glycoforms of human plasminogen (Plg I and Plg II), which differ in both the composition and extent of their glycosylation have been isolated from plasma based on their different affinities for lysine sepharose (Hayes and Castellino, 1979a; Hayes and Castellino, 1979b and Traas *et. al.,* 1984). Both glycoforms are glycosylated with an *o*-linked tetrasaccharide at Thr_{345} whereas glycoform I contains an additional *N*-glycosylation site at Asn_{289} (Hayes and Castellino, 1979a; Hayes and Castellino, 1979b; Hayes and Castellino, 1979c; Lijnen *et. al.,* 1981). Moreover, the *N*-linked glycosylation of glu-plasminogen increases its activation rate by urokinase (Takada and Takada, 1983). Lower concentrations of lysine analogues are required to induce a conformational change in glycoform I than glycoform II. The lysine-dependent transition of both glu-plasminogen glycoforms from the closed to open conformation I (Sugawara, *et. al.,* 1984) resulted in an increase in the activation rates of the molecules by urokinase (Takada *et. al.,* 1984). However, glycosylation at Asn_{289} (glycoform I) stabilises glu-plasminogen in the open conformation after a lysine-dependent interaction has occurred by significantly decreasing the rate of the reverse conformational change (i.e. the closing of the molecule via the removal of lysine) (Molgaard *et. al.,* 1997). This stablisation of the lysine-dependent, open conformation glu-plasminogen I would facilitate an increase in the activation rate of the molecule by allowing the plasminogen activators access to Arg_{560}-Val_{561} activation bond. Thus, glycosylation of glu-plasminogen affects both the stability and affinity of the protein to interact with lysine moieties as well as its subsequent activation rate.

Both neonatal glycoforms of glu-plasminogen have significantly more mannose and sialic acid than the adult glycoforms. This difference in the carbohydrate composition may be responsible for the decreased binding and activation rate observed for neonatal glu-plasminogen compared to adult glu-plasminogen (Edelberg *et. al.,* 1990). Hence, not only is the site of glu-plasminogen glycosylation important for the binding and activation of the molecule, but the composition of the carbohydrate moieties also impacts on the biology of glu-plasminogen.

Finally, circulating human plasminogen is a serine/threonine and tyrosine phospho-protein (Wang *et. al.,* 1997). Ser_{578} , located within the protease domain of the protein (figure 1.2), is a major phosphorylation site on circulating glu-plasminogen (Wang *et. al.,* 1997). Since Ser_{578} is present in the protease domain, phosphorylation at this site may affect plasmin activity. However, the role phosphorylation plays in plasminogen biology remains unknown.

1.1.1.8 Plasmin

The C-terminal 230 amino acids of the plasminogen comprise the protease domain (figure 1.2) containing the catalytic triad of plasmin (His_{602}, Asp_{645} and Ser_{740}). When activated by the mammalian plasminogen activators, uPA and tPA (Vassalli *et. al.,* 1992), plasminogen is cleaved at Arg_{560}-Val_{561} (Figure 1.2) and converted into twin-chain plasminogen (i.e. plasmin) thereby facilitating the formation of the trypsin-like protease catalytic triad (Robbins *et. al.,* 1967). The heavy and light chains of plasmin are covalently joined by two disulfide-bridges, between Cys_{548} of the heavy chain and Cys_{666} of the light chain, and between Cys_{558} of the heavy chain and Cys_{566} of the light chain (figure 1.2). Sedimentation coefficients indicate that the lys-plasmin has a more open conformation comparable to lys-plasminogen (Robbins *et. al.,* 1975). The binding of lysine analogues such as ε-ACA

and TA to the kringle domains of plasmin stabilises the structure of the protease domain and preserve the enzymatic activity of plasmin (Ueshima *et. al.,* 1996).

The physiological activation of glu-plasminogen may occur via two related pathways. Firstly, glu-plasminogen may be cleaved at Arg_{560}-Val_{561} by the plasminogen activators resulting in the formation of glu-plasmin, which can auto-catalytically remove its NTP forming lys-plasmin (i.e. the most active form of plasmin) (Summaria *et. al.,* 1973). Secondly, lys-plasminogen (formed by the removal of the NTP of glu-plasminogen by plasmin proteolysis) is cleaved at Arg_{560}-Val_{561} by plasminogen activators thereby forming lys-plasmin (Violand and Castellino, 1976).

Proteases, other than the plasminogen activators, such as factor XIa, factor XIIa, and kallikrein are able to activate glu-plasminogen *in vitro*. However, uPA is approximately 20,000 fold more active than kallikrein or factor XIa and 300,000 times more active than β-factor XIIa (Miles *et. al.,* 1983). Indirect plasminogen activation may also occur via the actions of both pancreatic and leukocyte elastases on glu-plasminogen which form val_{442}-plasminogen (lacks the LBS motifs of the parent molecule; Sottrup-Jensen *et. al.,* 1977), which in the presence of uPA and the plasmin-specific inhibitor α2-antiplasmin is converted to plasmin (Machovich and Owen, 1989). This form of plasmin is more resistant to α2-antiplasmin inhibition than native plasmin possibly due to the absence of the K1 LBS motif (Machovich and Owen, 1989), however the rate of fibrinolysis by val_{442}-plasmin is comparable to lys-plasmin (Ney and Pizzo, 1982). Thus, the inhibition of plasmin as opposed to its fibrinolytic rate is influenced by its LBS motifs.

In the blood vascular system, plasmin is primarily responsible for the degradation of fibrin polymers (Astrup, 1978) since plasminogen deficient mice are predisposed to severe thrombosis as well as fibrin depositions in numerous major

organs including the liver (Bugge *et. al.,* 1995; Ploplis *et. al.,* 1995). The specificity of plasmin is similar to that of trypsin (Robbins *et. al.,* 1981). Plasmin hydrolyses peptide bonds with exposed lysine or arginine residues in position P_1 (Keil, 1992) (i.e. on the C-terminal side of lysine or arginine residues; [K,R]↓X). Plasmin cleaves 11 out of 73 arginine residues and 20 out of 98 lysine residues of human fibrinogen (Henschen and Lottspeich, 1980). The cleaved residues are present in clusters within the Aα, Bβ and γ chains of human fibrinogen (Henschen and Lottspeich, 1980) (figure 1.8).

Plasmin is also able to degrade several extracellular matrix (ECM) basement membrane components such as fibronectin, laminin, vitronectin, and proteoglycans (Richardson *et. al.,* 1988; reviewed by Werb, 1997). However, plasmin alone is not sufficient for the complete degradation of the ECM. A significant proportion of the plasmin catalysed ECM degradation, is indirect and brought about by the plasmin-mediated activation of matrix pro-metalloproteases (pro-MMP's) such as pro-MMP1, -3, -9 and -14 (reviewed by Werb, 1997; Gordon *et. al.,* 1993). Thus, plasmin has been effectively used for the detachment and disaggregation of cells in tissue culture (Muranova *et. al.,* 1998).

Other substrates of plasmin include: the complement components C1, C3 and C5 (reviewed by Werb, 1997), tenascin-C (Gundersen *et. al.*,1997) and aggrecan (Poe *et. al.,* 1992). Plasmin is also responsible for the activation and release of growth factors such as transforming growth factor β (TGF-β; reviewed by Werb, 1997).

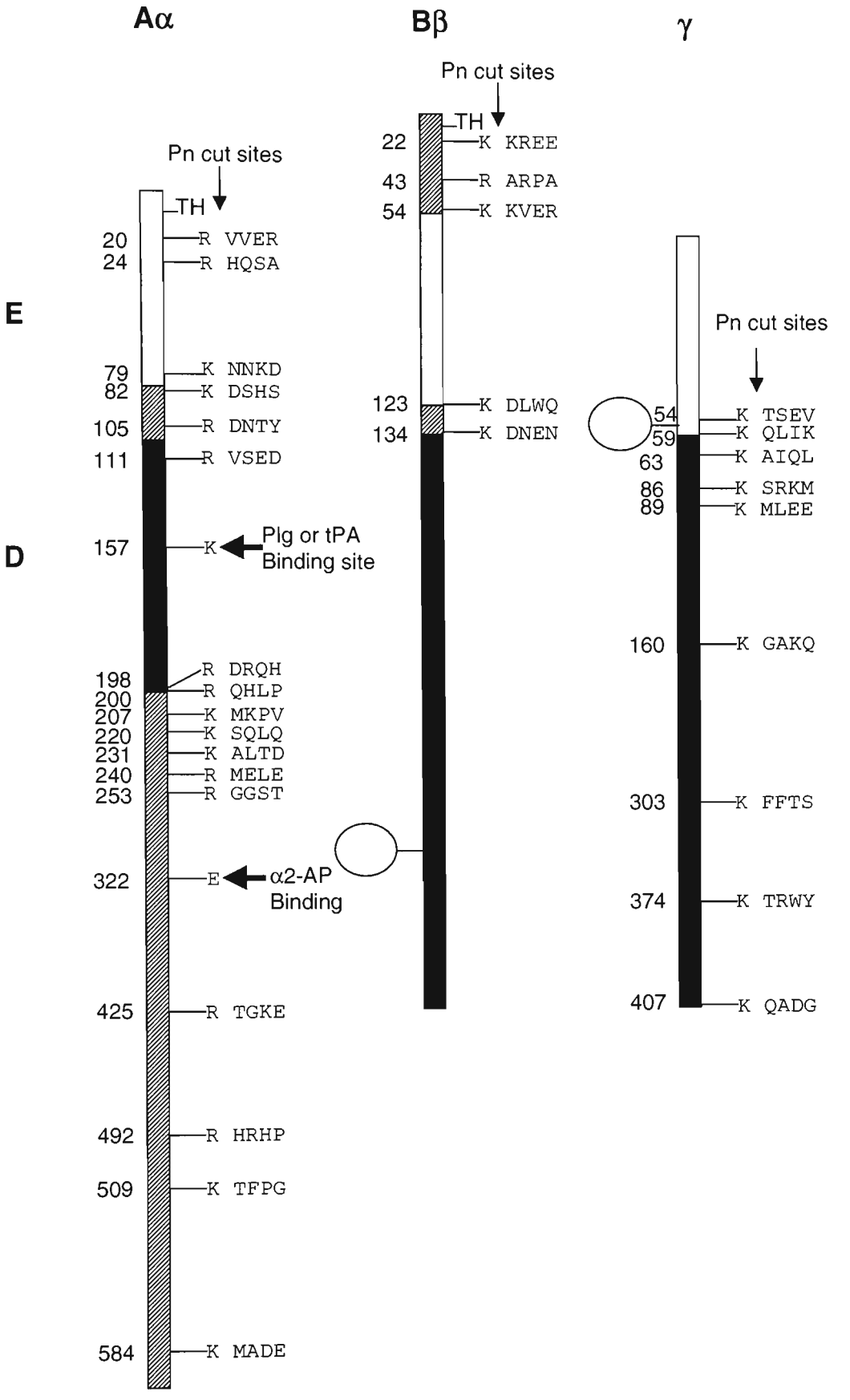

Figure 1.8: The location of the plasmin cut sites within the polypeptide chains of fibrinogen.

The arrows (↓) denote the plasmin cut sites within the chains of fibrinogen. Fragments of E (□) and D (■) of fibrinogen are white and black respectively. TH indicates the thrombin cleavage sites within fibrinogen. The plasminogen/tPA binding site and α2-antiplasmin transglutamination sites are denoted by ←. The glycosylation sites are denoted by O (modified by Henschen and Lottspeich, 1980).

1.1.1.9 Plasminogen summary

In summary, glu-plasminogen is a multi-domain protein. The heavy chain of glu-plasminogen is composed of the NTP and five kringle domains, which contain the LBS motifs of the protein. The NTP and the kringle LBS motifs of glu-plasminogen regulate the lysine-dependent conformation of zymogen and thus regulate its activation. The tertiary conformation of glu-plasminogen is very dynamic and dependent upon the interaction of lysine with the LBS motifs of the protein and Cl^-. The interaction of lysine residues with the kringle domains of glu-plasminogen stabilises their structures. The majority of the time glu-plasminogen exists *in vivo* in the closed activation resistant conformation. The interaction of glu-plasminogen with exogenous lysine residues via its LBS motifs increases the activation of glu-plasminogen by changing the conformation of the protein thereby converting it into a better substrate for the mammalian plasminogen activators. This results in the formation of the active protease plasmin. The closed "activation resistant" conformation of glu-plasminogen is physiologically beneficial, since this conformation retards the formation of broad-spectrum protease plasmin at inappropriate sites or times. Thus, the lysine-dependent binding of glu-plasminogen to either the cell-surface, or the fibrin clot may modulate the biological activity of this protease by inducing the above mentioned conformational changes in the molecule. In addition to modulating the conformation and activation of glu-plasminogen, the heavy chain of glu-plasminogen targets the trypsin-like proteolytic activity of the protease domain (light chain) to correct physiological substrates such as thrombi.

1.1.2 The Plasminogen Activators

Four major enzymes are responsible for the activation of plasminogen. These include two mammalian proteins: uPA and tPA, as well as the two bacterial plasminogen activators: streptokinase and staphylokinase. The mammalian

plasminogen activators will be reviewed briefly here. The mammalian plasminogen activators, uPA and tPA share approximately 40% amino acid sequence homology (Degen *et. al.,* 1986). Both of the mammalian plasminogen activators share the following functional domains: [1] an N-terminal growth factor-like domain, which is homologous to the receptor-binding regions of epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) (Gunzler *et. al.,* 1982; Derynck *et. al.,* 1984; Komoriya *et. al.,* 1984), [2] a single kringle structure and, [3] the C-terminal serine protease catalytic domain of the molecules (Strassburger *et. al.,* 1983). In addition, tPA has a second kringle domain, which is homologous to kringle 4 of plasminogen that it uses to bind to fibrin (Verheijen *et. al.,* 1986; Ichinose *et. al.,* 1986) as well as an additional 43 N-terminal amino acids.

1.1.2.1 Urokinase-type plasminogen activator.

The uPA gene is located on chromosome 8 (Rajput *et. al.,* 1985). Urokinase is synthesised and secreted as single chain zymogen termed sc-uPA and has a half-life in the plasma of approximately 10 minutes (Fletcher *et. al.,* 1965). Similarly, recombinant sc-uPA has a short half-life of only 3.5 min *in vivo* (Collen *et. al.,* 1984). Plasmin, (Wun *et. al.,* 1982; Blasi *et. al.,* 1987), kallikrein (Eaton *et. al.,* 1984), trypsin (Ichinose *et. al.,* 1986), factor XII (Wun *et. al.,* 1982) and human lung mast cell tryptase (Stack and Johnson, 1994) catalyse the activation of sc-uPA by cleaving its Lys_{158}-Ile_{159} activation bond (Marcotte *et. al.,* 1992). However, plasmin appears to be the most efficient (Wun *et. al.,* 1982; Andreasen *et. al.,* 1986). The light-chain (24 kDa) and heavy-chain (30 kDa) of activated twin chain-uPA (tc-uPA) are connected by a single disulfide bond. The proteolytic activity of tc-uPA is meditated by the catalytic triad of Asp_{275}, His_{224} and Ser_{276} (Pollenen *et. al.,* 1991). Further cleavage of tc-uPA by plasmin results in the active 33 kDa protein that lacks the growth factor-like domain (Barlow *et. al.,* 1981).

It is commonly accepted that tc-uPA is the active conformation of the enzyme since, it has the ability to hydrolyze small synthetic chromogenic substrates, incorporate diisopropylfluorophosphate (DFP) and react with physiological inhibitors (Andreasen *et. al.,* 1986; Wun and Reich, 1987; Wun *et. al.,* 1982; Nielsen *et. al.,* 1982). In contrast, sc-uPA is accepted as the zymogenic form of uPA since it does not significantly interact with the small chromogenic substrates (Wun *et. al.,* 1982), it does not bind DFP (Wun *et. al.,* 1982; Nielsen *et. al.,* 1982) and it does not interact with the physiological inhibitors of the plasminogen activators (Wun and Reich, 1987; Andreasen *et. al.,* 1986). However, evidence is emerging that sc-uPA may also catalyse the activation of glu-plasminogen under certain physiological conditions, even though the catalytic efficiency (k_{cat}/K_m) of tc-uPA is 200-1000 fold higher for plasminogen than sc-uPA (Gurewich, 1988a, 1988b). This intrinsic activity of sc-uPA and is substantially higher than other protease zymogens (Gertler *et. al,* 1974; Pannell and Gurewich, 1987). Lysine-dependent binding of sc-uPA to glu-plasminogen may be essential for the activation of glu-plasminogen by sc-uPA. ε-ACA, at concentrations that induce the open conformation of glu-plasminogen, facilitates the activation of glu-plasminogen by sc-uPA (Urano *et. al.,* 1988). In contrast, physiological levels of Cl^-, which maintain glu-plasminogen in a closed conformation, inhibit the activation of glu-plasminogen by sc-uPA (Urano *et. al.,* 1988). Thus, the open conformation of glu-plasminogen may be a pre-requisite for its activation by sc-uPA.

Single chain-uPA has no enzymatic activity when assayed by a low molecular weight peptide substrate, however, it does catalyse the activation of glu-plasminogen (Ellis *et .al.,* 1987). This suggests that elements, other than the activation bond of glu-plasminogen, may be required for the catalytic activity of sc-uPA. Thus, glu-plasminogen may act as both an effector and as a substrate for sc-uPA (Ellis *et. al.,*

1987). Glu-plasminogen can bind to sc-uPA, in a lysine-dependent manner, with an apparent K_d of 50 nM (Ellis *et. al.,* 1999). This lysine-dependent binding of glu-plasminogen to sc-uPA may induce a conformational change in glu-plasminogen necessary for the activation of glu-plasminogen by sc-uPA. The activation of glu-plasminogen by sc-uPA is mediated by three distinct phases based on the activation rate of glu-plasminogen (Liu *et. al.,* 1992). A slow, initial activation phase followed by an intermediate, rapid activation phase (most rapid phase) and the finally a less rapid activation phase (Liu *et. al.,* 1992). Taken together, these studies suggest that the activation of glu-plasminogen by sc-uPA may be dependent on both the lysine-dependent conformation of glu-plasminogen as well as the transition of uPA from single chain to twin chain. Thus, the activation of glu-plasminogen by sc-uPA under normal physiological condition may be dependent on a lysine-binding event that facilitates the open, activation-susceptible conformation glu-plasminogen. Single chain uPA may catalyse the initial activation of lysine-bound glu-plasminogen. Newly generated plasmin, is thought to act in a positive feedback amplification loop by activating sc-uPA thereby forming tc-uPA which, in turn, can activate more lysine-bound glu-plasminogen (figure 1.1).

At low sc-uPA concentrations, plasmin activates sc-uPA *in vitro* via a non-standard Michaelis-Menton model (Longstaff *et. al.,* 1992). The model predicts that plasmin may exist in two distinct conformations, firstly, a lower activity conformation where a LBS motif of plasmin is unoccupied or a higher activity conformation induced by the binding of sc-uPA to a LBS motif of plasmin (Longstaff *et. al.,* 1992). Thus, lysine-bound plasmin is more efficient at converting sc-uPA to tc-uPA.

Once formed plasmin, converts sc-uPA into the more catalytically active isomer tc-uPA by cleavage of the Lys_{158}-Ile_{159} activation bond of sc-uPA (Marcotte *et. al.,* 1992) resulting in lys_{158} becoming the C-terminal lysine residue of the A-chain of

uPA (lys-uPA). However, uPA, isolated from the urine has phenylalanine as the A-chain C-terminal residue (phe-uPA). There is a 1.6 fold decrease in the K_m for the *in vitro* activation of glu-plasminogen by lys-uPA compared to phe-uPA (Table 1.2; Lenich *et. al.,* 1991). This suggests that the binding of glu-plasminogen to the C-terminal lysine residue of tc-uPA induces a lysine-dependent conformational change to glu-plasminogen, which transforms it into a better substrate for activation. The lysine-dependent interactions of glu-plasminogen, which influences its conformation, modulate its activation by both sc-uPA, tc-uPA as well as the activation of uPA by plasmin.

1.1.2.2 Tissue-type plasminogen activator

Tissue-type plasminogen activator is a 68 kDa serine protease that displays high substrate specificity for plasminogen (Bachmann and Kruithof, 1984). The gene that encodes tPA is located on chromosome 10 (Rajput *et. al.,* 1985). Endothelial cells are the primary source of tPA and release the molecule into the blood where it is maintained at a concentration between 5-50 ng/ml (Rijken *et. al.,* 1984; Nilsson *et. al.,* 1985). Once secreted into the blood tPA has a half-life of approximately 2 mins (Matsuo, 1982; Fuchs *et. al.,* 1985; Emeis *et. al.,* 1985; Nilsson *et. al.,*1985).

In a similar manner to uPA, tPA exists as both a single chain (sc-tPA) and a twin chain (tc-tPA) isomer. Plasmin cleavage of the Arg_{275}-Ile_{276} peptide bond of sc-tPA concerts sc-tPA into tc-tPA. Other proteases such as factor Xa and tissue kallikrein can also convert sc-tPA into tc-tPA (Ichinose *et. al.,* 1984). In contrast to uPA, the catalytic efficiency of sc-tPA is only 5-10 fold lower than the catalytic efficiency tc-tPA (Tachias and Madison, 1996). Furthermore, sc-tPA is able to bind DFP and is active when assayed with synthetic tripeptide substrates (Wallen *et. al.,* 1983). Both sc-tPA and tc-tPA, in the absence of fibrin, display Michalis-Menton kinetics for the activation of glu-plasminogen, although tc-tPA possesses the greater

activity (table 1.2; Ranby, 1982; Bauchmann and Kruitof, 1984). However, at low glu-plasminogen substrate concentrations and in the absence of fibrin tc-tPA activates glu-plasminogen via non-linear (non-Michalis-Menton) activation kinetics (Nieuwenhuizen *et. al.,* 1988; Geppert and Binder, 1992). This suggests that glu-plasminogen may be modulating the enzymatic activity of sc-tPA. Glu-plasminogen may act as a modifying binding to the A-chain of tc-tPA via a single site with an apparent K_d of 0.1 μM (Geppert and Binder, 1992). This interaction between tPA and glu-plasminogen may be mediated by the lysine-dependent interaction of K5 since mini-plasminogen has comparable affinity for the A-chain of tPA (Geppert and Binder, 1992).

The contributions of both the single and twin-chain isoforms of the plasminogen activators may be important for the *in vivo* activation of glu-plasminogen, even though the involvement of the single-chain isomers remains controversial. Nevertheless, it is conceivable that both sc-PA may initially convert a small percentage of glu-plasminogen to plasmin resulting in an amplification feedback loop, especially when glu-plasminogen is in an activation-susceptible conformation.

1.1.3 The Plasmin Serine Protease Inhibitor (SERPIN): α2-Antiplasmin

α2-Antiplasmin (67 kDa) is the primary **ser**ine **p**rotease **in**hibitor (SERPIN) which inhibits plasmin (Mori and Aoki, 1976; Wiman and Collen, 1977). It has a circulating plasma concentration of approximately 1 μM (Mori and Aoki, 1976; Plow and Collen, 1991). α2-Antiplasmin contains 452 amino acids as determined by protein and DNA sequencing with the P1-P1' bond corresponding to Arg_{364}-Met_{365} (Wiman and Collen, 1979; Holmes *et. al.,* 1987; Hirosawa *et. al.,* 1988; Sumi *et. al.,* 1989). The α2-antiplasmin gene located on chromosome 17p13 is 16 kb and contains

10 exons and 9 introns (Hirosawa *et. al.,* 1988; Kato *et. al.,* 1993). There are four potential glycosylation sites at Asp_{87}, Asp_{256}, Asp_{270} and Asp_{277} and two disulfide bonds Cys_{64}-Cys_{104} and Cys_{31}-Cys_{113} (Holmes *et. al.,*1987). Circular dichorism studies of α2-antiplasmin in the far UV region suggests that it is composed of 16% α-helix, 18% β-structure and 66% random coil (Nilsson *et. al.,* 1982). A higher molecular weight α2-antiplasmin with an additional 12 amino acids at the N-terminal end of the molecule has been isolated from plasma and this may represent the full-length form of α2-antiplasmin (Sumi *et. al.,* 1989; Bangert *et. al.,*1993; Enghild *et. al.,* 1993). α2-Antiplasmin has three domains that are important for its function as a regulator of the plasminogen activation cascade: [1] the plasminogen binding site, [2] the fibrin cross-linking site and [3] the reactive centre loop (RCL).

The inhibition of plasmin by α2-antiplasmin is a two-step process that involves lysine-dependent binding followed by classic serpin inhibition of the protease. In the initial interaction, α2-antiplasmin binds to plasmin in a rapid, reversible, lysine-dependent, second-order reaction mechanism. The second step of the inhibition reaction involves the slower irreversible first-order reaction between the reactive centre loop (RCL) of α2-antiplasmin and the active site of plasmin (Wiman and Collen, 1978).

The initial lysine-dependent inhibition step of plasmin by α2-antiplasmin is mediated by the LBS of K1 binding to the C-terminal lysine residue (i.e. Lys_{491}) of α2-antiplasmin (Wiman *et. al.,* 1979). For example, incubation of a fibrin clot with glu-plasminogen, α2-antiplasmin, K1-3 and either uPA or tPA decreases the plasmin-mediated lysis time of the fibrin clot by competitively inhibiting the interaction between α2-antiplasmin and plasmin (Sugiyama *et. al.,* 1987). In contrast, incubation of the fibrin clot with plasminogen, α2-antiplasmin, either uPA or tPA in the presence

of K4 does not decrease fibrin clot lysis time (Sugiyama *et. al.,* 1987). Thus, α2-antiplasmin competitively inhibits the K1-3, lysine-dependent binding of glu-plasminogen binding to fibrin thereby delaying the onset of plasmin-mediated proteolysis (Aoki *et. al.,* 1978; Rakozi *et. al.,* 1978). This suggests that the K1 LBS plasminogen mediates the binding of plasminogen to α2-antiplasmin. However, K4 and K5 domains of plasminogen also bind to α2-antiplasmin with apparent K_d of 0.18 and 9 μM respectively (Wiman *et. al.,* 1979). In addition, Lys_{475} (internal lysine residue) and the C-terminal lysine residue of α2-antiplasmin both appear to be involved in the lysine-dependent binding of serpin to plasmin (Sugiyama *et. al.,* 1988). This suggests that the lysine-dependent interaction of α2-antiplasmin may not be attributed to just a single LBS of plasmin(ogen). Using midiplasmin (plasmin lacking K1-3), Christensen *et. al.,* (1995) has suggested that the lysine-dependent binding inhibition phase of bovine plasmin by bovine α2-antiplasmin may be mediated by both K4 and K5. Consequently, α2-antiplasmin appears to bind to plasmin(ogen) at the same sites as fibrin, ECM or cell-surface plasminogen binding proteins (Plow *et. al.,* 1986; Sugiyama *et. al.,* 1988; Hortin *et. al.,* 1989).

The inhibition rate of plasmin by α2-antiplasmin is modulated 10-fold by the lysine-dependent interaction of plasmin with α2-antiplasmin. The reversible lysine-dependent α2-antiplasmin inhibition constant of plasmin is $5x10^{-10}$ M (Edelberg and Pizzo, 1992). However, under conditions where the plasmin kringle-α2-antiplasmin interaction is blocked, the reversible α2-antiplasmin inhibition constant is increased from $5x10^{-10}$ to $5x10^{-9}$ M (Edelberg and Pizzo, 1992). Thus, lysine-dependent interactions of glu-plasminogen are important not only for the activation of glu-plasminogen but also for its inhibition.

1.1.4 Plasminogen Activator Inhibitor-type 1 and 2.

The plasminogen activator inhibitor serpins, PAI-1 and PAI-2, add a further dimension of control to the plasminogen activation system by specifically inhibiting the plasminogen activators uPA and tPA. PAI-1 is the predominant inhibitor of uPA and tPA which function in both fibrinolysis and tissue remodeling (Carmeliet *et. al.*, 1993a,b). It is a 52 kDa molecular weight glycoprotein (Colman *et. al.*, 1982; Loskutoff *et. al.* 1983; van Mourik *et. al.*, 1984) that has three potential N-linked glycosylation sites but no disulfide bonds. The reactive centre P1-P1' bond is Arg_{346}-Met_{347} (Ginsberg *et. al.*, 1986; Ny *et. al.*, 1986; Pannekoek *et. al.*, 1986; Andreasen *et. al.*, 1986).

The PAI-1 gene has been localised to chromosome 7q21.3-q22 (Ginsburg *et. al.*, 1986) and the protein is synthesised and secreted by endothelial cells, platelets, megakaryocytes and heptatocytes (Schleef and Loskutoff, 1984). The plasma concentrations of PAI-1 are approximately 20 ng/ml, but may be enhanced at sites of vascular damage by the release of the inhibitor from platelets (Erikson *et. al.*, 1984; Booth *et. al.*, 1988; Juhan-Vague *et. al.*, 1984). PAI-1 is able to inhibit sc-tPA, tc-tPA and tc-uPA, however, it cannot inhibit sc-uPA (figure 1.1; Kruithof *et. al.*, 1986). PAI-1 inhibit tc-tPA by only interacting with the protease domain of tPA (Bjorquist *et. al.*, 1994).

The PAI-2 gene is located on chromosome 18q21.3 and encodes a 415 amino acid protein with a characteristic SERPIN reactive centre (P1-P1') bond formed by Arg_{380}-Thr_{381} (Kruithof *et. al.*, 1986; Schleuning *et. al.*, 1987; Ye *et. al.*, 1987; Antalis *et. al.*, 1988; Kiso *et. al.*, 1988). Physiologically, PAI-2 is synthesised by numerous tissues including the placenta (reviewed by Kruitoff *et. al.*, 1995). Hence, the distribution of PAI-2 is widespread under normal physiological conditions. PAI-2 exists in two distinct molecular forms; an extracellular glycosylated 60 kDa protein

and the more abundant intracellular non-glycosylated 47 kDa protein (Genton *et. al.*, 1987; Christensen *et. al.*, 1982; Mikus *et. al.*, 1993). Both forms of PAI-2 efficiently inhibit the activity of tc-uPA (Mikus *et. al.*, 1993; Kruithof, 1988; Kruithof *et. al.*,1995). PAI-2 rapidly inhibits both tc-uPA and tc-tPA by forming SDS stable 1:1 protease/inhibitor complexes (figure 1.1; Kruithof *et. al.*, 1986). In contrast, PAI-2 reversibly inhibits solution-phase sc-uPA in a concentration-dependent, non-SDS-stable manner (figure 1.1; Schwartz, 1994).

1.1.5 Urokinase-type plasminogen activator receptor (uPAR)

Urokinase interacts at a single, saturable, specific site on the surfaces of numerous nucleated cell types (Vassille *et. al.*, 1985; Neilson *et. al.*, 1988; Chapman *et. al.*,1990; Stoppelli *et. al.*, 1985; Bajpai and Baker, 1985; Plow *et. al.*, 1986; Boyd *et. al.*, 1988). The urokinase plasminogen activator receptor (uPAR) binds both sc-uPA and tc-uPA with apparent high affinity (K_d 10^{-10}-10^{-9} M; Cubellis *et. al.*, 1986). The human 23 kb uPAR gene has been isolated to chromosome 19q13 (Borglum *et. al.*, 1992). The gene is transcribed into a 1.4 Kb mRNA which encodes a 313 amino acid polypeptide (Roldan *et. al.*, 1990). The 50-60 kDa highly glycosylated, single chain, cell-surface uPAR molecule (Neilson *et. al.*, 1988; Behrendt *et. al.*, 1990) is a glycosyl-phosphatidyl-inositol (GPI) linked glycoprotein that consists of three distinct functional domains. Post-translational modification of uPAR involves removal of 30 amino acids from the C-terminal of the molecule in the endoplasmic reticulum as well as the addition of the GPI moiety on residues 282, 283 or 284 (Plough *et. al.*, 1991). This hydrophobic GPI moiety is responsible for anchoring uPAR to the extracellular surface of the plasma membrane (Plough *et. al.*, 1991). An alternatively spliced variant of human uPAR mRNA (designated uPAR2) has been isolated from HT1080 cells which is devoid of GPI anchor domain and thus can not bind to cell surfaces (Pyke *et. al.*, 1993).

Secreted sc-uPA binds to its receptor via its growth factor domain (GFD) (residues 4-43), which is contained within the amino terminal fragment of uPA (ATF) (residues 1-135) in an autocrine manner where it can be activated by plasmin (Cubellis *et. al.,* 1986; Stoppelli *et. al.,* 1985; Stoppelli *et. al.,* 1986; Appella *et. al.,* 1987). The binding of uPA via its ATF to uPAR does not influence the activity of bound uPA (Ellis and Dano, 1991) since the ATF, kringle and protease domains display a high degree of independence (Bogusty *et. al.,* 1989; Oswald *et. al.,* 1989). Thus, the protease domain of receptor-bound uPA functions independently at the cell surface. However, the binding of sc-uPA to uPAR enhances the activation rate of the molecule by plasmin (Ellis *et. al.,* 1989). Oleic acid, which is the most abundant free fatty acid in plasma, inhibits the binding of sc-uPA to uPAR under physiological concentrations of oleic acid (Higazi *et. al.,* 1996).

Receptor-bound, active, tc-uPA is prone to inactivation by PAI-1 and PAI-2 (Andreasen *et. al.,* 1990; Baker *et. al.,* 1990; Ellis *et. al.,* 1990). In contrast, receptor-bound sc-uPA (which has low enzyme activity) is refractory to inhibition by PAI-2, whereas, solution-phase sc-uPA is susceptible to inhibition by PAI-2 (Schwartz, 1994). Therefore, the initial activation of glu-plasminogen by cell-surface sc-uPA may occur in a PAI-2 rich pericellular environment. Thus, cells that express surface uPAR have a mechanism whereby they can selectively capture sc-uPA, activate and inhibit the proteolytic activity of tc-uPA within a pericellular environment.

1.2 Plasminogen binding.

As described previously (section 1.1), the relationship between the conformation of glu-plasminogen and its activation rate has been established *in vitro* using small lysine analogues. Whilst lysine analogues provide good models that describe the type of lysine residues within proteins responsible for the binding and activation of glu-plasminogen, they do not provide information about the spatial

relationships of lysine residues within a protein required to facilitate the lysine-dependent conformational changes in glu-plasminogen. Physiologically, the lysine residues responsible for binding glu-plasminogen are located within proteins such as fibrin and the cell-surface plasminogen receptors.

Since the occupancy of the LBS motifs by lysine residues of the NTP maintains the closed conformation of solution-phase glu-plasminogen (section 1.1.1.5) the lysine-dependent binding of glu-plasminogen to proteins is hypothesised to involve a competition reaction between the lysine residues of these proteins and the lysine residues of the NTP of glu-plasminogen for the LBS motifs of the kringle domains of glu-plasminogen. Thus, the lysine-dependent binding of glu-plasminogen to the plasminogen binding proteins may result in disruption of the intramolecular lysine-binding network glu-plasminogen leading to an increase in its activation rate.

Proteins that bind glu-plasminogen in a lysine-dependent manner may be classified into two groups based on the types of lysine residues pre-existing within the protein. Group 1 plasminogen binding proteins possess a pre-existing C-terminal lysine residue, whilst group 2 plasminogen binding proteins do not have a pre-existing C-terminal lysine residue (e.g. intact fibrin). Whilst many ECM proteins are able to bind glu-plasminogen in a lysine-dependent manner, the following section will be limited to a detailed discussion of the plasma plasminogen binding proteins fibrin, tetranectin and histidine-rich glycoprotein as well as the cellular plasminogen receptors such as α-enolase. Each of these plasminogen binding proteins typically have low affinity for glu-plasminogen (K_d between 1-2 μM).

1.2.1 Fibrin

Fibrinogen (factor I; 330 kDa) is a soluble plasma glycoprotein present at 3 mg/ml and consisting of three non-identical pairs of polypeptide chains designated as Aα, Bβ and γ covalently linked by 29 disulfide bonds (reviewed by Doolittle, 1981;

Doolittle, 1984). The N-terminal regions of fibrinogen polypeptides are held in close proximity to each other by interchain disulfide bonds (central domain), whilst the C-terminal domains (terminal domains) of the fibrinogen polypeptide chains are separated by a distance of approximately 47.5 nm (figure 1.9). Thus, fibrinogen has an asymmetrical elongated structure. The N-terminal regions of the Aα and Bβ chains of fibrinogen are designated as fibrinopeptides A (FPA) and B (FPB), respectively, and are released after fibrinogen is cleaved by thrombin (reviewed by Doolittle, 1984). Removal of the FPA and FPB domains of fibrinogen by thrombin exposes fibrin binding sites that induce the spontaneous aggregation of fibrin monomers into a regularly staggered array forming the fibrin clot (reviewed by Doolittle, 1984).

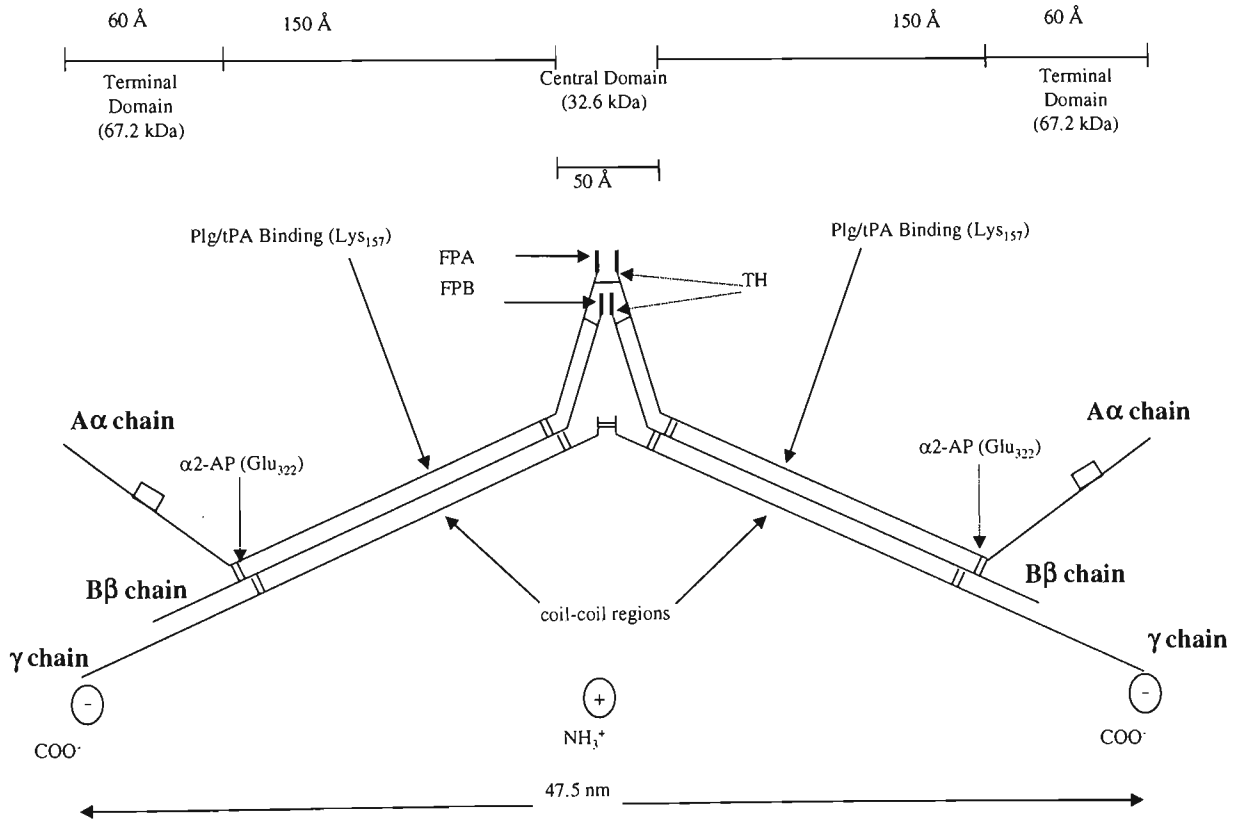

Figure 1.9: A schematic representation of fibrinogen.

A schematic representation of fibrinogen (not drawn to scale) showing pairs of Aα, Bβ and γ chains linked by disulfide bonds. Fibrinopeptide A (FPA) and fibrinopeptide B (FPB) are cleaved from fibrinogen by thrombin (Th) from the Aα and Bβ chains respectively. Plg/tPA and α2-AP denotes the plasminogen/tPA binding site of Lys_{157} and the Glu_{322} transglutamination site of the Aα chain respectively (modified from Murry *et. al.*, 1990).

Glu-plasminogen binds with low affinity to intact fibrin *in vitro* in a lysine-dependent manner with an apparent K_d of either 45 or 24 μM (Suenson and Thorsen, 1981). Both small lysine analogues and proteolytic fragments of plasminogen (K1-3, K4, K5-protease domain (mini-plasminogen) and protease domain (micro-plasminogen), have been used to identify the kringles domains of plasminogen responsible for binding intact fibrin (Thorsen *et. al.,* 1981; Suenson and Thorsen, 1981; Wu *et. al,* 1990). The LBS of K4 does not significantly bind to either intact fibrin (Thorsen *et. al.,* 1981) or CNBr fragments derived from fibrin (Wu *et. al.,* 1990) and therefore probably does not mediate the initial interaction of glu- or lys-plasminogen with fibrin. The two lysine-dependent fibrin binding sites located in K1-3 and mini-plasminogen bind to intact fibrin (Thorsen *et. al.,* 1981; Suenson and Thorsen, 1981; Wu *et. al.,* 1990). However, mini-plasminogen binds with high affinity to both intact (Thorsen *et. al.,* 1981; Suenson and Thorsen 1981) and CNBr fragments of fibrin (Wu *et. al.,*1990). Furthermore, mini-plasminogen inhibits the binding of glu- or lys-plasminogen to fibrin (Wu *et. al.,* 1990). Thus, K5 of plasminogen may represent the major LBS responsible for localising glu-plasminogen to intact fibrin. The K1-3 fragment of plasminogen binds with low affinity to both intact and CNBr fragments of fibrin. However, K1-3 did not effectively inhibit the binding of glu- or lys-plasminogen to fibrin (Wu *et. al.,* 1990). These studies suggest that K1-3 and K5 mediate the binding of glu-plasminogen to fibrin, with the K5 LBS contributing the most to the lysine-dependent interaction of glu-plasminogen to intact fibrin.

The Aα, Bβ and γ chains of intact fibrin/fibrinogen do not contain C-terminal lysine residues (Henchen and Lottspeich, 1980) and as such may be catagorised as group 2 plasminogen binding proteins. The LBS of K5 displays a preference for small lysine analogues that mimic internal lysine residues of proteins and, as suggested

above, may be responsible for the initial interaction of glu-plasminogen with fibrin via an internal lysine residue (table 1.1; Christensen, 1984; Ponting *et. al.,* 1991). Lys-plasminogen and active site inhibited glu-plasmin and lys-plasmin, in which the K5 LBS motifs are unoccupied and available (Marshall *et. al.,* 1994), all have higher lysine-dependent affinity for fibrin than glu-plasminogen (Lucas *et. al.,* 1983; Suenson and Thorsen, 1981).

At least two sites on polymeric fibrin are involved in the enhancement of glu-plasminogen activation, a site on the Aα-chain between amino acids 148-160 and a site on the γ-chain between amino acids 311-379 (Nieuwenhuizen, 1994). These sites are not accessible in fibrinogen but are exposed upon conversion of fibrinogen into fibrin (Nieuwenhuizen, 1994). The presence of Lys_{157} of the Aα chain of fibrin enhances the tPA mediated glu-plasminogen activation rate (Voskuilen *et. al,* 1987). Thus, Lys_{157} of the Aα of fibrin may represent an internal lysine residue candidate responsible for the initial interaction of glu-plasminogen via K5 or the lysine-dependent binding of tPA to intact fibrin (Voskuilen *et. al.,* 1987; figures 1.8 and 1.9). Thus, the low affinity LBS of K5 is probably responsible for the initial lysine-dependent interaction of glu-plasminogen with fibrin.

The lysine-dependent binding of glu-plasminogen to fibrin is complicated by the fact that partial plasmin degradation of fibrin creates new C-terminal lysine binding sites for glu-plasminogen. The fibrin fragments released by plasmin digestion of intact fibrinogen (340 kDa) have been determined by SDS-PAGE analysis (figure 1.10). Fragment X (250 kDa) is the first fragment observed, further digestion in the coil-coil region of fragment X induces the removal of a 10 kDa fragment and results in the production of fragment Y (150 kDa) and fragment D (90 kDa). Continued plasmin digestion of the coil-coil region of fragment Y results in the formation of another fragment D (90 kDa) and the 50 kDa E fragment (figure 1.10 Doolittle, 1984;

Budzynski and Marder, 1977). Thus, the end-products of this asymmetric plasmin degradation pathway is the formation of two fragments of D and one fragment of E molecules from each molecule of fibrinogen.

A detailed map of the plasmin cut sites of fibrinogen has been developed by comparing the plasmin digestion products of fibrinogen characterised by N-terminal amino acid sequencing from numerous studies to the sequences of the Aα, Bβ and γ chains of intact fibrinogen (Henschen and Lottspeich, 1980; figure 1.8). Due to the

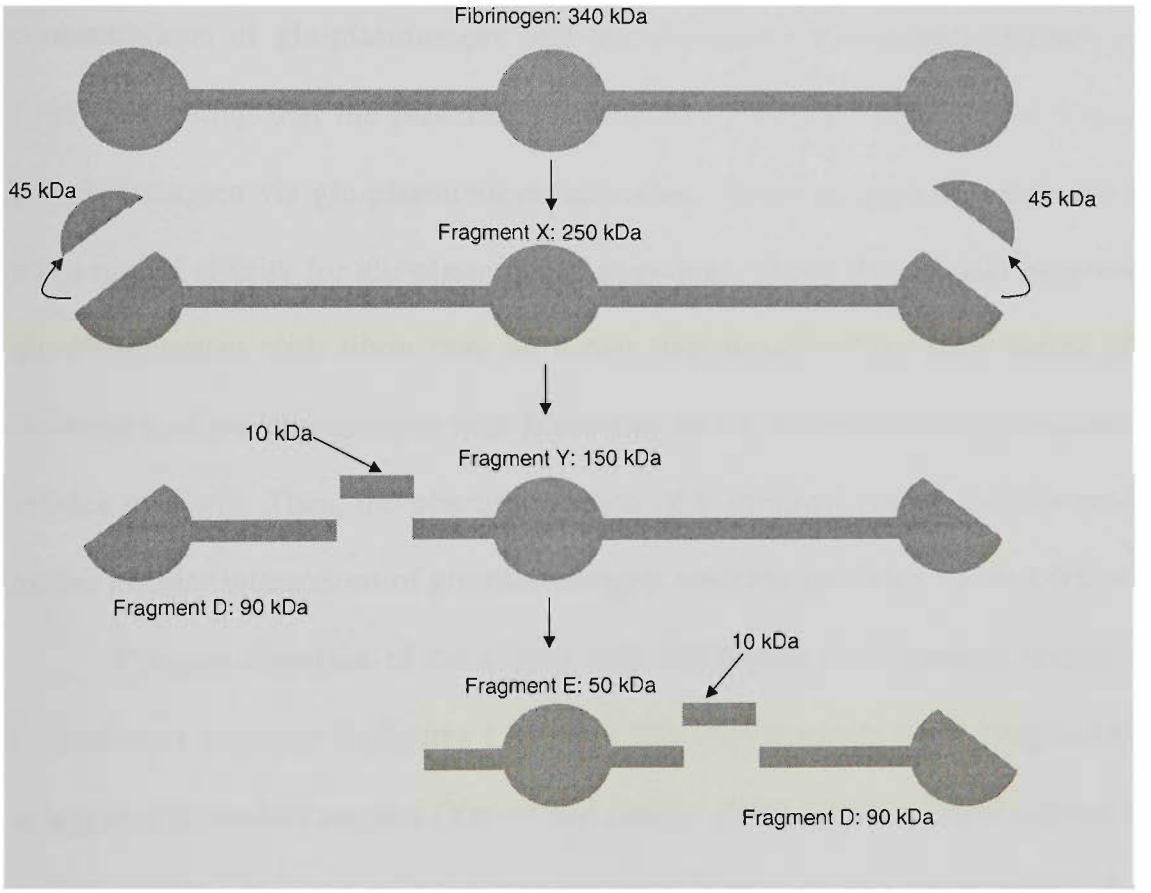

Figure 1.10: The plasmin cleavage fragments of fibrin(ogen). Reproduced from Doolittle, 1981.

coil-coil structure of fibrinogen, clusters of multiple plasmin digestions are required for the fragmentation of fibrinogen. The early plasmin cleavages of the Aα, Bβ and γ-chains of fibrin(ogen) result in the formation of new C-terminal lysine residues within the protein but may not result in the fragmentation of fibrin(ogen). Thus, plasmin cleavages, insufficient for the fragmentation of fibrin(ogen) would create new C-

terminal lysine residues that may bind the LBS motifs of glu-plasminogen which display a preference for C-terminal lysine analogues such as K1. These newly formed C-terminal lysine residues are essential for the high affinity binding of glu-plasminogen to partially plasmin-degraded fibrin (Christensen, 1985). Plasmin digestion of fibrin decreases the apparent K_d of glu-plasminogen binding whilst increasing the total number of plasminogen binding sites on fibrin (Fleury and Angles-Cano, 1991). Furthermore, both tPA and uPA induce an increase in the binding capacity of fibrin that was dependent upon the incubation time as well as the concentrations of glu-plasminogen and the plasminogen activators (Harpel *et. al.,* 1985) suggesting that the plasminogen activators indirectly regulate the binding of glu-plasminogen via glu-plasminogen activation. Therefore partially degraded fibrin has a higher affinity for glu-plasminogen than intact fibrin. The overall interaction of glu-plasminogen with fibrin may be a two step process. The initial lower affinity interaction of glu-plasminogen with fibrin may be via K5 binding to an internal lysine residue of fibrin. Thus, the plasmin creation of C-terminal lysine residues results in higher affinity interactions of glu-plasminogen. possibly mediated by the LBS of K1.

Plasmin digestion of the central coil-coil region of fibrinogen results in the formation of fragment E (figures 1.8, 1.9, 1.10). This fragment binds lys-plasminogen in a lysine-dependent manner (Varadi and Patthy, 1984). Thus, a lysine residue in this domain is hypothesised to bind plasminogen. However, the **t**hermo**s**table **d**omain (TSD) of fibrinogen, a fragment consisting of the terminal part of the E fragment connected to the D fragment of the fibrinogen γ-chain (Medved *et. al.,* 1982; Doolitle *et. al.,* 1978; figures 1.8, 1.9, 1.10) binds the K1-3 but not the K4 or mini-plasminogen fragments of plasminogen (Lezhen *et. al.,* 1986). This suggests that the K1-3 LBS of glu-plasminogen binds to a lysine residue within the TSD domain of fibrin.

The transition of fibrin from a class 2 plasminogen binding protein (no C-terminal lysine residues) to a class 1 plasminogen binding protein (C-terminal lysine residues) is associated with an increase in its affinity for glu-plasminogen that induces an acceleration of its activation rate. Both glu-plasminogen and tPA (via K2) bind to the fibrin clot in a lysine-dependent manner. In addition, tPA also binds to fibrin via its fibronectin finger (Pennica *et. al.,* 1983; de Vries *et. al.,* 1989). The lysine-dependent binding of glu-plasminogen to fibrinogen or CNBr-fragments of fibrinogen, in the presence of tc-tPA, increases the activation rate of glu-plasminogen by increasing the k_{cat} of the reaction (table 1.2; Hoylaerts *et. al.,* 1982; Stack *et. al.,* 1990). Thus, the C-terminal independent binding of glu-plasminogen to fibrinogen increases the k_{cat} thereby accelerating the activation of the zymogen. In contrast, the lysine-dependent binding of glu-plasminogen to fibrin decreases the K_m of tc-tPA (table 1.2; Ranby, 1982; Tachias *et. al.* 1996) induced glu-plasminogen activation and may be associated with a more complete change to the conformation of glu-plasminogen. The magnitude of glu-plasminogen activation rate enhancement is dependent on the species of fibrinogen present (i.e. fragment D> fragment E> fibrin> fibrinogen) (Allen and Pepper, 1981; Hoylaerts *et. al.,* 1982; Lucas *et. al.,* 1983). This suggests that plasmin fragments of fibrin with C-terminal lysine residues present may induce the fully open, activation-susceptible conformation of glu-plasminogen. The fundamental reason the fibrin clot acts to stimulate the glu-plasminogen activation cascade is because it can efficiently co-localise in a lysine-dependent manner both glu-plasminogen, in an activation susceptible conformation, and tPA. Thus, lysine-dependent binding events are essential to the correct physiological outcome of fibrinolysis.

Glu-Plasmin is probably the major plasmin species present on the fibrin clot surface as a result of *in vivo* plasminogen activation. However, Rouy and Angles-

Cano (1990), using plasminogen-depleted plasma and an artificial system consisting of tPA and α2-antiplasmin have shown that glu-plasmin is responsible for the lysis of the clot. Furthermore, complexes of glu-plasmin and α2-antiplasmin are found in the clot lysis solution (Rouy and Angles-Cano, 1990; Harpel *et. al.*, 1985). Hence, the presence of α2-antiplasmin in the fibrin clot milieu prevents the plasmin-mediated over processing of glu-plasmin to lys-plasmin. Harpel *et. al.*, (1985) that glu-plasminogen was the molecular form of plasminogen bound to fibrin in plasma. Similarly, only low levels of lys-plasminogen were consistently detected in the plasma of patients that were receiving therapeutic doses of tPA (Holvoet *et. al.*, 1985). Thus, under physiological conditions, glu-plasmin is the major fibrinolytic agent on the fibrin clot surface and lys-plasminogen is not a major physiological intermediate required for the formation of plasmin at the surface of a blood clot.

1.2.1.1 Inhibitors of fibrinolysis

Agents that modulate fibrinolysis include the serpins α2-antiplasmin, PAI-1 and PAI-2. Plasma and platelets trapped within the blood clot are the primary sources of PAI-1, α2-antiplasmin and factor XIII available to the thrombus for the inhibition of fibrinolysis (Reed *et. al.*, 1992). Other agents responsible for modulating fibrinolysis by inhibiting the binding of glu-plasminogen to fibrin include histidine-rich glycoprotein, tetranectin and carboxypeptidases (figure 1.11).

α2-Antiplasmin regulates fibrinolysis by directly inhibiting glu-plasmin in a lysine-dependent manner since ε-ACA significantly reduces the inhibition of plasmin by α2-antiplasmin (Wiman and Collen, 1978; Longstaff and Gafney, 1992). This suggests that lysine-dependent binding of plasmin to a substrate decreases the inhibitory ability of α2-antiplasmin for plasmin. Thus, for the efficient inhibition of plasmin, α2-antiplasmin must bind in a lysine-dependent manner to the protease.

Therefore, plasmin bound to fibrin via K1 is refractory to inhibition by α2-antiplasmin since the α2-antiplasmin LBS motif of plasmin is occupied (Plow *et. al.*, 1986; Sugiyama *et. al.*, 1988; Hortin *et. al.*, 1989). Furthermore, α2-antiplasmin may also compete with fibrin for the K1 LBS motif of plasmin. However, approximately 30% of α2-antiplasmin from plasma does not have the C-terminal lysine residue required to bind plasminogen/plasmin and may represent a pool of enzyme that has

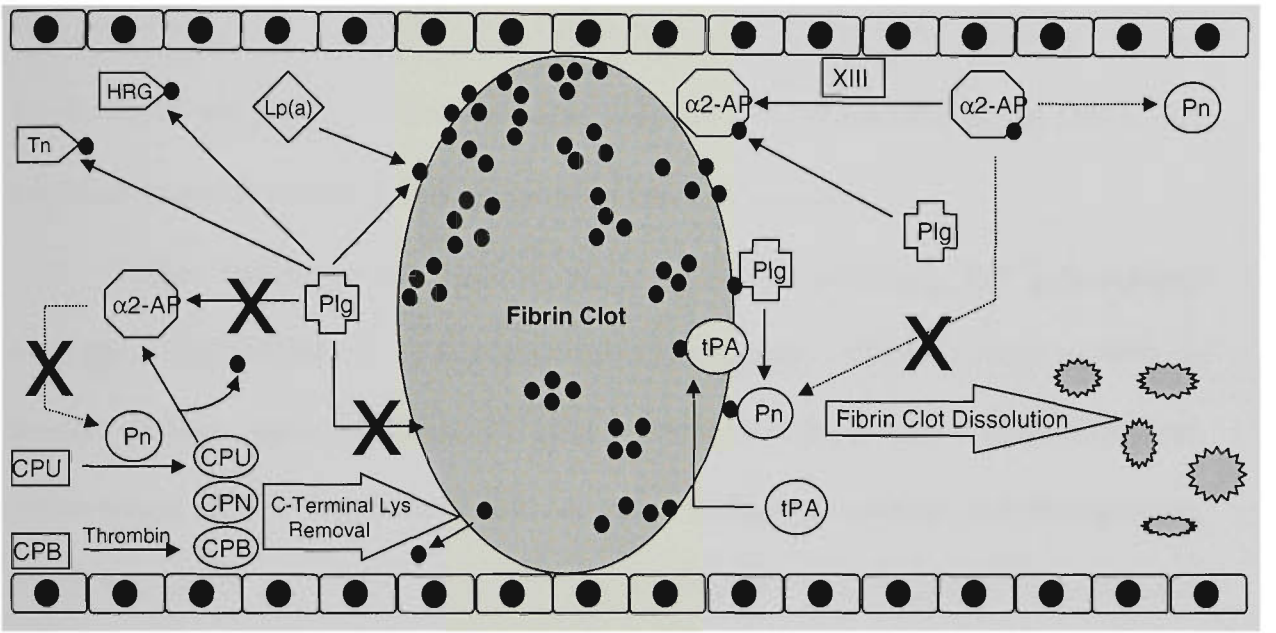

Figure 1.11: The blood vascular fibrinolytic cascade.

Both glu-plasminogen (Plg) and tPA are able to bind to the thrombus via the fibrin-associated lysine residues of the blood clot (●). The plg-tPA-fibrin ternary complex provides an ideal environment for the activation of plasmin (Pn). Once formed plasmin readily degrades the fibrin clot which results in the restoration of vascular integrity. After vascular integrity has been restored, fibrin-dissociated plasmin is rapidly inhibited by circulating α2-antiplasmin (α2-AP) in a lysine-dependent manner. The lysine-dependent binding of glu-plasminogen to the surface of the fibrin clot shields bound plasmin from inactivation by α2-antiplasmin. However, α2-antiplasmin may be N-terminally transglutaminated to the clot surface by factor XIII and thus may shield the clot from dissolution by plasmin. Lipoprotein (a) (Lp(a)) may compete with plasminogen for lysine residues of the thrombus, thereby reducing the amount of plasminogen bound to the blood clot. Other modifications to the thrombus that stabilise it against degradation by plasmin include the removal of thrombus-lysine residues (●) by circulating carboxypeptidases (CPN, CPB, CPU). Finally, circulating plasminogen binding proteins such as histidine-rich glycoprotein (HRG) and tetranectin (TN) may modulate fibrinolysis by sequestering plasminogen from the plasma in a lysine-dependent manner.

reduced plasmin inhibitory capacity (Clemmensen *et. al.*, 1981 and Kluft and Los, 1981).

α2-Antiplasmin may also retard fibrinolysis by binding to the fibrin clot and shielding it from digestion by inhibiting plasmin. Approximately 25% of circulating

α2-antiplasmin is transglutaminated to Glu_{322} of the Aα chain of fibrin (figures 1.8, 1.9; 1.11) by factor XIII (Sakata and Aoki, 1980; Sakata and Aoki, 1982; Tamaki and Aoki; 1981; Tamaki and Aoki; 1982) via Glu_2 residue of the serpin (Ichinose *et. al.*,1981). However, Lys_{303} within the Aα chain of fibrin has been identified as another α2-antiplasmin transglutamination site (Kimura and Aoki, 1986). In this orientation, the C-terminal lysine residue of α2-antiplasmin may be able to compete with fibrin for glu-plasminogen binding. Upon activation, plasmin may be inhibited by clot localised α2-antiplasmin. Thus, transglutamination of α2-antiplasmin to fibrin may establish an anti-plasmin protective shield at the clot surface.

PAI-1 decreases the rate of fibrinolysis by inhibiting the plasminogen activators responsible for activating glu-plasminogen. Fibrin bound sc-tPA is protected from rapid inhibition by PAI-1 (Chmielewska *et. al.,* 1988). However, fibrin-bound PAI-1 may inhibit tPA-mediated fibrinolysis (Reilly and Hutzelmann, 1992; Wagner *et. al.,* 1989). Thus, the presence of PAI-1 will inhibit glu-plasminogen activation by inhibiting the action of tPA.

Another possible mechanism for modulating glu-plasminogen activation during fibrinolysis is by influencing the amount of glu-plasminogen that is available to bind the fibrin clot (i.e. the amount of glu-plasminogen that is available for activation and therefore fibrinolysis). Molecules such as histidine-rich glycoprotein and tetranectin, which reduce the amount of unbound glu-plasminogen in plasma may modulate fibrinolysis by influencing the availability of glu-plasminogen for binding to fibrin. Approximately 60% of the circulating levels of glu-plasminogen is available for activation *in situ.* The remainder is bound in a reversible, lysine-dependent manner to HRG (Lijnen *et. al.,* 1980). The use of the anabolic steroid stanozolol (Kluft *et. al.,* 1984c) induce a decrease in the circulating levels of HRG, thereby resulting in an increase in the amount of glu-plasminogen available for activation.

However, a blood clot is estimated to sequester only about 4% of soluble glu-plasminogen from the circulation (Rakoczi *et. al.,* 1978). Due to the small amount of glu-plasminogen that is sequestered by a thrombus it remains unclear whether the other blood glu-plasminogen binding proteins influence the fibrinolytic rate of the thrombus.

Approximately 13-17% of the tetranectin present in human plasma does associate with the fibrin clot in a calcium dependent manner (Kluft *et. al.,* 1989a). Furthermore, the binding of tetranectin to the fibrin clot is independent of both glu-plasminogen and the transglutaminase, factor XIII (Kluft *et. al.,* 1989). In the presence of poly-D-lysine, tetranectin enhances the rate of glu-plasminogen activation by t-PA (Clemmensen *et. al.,* 1986), suggesting that a quaternary complex between fibrin, tPA, tetranectin and glu-plasminogen mediates this effect. Sequestration of glu-plasminogen by soluble blood plasminogen binding proteins effects the rate of plasminogen activation at the thrombus surface in a manner dependent on the local milieu concentration of these factors.

Finally, inhibition of the ability of the fibrin clot surface to bind both glu-plasminogen and tPA down-regulates the clot associated activation of glu-plasminogen. Modification of fibrin by plasma carboxypeptidases decreases the amount of lysine residues available for glu-plasminogen and tPA binding, thereby stabilising the thrombus (Felez, 1998). For example, the inactive precursor of caboxypeptidase U circulates bound to glu-plasminogen in the plasma (Wang *et. al.,* 1994). Once activated by plasmin caboxypeptidase U can degrade C-terminal lysine residues, however, active carboxypeptidase U is very unstable (Wang *et. al.,* 1994).

The regulation of fibrinolysis is multi-facetted, however, central to the activation of glu-plasminogen is the formation of a lysine-dependent quaternary complex between the activator, the zymogen, the inhibitor and the substrate. Factors

that modulate these binding processes fine tune the amount of plasmin activity associated with the thrombus, thereby permitting a physiologically appropriate fibrinolytic rate.

1.2.2 The Cell Surface Plasminogen Receptor Candidates

Because many cellular processes (ovulation, blastocyst implantation, embryonic development, inflammation, wound healing and angiogenesis) require the utilisation of plasmin by particular cell-types, another major compartment where plasminogen activation occurs is the cell-surface.

Plasminogen or plasmin can bind both proteinaceous and non-proteinaceous moieties in a lysine-dependent manner. Non-proteinaceous molecules that have plasminogen binding capacity include gangliosides (Miles *et. al.,* 1989) and free fatty acids (Higazi *et. al.,* 1992). Gangliosides are able to bind glu-plasminogen in a saturable concentration-dependent manner. However, individual components of the gangliosides are unable to inhibit plasminogen binding to isolated gangliosides or the plasma membrane of U937 cells (Miles *et. al.,* 1989). This suggests that the plasminogen binding site of the gangliosides may be a combination of the individual components of the molecule.

Oleic acid increases the activation rate of glu-plasminogen by uPA (Higazi *et. al.,* 1992) and is able to specifically bind plasmin and increase its amidolytic activity in a saturable and concentration-dependent manner. However, the plasmin stimulatory activity of oleic acid can be suppressed by the lysine analogue ε-ACA (Higazi *et. al.,* 1992).

The cell-surface plasminogen binding proteins have several properties, namely; saturablility, low affinity, lysine-dependent binding of plasminogen, acceleration of glu-plasminogen activation and protection of receptor bound plasmin from inactivation by α2-antiplasmin. The final criteria these plasminogen binding

proteins must satisfy to be classified as cellular plasminogen receptors is their localisation and orientation at the cell-surface such that they can bind extracellular plasminogen in a form that increases the activation rate of glu-plasminogen by cell-surface-associated plasminogen activators. Because cell-surface plasminogen binding proteins do modulate the rate of glu-plasminogen activation, it is possible that these receptors facilitate this by lysine-dependent unfolding in a manner similar to small lysine analogues.

To date nine, mammalian cellular plasminogen receptors and three bacterial plasminogen receptors have been identified. However, only the mammalian plasminogen receptors will be discussed below. They typically have a K_d for glu-plasminogen ranging from 0.5 to 1.5 μM (table 1.4). These receptor candidates are a diverse group of proteins with the only unifying parameter being that they bind plasminogen in a lysine-dependent manner. Furthermore, only two of the nine known plasminogen receptors contain a pre-formed C-terminal lysine residue (i.e. cytokeratin 8 and α-enolase; table 1.4). Many of the plasminogen receptors isolated from the surfaces of mammalian cells have alternative and often more classical functions (with the notable exception of tetranectin).

1.2.2.1 Actin

Actin is present in all eukaryotic cells (Korn, 1982) and primarily functions as a cytoskeletal stress fiber in cells. Actin acts as a both an extracellular and cell-surface plasminogen binding protein (table 1.4). Extracellular actin has been identified as a

Table 1.4: The plasminogen binding properties of the mammalian cellular plasminogen receptor candidates.

Plg Binding Molecules	M_r (kDa)	K_d (M)	C-terminal lysine	Inhibitor	Enhanced Plg Activation	Binds tPA	Reference
Actin	42	N/D[1]	No	ε-ACA	Yes	Yes	Lind and Smith, 1991
Amphoterin	30	N/D[1]	N/D[1]	ε-ACA	N/D[1]	N/D[1]	Parkkinen and Rauvala, 1991
Annexin II	40	1.6×10^{-7}	No	ε-ACA	Yes	Yes	Cesarman *et. al.*, 1994
Cytokeratin 8	59	N/D[1]	Yes	ε-ACA	Yes	N/D[1]	Hembrough *et. al.*, 1996
α-enolase	54	1.4×10^{-6}	Yes	ε-ACA	Yes	N/D[1]	Miles *et. al.*,1989; Redlitz *et.al.*1995
Glycoprotein IIb-IIIa	115 97		No		Yes	N/D[1]	Miles and Plow, 1986
Megalin	330	N/D[1]	No	Benz[2]	N/D[1]	N/D[1]	Kanalas and Makker 1991
Osteonectin (Platelet) (Bone)	32.7	 1.2×10^{-7} 4.7×10^{-8}	No	ε-ACA	Yes	N/D[1]	Kelm *et. al.*, 1994
Tetranectin	17	5×10^{-7}	No	ε-ACA	Yes	Yes[3]	Christensen and Clemmensen, 1991

[1] Not determined
[2] Western Blot determination
[3]Requires the presence of poly-L-lysine

glu-plasminogen binding protein which is able to decrease the rate of fibrinolysis possibly by competing with fibrin for the kringle LBS motifs of glu-plasminogen (Lind and Smith, 1991). Cell-surface actin accounts for approximately 45% and 46% of the total glu-plasminogen and t-PA binding capacity of endothelial cells, respectively (Dudani and Ganz, 1996), even though intact actin does not contain a C-terminal lysine residue (table 1.4).

Actin stimulates plasmin formation by lowering the K_m for the activation of glu-plasminogen in a lysine-dependent manner (table 1.4; Lind and Smith, 1991). Actin has a K_d of 0.55 μM for tPA. Partial plasmin digestion of actin facilitates the simultaneous binding of both glu-plasminogen and t-PA to the molecule presumably by creating a C-terminal lysine residue at Lys_{373} (Lind and Smith, 1993; Mornet and Ue, 1984). Hence, plasmin treated actin may act as a receptor for both glu-plasminogen and t-PA thereby facilitating plasminogen activation in a type of positive feedback loop. Despite this, actin does not increase the proteolytic activity of t-PA (Lind and Smith, 1993) during plasminogen activation. Although actin does not bind to tc-uPA, the presence of actin facilitates the activation of glu-plasminogen by tc-uPA. In contrast to glu-plasminogen, actin does not increase the uPA-mediated activation rate of lys-plasminogen (Lind and Smith, 1993). Therefore it appears that actin (as with the other plasminogen receptor candidates) facilitates glu-plasminogen activation by inducing a conformational changes in glu-plasminogen from the activation resistant to the activation susceptible isomer of the molecule.

1.2.2.2 Amphoterin

Amphoterin is a 30 kDa heparin-binding, sulfoglycolipid-binding (Mohan *et. al.,* 1992) protein that has multiple biological functions primarily associated with the nervous system. It is a member of the high mobility group-1 (HMG-1) proteins and is found throughout the different compartments of cells, including the nucleus and the

substrate-attached material (Merenmies *et. al.,* 1991). Nuclear amphoterin is involved in DNA transcription regulation (Maher and Nathans, 1996). In cells that are actively spreading, amphoterin is localised to the extending filopodia of the advancing plasma membrane (Merenmies *et. al.,* 1991) of highly motile cells (Parkkinen *et. al.,* 1993). Amphoterin is also a cell-surface antigen in breast tissue (Salmivirta *et. al.,* 1992).

Amphoterin is localised on the extracellular surface of non-permeabilised cells (Parkkinen *et. al.,* 1993). Amphoterin and tPA are co-localised on the leading edge filopodia of the N18 neuroblastoma cell line and to the substrate-attached material (Parkkinen and Ravala, 1991). *In vitro* plasminogen binding studies demonstrate that both tPA and glu-plasminogen bind amphoterin in a lysine-dependent manner (Parkkinen and Ravala, 1991). Amphoterin-bound tPA accelerates the activation rate of glu-plasminogen (Parkkinen and Ravala, 1991). Hence, amphoterin is a plasminogen binding protein which may be localised to the cell-surface of neural cells and possibly breast tissue.

1.2.2.3 Annexin II

A 40 kDa protein that functions as a plasminogen and tPA receptor on the surfaces of endothelial cells (Hajjar and Hamel, 1990; Hajjar, 1990) was identified as annexin II (Hajjar *et. al.,* 1994; Cesarman *et. al.,* 1994). Annexin biology has been extensively reviewed by Gerke and Moss (1997) and Waisman (1995) and will not be discussed here in detail. Annexins are a family of calcium binding proteins defined by a 70 amino acid calcium binding structural motif known as the annexin core (Donnelly and Moss, 1997). Currently, there are nine proteins within this family. Since annexin II has been identified as a major plasminogen receptor, the remainder of the discussion will focus on this member of the family. Annexin II, also known as lipocortin II and can exist as either a monomer or as a hetrotetramer. The latter is composed of two annexin II (p36) subunits and two S100A10 (p11) subunits (a

member of the calcium binding S100 protein family). Monomeric annexin II (36 kDa) can undergo polymerisation in a calcium-dependent manner forming heterotetrameric annexin II and is present on the plasma membranes of numerous cell types (Gerke and Moss 1995; Waisman 1995).

Human tetrameric annexin II is a class 2 plasminogen binding protein since it does not posses a C-terminal lysine residue and partial degradation of annexin II is required for maximal plasminogen binding (Hajjar *et. al.,* 1994). The tPA/glu-plasminogen binding protein annexin II tetramer substantially inhibits the fibrinolytic activity of plasmin but, it does not inhibit the collagenolytic activity of plasmin (Choi *et. al.,* 1998). Thus, the plasmin inhibitory activity of annexin II tetramer is dependent on the presence of fibrin. Choi *et. al.,* (1998) suggests that the fibrin dependent plasmin inhibitory activity of annexin II tetramer is due to a ternary complex between plasmin, annexin II tetramer and fibrin (or possibly a quaternary complex between glu-plasminogen, annexin II tetramer, tPA and fibrin), since fibrin can also interact with annexin II tetramer.

1.2.2.4 Cytokeratin 8

Monomeric cytokeratin 8 (55 kDa), a cytoplasmic intermediate filament protein has been identified as a class 1 plasminogen binding protein found on the surfaces of hepatocytes (Hembrough *et. al.,* 1985) and MCF-7 breast cancer cells (Hembrough *et. al.,* 1995, 1996). The binding of glu-plasminogen to cytokeratin 8 is lysine dependent. However, the physiochemical properties of plasminogen binding to cytokeratin 8 remain to be characterised.

1.2.2.5 α-Enolase

Enolases (E.C.4.2.1.11) are dimeric, glycolytic, cytoplasmic enzymes with an apparent molecular weight of approximately 90,000 kDa. They catalyse the

interconversion of 2-phospho-D-glycerate (2-PGA) and phosphoenolpyruvate (PEP) in the glycolytic/gluconeogenic pathway. These enzymes have three discrete isozymes designated α (Mr: 46, 000), β (Mr: 44,000) and γ (Mr: 46,000). Five forms of the enzyme have been found (Rider and Taylor, 1974; Fletcher *et. al.,* 1976; Marangos *et. al.,* 1978), namely, the three homodimers αα, ββ and γγ as well as the two hybrid forms of enolase composed of αγ and αβ. The α-enolase isoform (non-neuronal enolase) is known as "liver" enolase and is ubiquitous, occurring in most adult tissues and probably the sole isoform present during the early stages of fetal development (Fletcher *et. al.,* 1978; Schmechel *et. al.,* 1980). A second isozyme of human α-enolase from the lung has been cloned and sequenced. Human lung-specific α-enolase is devoid of a C-terminal lysine (Verma and Kurl, 1993), and therefore may not bind plasminogen. The β isoform is found predominantly in muscle (muscle-specific enolase); as opposed to γ-enolase which is characteristic of neurons and the amine-precursor uptake and decarboxylation (APUD) cells (neuronal enolase) (Schmechel *et. al.,* 1978a, b).

While the primary physiological role of α-enolase involves energy metabolism via the glycolytic and gluconeogenic pathways of the cell, it has been shown to have other potential functions. In the lens, α-enolase is identical to τ-crystallin, a major lens component of birds and reptiles (Wistow *et. al.,* 1988). The lens utilises α-enolase as a structural protein since its glycolytic enzyme activity is abrogated.

Secondly, an α-enolase-related molecule (ERM) has been identified as a candidate cell-surface plasminogen receptor molecule on both the monocytoid cell line U937 (Miles *et. al.,* 1991; Redlitz *et. al.,* 1995) and rat neuronal cells (Nakajima *et. al.,* 1994) which is capable of binding plasminogen via its C-terminal lysine

residue. Purified α-enolase interacts with glu-plasminogen with a K_d of approximately 1.4 μM in a lysine-dependent manner and removal of the C-terminal lysine residue from α-enolase abrogates its glu-plasminogen binding capacity (Redlitz *et. al.*, 1995). Moreover, the lysine-dependent binding of glu-plasminogen by α-enolase increases the activation rate of the molecule by tPA and α-enolase competes with α2-antiplasmin for the LBS motifs of plasminogen (Redlitz *et. al.*, 1995).

α-Enolase is expressed at high levels in numerous human tumours (Ishiguro *et. al.*, 1984; Royds *et. al.*, 1982; Oka *et. al.*, 1989; Royds *et. al.*, 1982; Yoneda *et. al.*, 1987). Although the subcellular location of α-enolase in these tumours has not been characterised, the high levels of the enzyme may be due to the energy requirements of the tumour cells and thus α-enolase would be associated with the cytoplasm. Nevertheless, a fraction of α-enolase may be associated with the cell surface especially in the neurological tumours where it may function as a plasminogen receptor (Nakajima *et. al.*, 1994)

1.2.2.7 Glycoprotein 330

The autoantigen associated with Heymann nephritis, namely gp330, is also a cellular plasminogen binding protein. The binding of glu-plasminogen to gp330 induces an increase the activation rate of the zymogen by uPA (Kanalas and Makker, 1988; Kanalas and Makker, 1991; Kanalas, 1992). During active Heymann nephritis, autoantibodies are produced against both gp330 and plasminogen, with the latter antibodies capable of blocking the activation of the glu-plasminogen (Kanalas, 1993).

1.2.2.8 Tetranectin

Tetranectin is a homotrimeric C-type lectin (Holtet *et. al.*, 1997) that binds to K4 of plasminogen in a lysine-dependent manner with an apparent affinity of 0.5 μM

(Clemmensen *et. al.,* 1986; Kluft *et. al.,* 1989b). It has been isolated from human plasma (Clemmensen *et. al.,* 1986) and has also been localised to lung, liver, spleen, kidney and pancreas (Wewer and Albrechtsen, 1992) endothelial cells, numerous epithelial cells from the intestine, in many of the immune system cells such as mast cells, macrophages and granulocytes (Cristensen and Clemmensen, 1989) and various endocrine tissues such as chromophils of the pituitary gland and follicular and parafollicular cells of the thyroid gland (Christensen *et. al.,* 1987). However, the biological role of tetranectin remains unknown. Its concentration range in human plasma is 120-140 nM, and varies slightly with age and sex (Jensen *et. al.,* 1987) and oral contraceptives (Kluft *et. al.,* 1989c). Each of the non-glycosylated, 20 kDa, subunits of tetranectin contain three intrachain disulfide bonds (Fuhlendorff *et. al.,* 1987). The crystal structure for human tetranectin has been solved (Nielsen *et. al.,* 1997) and demonstrates that tetranectin is trimeric.

Tetranectin, apart from binding K4 of glu-plasminogen is also able to specifically interact with sulfated polysaccharides such as chondroitin and heparin sulfates (Clemmensen, 1989), fibrin in a calcium-dependent manner (Kluft *et. al.,* 1989a) and to the K4 containing protein apo-lipoprotein (a) (Kluft *et. al.,* 1989b). In the presence of poly-D-lysine, tetranectin enhances the rate of plasminogen activation by t-PA (Clemmensen *et. al.,* 1986). This suggests that the binding of glu-plasminogen to tetranectin via K4 alone is not sufficient to induce the activation-susceptible conformation of glu-plasminogen.

Tissue tetranectin is primarily associated with the ECM of the cells (e.g. human embryonal fibroblasts) that secrete it and its association with the ECM may be due to its ability to bind to sulfated carbohydrates (Clemmensen *et. al.,* 1991). Both normal colon mucosal cells and colonic tumour cells does not express tetranectin, whereas, tetranectin is expressed and secreted into the ECM by the colonic tumour

stromal cells where it is co-distributed with plasminogen (Wewer and Albrechtsen, 1992).

1.2.3 Glu-plasminogen and the cell-surface.

Many of the plasminogen receptors described above are also able to bind tPA in a lysine-dependent manner. Thus, glu-plasminogen, its receptor and tPA may form a ternary activation complex that facilitate the formation of plasmin at the cell surface in a manner comparable to plasminogen activation on a fibrin clot.

The ability of plasminogen to interact with the cell surface in a time-, concentration- and lysine-dependent manner was first demonstrated on the surfaces of platelets (Miles and Plow, 1985) and subsequently on many nucleated eukaryotic cells (table 1.5). The general properties of plasminogen binding to a cell-surface include: [1] Saturable, low affinity, lysine-dependent, interactions with a high number of binding sites/cell (10^5-10^7 sites/cell). [2] The facilitation of glu-plasminogen activation. [3] Protection of cell-surface plasmin from inactivation by α2-antiplasmin (Plow and Miles, 1990; Hall *et. al.,* 1991). [4] Increased catalytic efficiency of cell-surface plasmin compared to solution-phase plasmin (Gonzalez-Gronow *et. al.,* 1991).

Table 1.5: Plasminogen binding properties of mammalian cells.

Cell	Cell Type/ Characteristic	K_d (M)	N° Binding Sites/Cell	References
SW 1116	Colonic Adenocarcinoma	$5x10^{-6}$ (Plg); $6x10^{-8}$ (Pn)	10^4-20^4 (Plg); $5x10^4$-10^5 (Pn)	Burtin , Fondaneche, 1988
L2	Rat yolk sac carcinoma	$1.3x10^{-6}$ (Plg)	$1.54x10^7$	Kanalas, 1995
Platelet	Thrombin Stimulated	$2.6x10^{-6}$	$1.9x10^5$	Miles , Plow, 1985
Platelet	Unstimulated	$1.9x10^{-6}$ (Plg)	$3.7x10^4$	Miles , Plow, 1985
WEHI-3B	Murine Leukemic Cells	$3.8x10^{-5}$ (Plg)	$4.0x10^4$	Martinez , Santibanez, 1994
PROb	Rat Carcinoma	10^{-7} (Plg)	$3.6x10^6$	Durliat *et.al.*,1992
REGb	Rat Carcinoma	$5.0x10^{-7}$ (Plg)	$0.9x10^6$	Durliat *et.al.*,1992
HT-1080	Fibrosarcoma	$5.4x10^{-9}$ (Pn)	$6x10^5$	Pollanen, 1989
U937	Monocytoid	$9x10^{-7}$ (Plg)	$5.5x10^4$	Plow *et. al.*, 1986
GM1380	Fibroblast	$8x10^{-7}$ (Plg)	$8.6x10^4$	Plow *et. al.*, 1986
HUVEC	Endothelial	$3.1x10^{-9}$ (Plg)	$1.4x10^6$	Hajjar *et al.*, 1986
Hepatocyte	Rat	$5.9x10^{-7}$ (Plg)	$1.4x10^7$	Gonias *et.al.1989*
THP-1	Monocytoid	$1.8x10^{-6}$ (Plg)	$1.9x10^6$	Felez *et. al.*, 1990
C6	Rat Glioma	$2.2x10^{-6}$ (Plg1); $1.5x10^{-6}$ (Plg2)	$2.8x10^6$; $3.7x10^6$	Hall *et. al.*, 1990
Heptaocyte	Rat	$3.2x10^{-6}$ (Plg1); $1.9x10^{-6}$ (Plg2)	$9.4x10^7$(Plg1) $9.8x10^7$ (Plg2)	Hall *et.al.*, 1990
HUVEC	Endothelial	$1.4x10^{-10}$ (lys-plg); $1.2x10^{-7}$ (lys-plg)	$2x10^4$ (lys-Plg); $3.9x10^5$(lys-Plg)	Hajjar &Nachman, 1988
MCF-7	Breast adenocarcinoma	N/D	$1.5x10^6$	Hembrough *et. al.*, 1996
Endothelial	Aortic	$1.4x10^{-9}$ (Pn); $2x10^{-8}$ (Pn)	$1.2x10^4$ (Pn); $3.2x10^4$ (Pn)	Bauer *et. al.*, 1984

Glu-plasminogen (Plg), lys-plasminogen (Lys-Plg), glyco-isoform 1 of plasminogen (Plg1), glyco-isoform 2 of plasminogen (Plg2) and plasmin (Pn). Not Determined (N/D)

Apart from these four general properties of plasminogen binding and activation at the cell surface, plasmin may mediate other cellular functions in an agonist, protease-independent manner (refer to section 1.2.7).

The high number of plasminogen binding sites/cell suggests that more than one receptor is responsible for localising glu-plasminogen to the cell surface. The amount of glu-plasminogen that α-enolase binds on the surface of U-937 cells is approximately 10% of the total amount of glu-plasminogen bound by these cell (Redlitz, *et. al.,* 1995). Whereas, actin accounts for approximately 45% of the plasminogen binding capacity of endothelial cells (Dudani and Ganz, 1996). These data suggest that it is likely that more than one proteinaceous plasminogen receptor candidate and/or non-proteinaceous moiety is responsible for binding glu-plasminogen at the surface of nucleated cells. The affinity of glu-plasminogen for the cell surface and the degree to which the cell binds this protein is dependent upon the variant of plasminogen investigated and is probably due to differences in the conformations of these variants. The affinity of glu-plasminogen binding to the cell-surface is approximately 1 μM (table 1.5). In contrast, lys-plasminogen (Burtin and Fondaneche, 1988; Pollanen, 1989) and plasmin (Bauer *et. al.,* 1984) bind with higher affinity to cells (table 1.5). This differential affinity of plasminogen variants for the cell surface is probably due to the conformational differences between glu- and lys-plasminogen in which the latter exists in a more open conformation with the LBS of K5 uncomplexed and available for binding (Marshall *et. al.,* 1994). Cells may be able to take advantage of the higher affinity that lys-plasminogen has for the cell surface. For example, incubation of glu-plasminogen with either endothelial or U937 cells induces the formation of lys-plasminogen (Hajjar and Nachman, 1988; Silverstein *et. al.,* 1988) which has a more open conformation. The authors of these studies suggested that the pericellular conversion of glu-plasminogen into lys-plasminogen may provide a mechanism for the increase in plasmin generation at the cell surface. However, physiological pericellular

proteolysis of glu-plasminogen into lys-plasminogen may not enhance the capture of plasminogen by the cell-surface since lys-plasminogen (in the open conformation) would be in equilibrium with its proteolytically detached pre-activation domain. This equilibrium dictates that two conformational forms of plasminogen (open and closed) are present in the pericellular environment due to the lysine-dependent interaction between the NTP and the kringle LBS motifs of lys-plasminogen (Sjoholm *et. al.,* 1973). A slight modification of this speculation is that receptor-bound glu-plasminogen may be converted into receptor-bound lys-plasminogen by cell-surface plasmin. Alternatively, after activation, receptor-bound glu-plasmin may undergo auto-catalysis yielding receptor-bound lys-plasmin plus unbound NTP. However, the mechanism of pericellular/cellular lys-plasminogen formation remains unclear.

Another parameter that influences cell-surface glu-plasminogen binding is the glycosylation status of the zymogen. The more heavily glycosylated glu-plasminogen (glycoform I) binds with a slightly lower affinity than glycoform II (table 1.3) (Hall *et. al.,* 1990) possibly due to a glycosylation induced steric hindrance of the LBS motif(s):cell-surface receptor interaction.

Binding studies using elastase fragmented plasminogen identified K1-3 as a primary recognition site for plasminogen on stimulated and unstimulated platelets (Miles *et. al.,* 1988). However, lower amounts of isolated K4 and mini-plasminogen (K5-protease domain) can also bind to stimulated and unstimulated platelets (Miles *et. al.,* 1988). Thus, the LBS motifs of K1-3, K4 and K5 have the potential to bind to the cell-surface. Isolation of plasminogen fragments by elastase digestion would destroy the intramolecular lysine binding network of glu-plasminogen and thus its conformation. This loss of glu-plasminogen conformation may confound the importance of which kringle LBS motifs are responsible for localising glu-plasminogen to the cell surface and the results may simply reflect the higher affinity LBS motif present in the K1-3 fragment. Using fragments of plasminogen, ε-ACA

and benzamidine, Burge *et. al.*,(1992) demonstrated that glu-plasminogen binding to basal cell layers of the epidermis was primarily mediated by the K5 LBS motif as well as by a second ε-ACA-dependent site. This suggested that the binding of glu-plasminogen to cell surfaces may involve multiple kringle domains.

Many of the cellular plasminogen receptors are proteins that have pre-existing intracellular biological roles when expressed at locations other than at the cell surface. If the analysis of the plasminogen binding experimental data does not include a procedure for the discrimination of the plasminogen bound by viable cells as opposed to binding by non-viable cells, then the specific number of binding sites per cell may be overestimated, since many of the plasminogen receptors are intracellular proteins. Thus, the viability status of a cell may influence its glu-plasminogen binding capacity.

Finally, the binding of glu-plasminogen to the cell-surface may be species specific, for example, bovine endothelial or smooth muscle cells bind lower amounts of human glu-plasminogen than their human counterparts (Hajjar *et.al.,* 1986).

1.2.4 Cellular plasminogen activation.

The lysine-dependent binding of glu-plasminogen to a cell-surface enhances the activation rate of glu-plasminogen by the mammalian plasminogen activators (Namiranian *et al.,* 1995). If the interaction of glu-plasminogen with the cell surface induces a lysine-dependent conformational change in glu-plasminogen similar to the change in conformation the lysine analogues induce, then binding should facilitate the rate of cell-surface plasmin formation. For example, both the apparent K_d (table 1.5; Plow *et. al.,* 1986) for the binding of glu-plasminogen to U937 cells and the apparent K_m (table 1.2; Felez *et. al.,* 1996) for the U937 cell-surface bound glu-plasminogen activation are 0.9 μM. This suggests that saturation of the cell surface with glu-plasminogen induces the maximal rate of plasminogen activation. Thus, the activation of cell-bound glu-plasminogen by tPA or receptor-bound uPA is due to a

decrease in the apparent K_m of these activators for glu-plasminogen (table 1.2; Hajjar *et. al.*, 1986; Schafer *et.al.*, 1989; Ellis *et. al.*, 1991). These apparent decreases in the K_m of the activation reactions suggest that at physiologically relevant concentrations cell-surface-bound glu-plasminogen acts as better substrate for the plasminogen activators than solution-phase glu-plasminogen (table 1.2).

Another consequence of plasminogen binding to the cell surface is protection from inhibition by α2-antiplasmin. In contrast to uPA, plasmin is refractory to inhibition by its extracellular serpin, α2-antiplasmin when bound in a lysine-dependent manner to its cell-surface receptor (Plow and Miles, 1990; Hall *et. al.*,1991). Cell-bound plasmin treated with α2-antiplasmin results in the dissociation of plasmin from the cell surface and the formation of plamin:α2-antiplasmin complexes (Hall *et. al.*, 1991). This suggests that α2-antiplasmin can compete with the plasminogen receptors for plasmin. Thus the cell-surface, with respect to α2-antiplasmin inhibition of plasmin, seems to be homologous to the surface of a fibrin clot.

1.2.5 Regulation of cell-surface plasminogen binding.

To date the biochemical pathways and mechanisms responsible for the regulation of cell-surface plasminogen receptors remains to be characterised. Since there are several cellular plasminogen receptor molecules there may be more than one regulatory pathway responsible for localising these molecules to the cell-surface. Regulation of plasminogen binding proteins may involve both intracellular and extracellular processes. The extracellular regulation of the plasminogen binding capacity of a tissue may be similar to the regulation of plasminogen binding of fibrin. Down regulation of the plasminogen binding capacity of a cell may result from carboxypeptidase (enzymes that cleave basic carboxyl-terminal residues such as lysine) proteolysis of cell surface proteins (Plow *et. al.*, 1995; Redlitz *et. al.*, 1995; figure

1.12). In contrast, cell-surface plasmin may cleave pre-existing, cell-surface proteins between [K,R]$^{\downarrow}$X residues thereby exposing cryptic C-terminal lysine residues (figure 1.12; Camacho *et. al.*, 1989 and Gonzalez-Gronow *et. al.*, 1991; Plow *et. al.*, 1995).

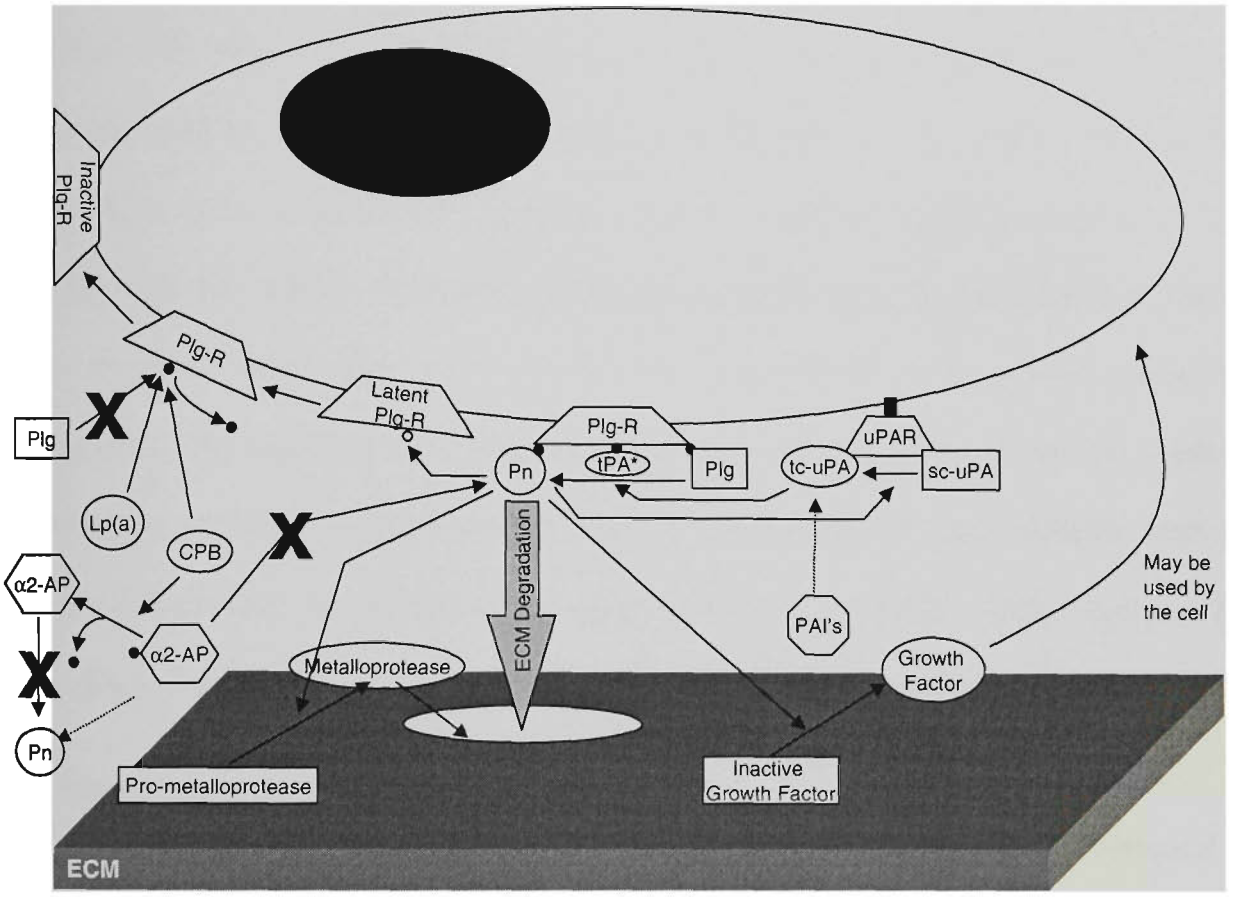

Figure 1.12: The cell-surface plasminogen activation cascade.

Organisation of the components of the cell-surface plasminogen activation cascade emphasising the role of the plasminogen receptors. ● represents a pre-existing C-terminal lysine residue from a pre-formed or class 1 plasminogen receptor. O represents a plasmin cleavable lysine residue from a latent or class 2 plasminogen receptor. *Note: selected plasminogen receptors can bind tPA and plasminogen simultaneously. Solid arrows represent activation reactions or binding events whereas, broken arrows represent inhibition events.

Therefore, plasmin may act in a positive feedback loop to activate the glu-plasminogen binding capacity of pre-existing, latent cell-surface plasminogen binding proteins.

A relationship between glu-plasminogen binding and activation capacity and the apopotic status of a U937 cells has been established (O'Mullane and Baker, 1998, 1999). Glu-plasminogen activation occurs on the surface of these apopotic cells due to a 100-fold increase in the both their lysine-dependent plasminogen binding capacity and endogenous

uPA levels in the presence of the protein synthesis inhibitor cycloheximide. Thus, the glu-plasminogen binding capacity of monocytes may be coupled to the apopotic processes of these cells (O'Mullane and Baker, 1998).

Another study by Kim *et. al.,* (1996) has suggested that the adhesion of the human monocytoid cell lines, THP-1 and U937, to extracellular matrix proteins promotes an increase in the expression of cell-surface glu-plasminogen binding (Kim *et. al.,* 1996). The increase in the binding of plasminogen to the surfaces of these adherent monocytoid cell lines was due to an increase in the number of binding sites and not an increase in the affinity of the cell-surface for plasminogen (Kim *et. al.,* 1996). This augmentation of the number plasminogen binding sites was rapid and reversible, involved pre-existing proteins of the cell, promoted plasminogen activation and dependent upon an interaction between adherent and non-adherent cell populations as well as β1-integrin (Kim *et al.,* 1996). Taken together, these studies suggest that glu-plasminogen binding does not require new protein synthesis.

1.2.6 Physiology of plasminogen binding.

Plasminogen binding plays a central role in the balance between physiological and pathological consequences of proteolysis. Since the concentration of glu-plasminogen in the blood is approximately 2 μM, a proportion of cell-surface plasminogen receptors would be occupied by glu-plasminogen. This equilibrium may result in the compartmetalisation of glu-plasminogen into two metabolically distinct pools: firstly, solution-phase, activation-resistant glu-plasminogen and secondly cell-surface-, ECM- or fibrin clot-bound, activation-susceptible, glu-plasminogen. Hence, physiologically inappropriate over-expression of glu-plasminogen binding proteins or cellular plasminogen receptors, and thus increased glu-plasminogen binding may increase the pool of activatable glu-plasminogen resulting in destructive proteolytic activity. Conversely, inappropriate down-regulation of cellular plasminogen receptors may hamper beneficial proteolytic activity. Within the bound-glu-

plasminogen metabolic pool there may be a specific hierarchy of plasminogen interactions based on binding protein affinities. The ECM proteins have approximately two orders of magnitude lower dissociation constants than cell-surface plasminogen receptors (table 1.4; Stack *et. al.,* 1994; Stack *et. al.,* 1992). This suggests that the binding of glu-plasminogen to cell-surface receptors (large capacity, low affinity) may represent a glu-plasminogen transportation mechanism that carries the protein from the blood to the tissues under certain physiological/pathological conditions. For example, Martinez and Santibanez (1994) provided circumstantial experimental evidence for this transportation process using WEHI-3B leukemic cells and collagen I.

1.2.7 Other plasmin-mediated cellular functions

The majority of cell-types associated with the blood vascular system, such as polymorphonuclear neutrophils (PMN's), platelets, endothelial cells and monocytes bind plasminogen in a lysine-dependent manner that facilitates the activation of the zymogen and protection of cell-surface associated plasmin from inactivation. However, some studies have reported that plasmin may also mediate a protease-independent cellular function via the initiation of intracellular signal transduction cascades within these cells in an agonist-dependent manner. (Ryan *et. al.,* 1992; Schafer and Adelman, 1985; Schafer *et. al.,* 1986; Chang *et. al.,* 1993; Syrovets *et. al.,* 1997). Lysine-dependent binding of plasmin, but not active-site blocked plasmin or plasminogen induces the synthesis of the intracellular second messenger molecule cyclic guanosine monophosphate (cGMP) in monocytes (Syrovets *et. al.,* 1997). Analogue competitors of cGMP inhibited plasmin-mediated chemotaxis of monocytes *in vitro*. This suggests that plasmin, produced at the site of inflammation, may act as a chemoattractant, thereby recruiting monocytes to inflammatory sites within the body (Syrovets *et. al.,* 1997).

Another key event in acute inflammation is the adhesion of neutrophils to vascular endothelial cells during the extravascation of these cells possibly via the neutrophil cell-surface glycoprotein CD18 (Pohlman *et. al.,* 1986; Anderson *et. al.,* 1987). Plasmin may also mediate the adherence of neutrophils to arterial endothelium in a lysine- time- and concentration-dependent manner and is due to an increase in the functional activity of CD18 (Lo *et. al.,* 1989). This adhesion event was specific for plasmin, but not dependent on the proteolytic activity of plasmin. Lys-plasminogen induced less neutrophil adherence than plasmin, whilst, glu-plasminogen is unable to mediate adherence. Furthermore, the K1-3 and K4 fragments of plasminogen mediate neutrophil adherence, whereas, miniplasminogen has a minimal effect on neutrophil adherence (Lo *et. al.,* 1989). This induction of neutrophil adherence may be due to a plasmin-induced, lysine-dependent signaling pathway via a cell-surface receptor.

1.3 Normal Breast tissue, carcinogenesis and plasminogen activation

Growth of breast tissue is the result of multiple hormones such as the female sex hormones (e.g. oestrogen and progesterone) and epidermal growth factors. Breast cancer is an example of a hormone-dependent malignancy (reviewed by Manni, 1999). Two well established antigenic markers of breast tumours are the oestrogen receptor (ER) and the oncogenes EGFR/c-*erb*B-2. Approximately 20-30% of all human breast cancers over-express two closely related receptor tyrosine kinases, namely epidermal growth factor receptor (EGFR) and/or c-*erb*B-2. This phenotype is associated with more aggressive tumour growth and reduced patient survival (Singleton and Strickler, 1992). In contrast, breast cancer cell lines that are ER positive and have little or no EGFR, have a non-metastatic phenotype (Lee *et al,* 1990).

Breast cancer invasion and metastasis are highly orchestrated pathological processes involving dynamic but aberrant relationships between the malignant cells and surrounding

host stromal cells. The co-ordinate expression of molecules of several perturbed biochemical pathways is required for the adhesion, proteolysis and cell motility of tumour cells. Some of these biochemical pathways include the cell-cell and cell-ECM interactions mediated by the cadherins and integrins as well as ECM proteolysis (reviewed by Jones and Walker, 1997). Essentially, two classes of proteases (plasmin and MMP's) have been identified as important determinants for breast cancer metastasis. The role of the pericellular plasminogen activation cascade in the process of breast cancer invasion is discussed below.

1.3.1 The plasminogen activation system and breast cancer

The role of the plasminogen activation system in migration, wound healing, tumour cell invasion and metastasis have recently been reviewed (Plow *et. al.,* 1999; Dano *et. al.,* 1999; Duffy *et. al.,* 1999; Schmitt *et. al.,* 1997). Evidence has accumulated that support the contention that invasion and metastasis of solid tumours requires the action of tumour-associated proteases. These proteases are responsible for the degradation of extracellular matrices that must be breached by the migrating cell to achieve colonisation of a distant tissue.

Components of the plasminogen activation proteolytic cascade are also linked to the pathology of breast cancer growth and metastasis (Duffy, 1993) and have been termed metastatic factors. Differentiated, ER positive breast cancer cells express low levels of uPA and uPAR (Long and Rose, 1996; Connolly and Rose, 1997). In contrast, EGFR stimulation or c-*erb*B-2 over-expression in aggressive human breast cancer cells enhances the expression and secretion of uPA and expression of uPAR (Long and Rose, 1996; Connolly and Rose, 1997). It is apparent that breast cancer cell lines such as MDA-MB-231 cells, that are highly tumourogenic and metastasise in nude mouse models and/or are invasive in *in vitro* models of metastases (Thompson et al, 1992), tend to be EGFR(+)/*erb*B-2 protein(+) and ER(-) (Lee et al, 1990), as well as uPA/uPAR(+) (Holst-Hansen et al, 1996). Combined over-expression of

uPA, uPAR and PAI-1 is associated with decreased disease-free periods and reduced patient survival times (Janike *et. al.* 1993; Christensen *et. al.,* 1996; Costantini *et. al.,* 1996; Neilsen *et. al.,* 1996), whilst elevated levels of PAI-2 are associated with a favourable prognosis in primary breast cancer (Foekens *et. al.,* 1995). As well as plasminogen activator antigen levels Yamashita *et. al.,* (1993) examined plasminogen activator activities associated breast cancer metastasis from patients presenting with primary metastases in the bone only, bone and lung, lung only or disease-free subjects in terms of lymph node status. The authors found that tPA activity and antigen levels are low in patients with bone-only metastasis from node-negative breast cancers (Yamashita *et. al.,* 1993). In contrast, uPA activity, as opposed to tPA activity, is expressed by node-positive breast cancer, which may progress into lung metastases (Yamashita *et. al.,* 1993). The *in vitro* invasive capacities of human breast cancer cell lines is dependent on both uPA and plasmin activity (Holst-Hanson *et. al.,* 1996).

Cell-surface localised plasminogen may also be involved with breast cancer metastasis. Plasminogen binds in a lysine-dependent manner to infiltrating grade II or III ductal carcinomas (Burtin *et. al.,* 1993). However, the plasminogen receptors of these breast cancer cells are not saturated *in vivo* and bind additional amounts of plasminogen *in vitro* (Burtin *et. al.,* 1993). The above studies all present evidence that aggressive, invasive breast cancer is associated with the expression of high levels of the plasminogen activation cascade components. These studies indirectly support the contention that metastasising breast cancer cells acquire active plasmin at their surfaces that can be used by the cells to degrade physiological barriers. However, the precise mechanism of glu-plasminogen acquisition and activation by these cells remains poorly defined.

1.4 Rationale and Aims

The fibrin clot acts to focus proteolytic activity at its surface by binding the proteases of the plasminogen activation cascade in a ternary complex. Similarly, the cell-surface can bind plasminogen activators and glu-plasminogen in a ternary complex. Thus, the process of glu-plasminogen activation at the cell-surface may be analogous to glu-plasminogen activation during fibrinolysis and requires the cell-surface-association of both plasminogen activators and glu-plasminogen. The activity of both inhibitors of plasminogen activation and modifying agents help regulate the activation of glu-plasminogen at the cell surface. Thus, plasminogen activation at the cell-surface is a balance between activators, inhibitors and modifying agents.

Cellular plasminogen receptors are not as well characterised as the other components of the plasminogen activation cascade. To date nine cellular plasminogen receptor candidate proteins have been identified (table 1.4). However, detailed mechanistic knowledge about the interaction of glu-plasminogen with this class of protein is rudimentary. Therefore, the overall objective of this thesis was to define a mechanism for the binding of glu-plasminogen to its plasminogen receptors that accounted for the observed increase in the activation rate of receptor-bound glu-plasminogen.

It is hypothesised that the lysine-dependent binding of glu-plasminogen to its receptor is mediated by a competition reaction between the internal lysine residues of glu-plasminogen and the lysine residues of a receptor for the LBS motifs of glu-plasminogen. The result of such reactions would disrupt the intramolecular lysine-binding network of glu-plasminogen facilitating the open, activation-susceptible conformation of the zymogen. α-Enolase was selected as the plasminogen receptor molecule of choice since it had previously been shown to bind glu-plasminogen in a lysine-dependent manner that increases the activation rate of the zymogen. Another reason for the selection of α-enolase as a model plasminogen receptor

candidate to study the mechanism of plasminogen binding was that it possesses a C-terminal lysine residue.

The specific aims of this thesis were to:

[1] Determine if the human gene product of ENO1 (namely recombinant α-enolase) did encode an authentic plasminogen receptor protein and to produce antibodies against this protein.

[2] Use α-enolase as a model plasminogen receptor protein to determine a physiologically relevant mechanism for its interaction with glu-plasminogen.

[3] Examine the cell surface glu-plasminogen binding events in three human breast cancer cell lines in relation to their metastatic potentials.

Chapter 2

The human ENO1 gene product (recombinant human alpha-enolase) displays many characteristics required for an authentic plasminogen binding protein.

2.1 Introduction

Glu-plasminogen interacts with cells via both proteinaceous and non-proteinaceous (e.g. gangliosides) cell-surface moieties (Miles *et. al.*, 1989) in a lysine-dependent manner. To date, nine candidate cell-surface plasminogen binding proteins have been identified and six other proteins (apparent Mr 40-60 kDa) have been reported but remain essentially unidentified and uncharacterised (Lopez-Alemany *et. al.*, 1995). One of these plasminogen receptor candidates, a 54 kDa protein has been isolated from the plasma membrane of monocytoid cell line U-937 and identified as human α-enolase by partial amino-acid-sequence homology (Miles *et. al.*, 1991) and is known as α-enolase-related molecule (ERM; Section 1.3.5).

The 54 kDa ERM has all the properties required for classification as a cell-surface plasminogen binding protein, in that it is able to bind glu-plasminogen in a saturable, specific and lysine-dependent manner with a K_d of approximately 1.4 μM and once bound to this molecule, the activation rate of glu-plasminogen is significantly increased. The ERM bound glu-plasminogen is also partially shielded from inactivation by α2-antiplasmin by competing with α2-antiplasmin for the lysine binding sites of plasminogen (Redlitz *et. al.*,1995).

The molecular weight of cytoplasmic α-enolase is 47 kDa, whereas the molecular weight of ERM is estimated at 54 kDa. Thus, ERM may be encoded by a separate gene. The purpose of this chapter was to assess whether the human ENO1 gene encoded a glu-

plasminogen binding protein. Data within this chapter indicated that recombinant human α-enolase (r-α-enolase), as encoded by the ENO1 cDNA, was an authentic plasminogen binding protein. Furthermore, the plasminogen binding capacity of human α-enolase is not due to a post-translational modification of the protein.

2.2 Material and Methods

2.2.1 Materials

Plasminogen, α2-antiplasmin, the plasminogen specific substrate Spectrozyme-PL and monoclonal antibody #3641 and #3642 which specifically recognise epitopes within the NTP and K1-3 domains of glu-plasminogen were all kindly donated by Dr Richard Hart (American Diagnostica Inc., Greenwich, CT., U.S.A.). Plasmid HHCME18, which encoded the human ENO1 gene, was purchased from ATCC. Plasmid pQE-30 and Ni^{2+}-NTA-Agarose were from QIAGEN Inc. Hilden, Germany. Lysine Sepharose 4B, G75-Superdex and PD-10 columns were obtained from Pharmacia, Uppsala, Sweden. RPMI 1640, L-glutamine were purchased from Trace Biosciences Castle Hill, NSW, Australia. Fetal calf serum was obtained from CSL Parkville, Vic, Australia. Active human tc-uPA was from Serono, Sydney NSW, Australia. Plastic tissue culture flasks were purchased from Corning, Corning, NY, USA. Microtitre 96 well plates were from Titre-Tek Flow Laboratories, The Netherlands. Trasylol was bought from Bayer, Leverkusen, Germany. Carboxypeptidase B was obtained from Boehringer Mannheim, Mannheim Germany. Horse radish peroxidase conjugated avidin (avidin-HRP), alkaline phosphatase conjugated avidin (avidin-AP). Molecular weight protein standards were obtained from Novex (Sydney, Australia). All other reagents were of the highest quality commercially available and all reagents were made using double de-ionised water purified by a Millipore water purification system.

2.2.2 Purification and biotinylation of human glu-plasminogen

Human glu-plasminogen was also purified from human plasma using lysine Sepharose-4B affinity chromatography as described below. Briefly, 400 ml of plasma containing 1 mM PMSF, 5 mM EDTA and 0.02% NaN_3, was loaded onto a lysine Sepharose-4B column (60 ml) for 24 h at 4°C at a flow rate of 30 ml/h. The column was washed overnight with PBS (pH 7.4) supplemented with 1 mM PMSF, 5 mM EDTA and 0.02% NaN_3 at 4°C at a flow rate of 30 ml/h until baseline was achieved. The column was washed with wash buffer (50 mM Na-phosphate (pH 7.4), 500 mM NaCl, 1 mM PMSF, 5 mM EDTA and 0.02% NaN_3) at a flow rate of 30 ml/h until baseline was achieved. The plasminogen was competitively eluted with PBS (pH 7.4) containing 200 mM of the lysine analogue ε-ACA at a flow rate of 30 ml/h. The fractions were analysed by non-reducing 12% SDS-PAGE and western blotting using monoclonal antibodies #3641 and #3642 which are specific for the NTP and K1-3 domains of plasminogen, respectively. The plasminogen was dialysed against PBS (pH 7.4) for 24 h at 4°C with 3 changes of buffer.

2.2.3 Modification of glu-plasminogen.

Human glu-plasminogen was biotinylated by first increasing the pH above 8 by the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). A 40 molar excess of biotin-X-NHS was added and the reaction mixture incubated overnight at 4°C with rocking. Human glu-plasminogen in PBS was conjugated with fluorescein isothiocyanate isomer I (FITC) as described by Goding (1976). Both unconjugated biotin-X-NHS and FITC were separated from the conjugated protein by gel filtration using a PD-10 gel filtration column equilibrated with PBS/0.1% azide. The lysine-dependent binding capacity of modified glu-plasminogen was determined by batch lysine-Sepharose-4B affinity chromatography.

2.2.4 Production of recombinant human 6xHis-α-enolase protein

The human α-enolase [Human ENO1 (HHCME18) ATCC] coding domain within a 1.23-kb *Sac* 1-*Xho* 1 cDNA fragment was ligated downstream and in-frame to the 6-His affinity tag in the QIAexpress pQE-30 expression vector (QIAGEN Inc.). The resulting construct pQE-ENO1 was transformed into JM109 *E.coli* host cells. The expression of the resultant pQE-ENO1 construct creates an enolase fusion protein with a 6-His N-terminal affinity tag and a predicted protein size of approximately 42 kDa. This recombinant human α-enolase protein will be referred to throughout this thesis as r-α-enolase.

2.2.5 Non-denaturing purification of r-α-enolase

The procedure for the large scale purification of r-α-enolase under non-denaturing conditions was followed as outlined by the QIAexpress protocol with minor modifications. Briefly, transformed JM109 cells were pelleted and resuspended in sonication buffer (50 mM Na-phosphate (pH 8.0), 300 mM NaCl, 1mM PMSF, 4 mM $MgCl_2$ and 0.2% Tween-20). After 2 freeze-thaw cycles in liquid nitrogen, the cells were treated with 1 mg/ml lysozyme for 30 min on ice followed by treatment with RNase (10μg/ml) and DNase (5 μg/ml) for 15 min at 4°C and sonicated (Branson Sonifier 250) for three 1 min bursts on ice. The preparation was centrifuged at 14,000g for 15 min at 4°C and the supernatant batch loaded onto Ni^{2+}-NTA-agarose resin for 1 h at 4°C. This was then poured into a column and washed with 10 column volumes of sonication buffer and 10 column volumes of wash buffer (50 mM Na-phosphate, pH 6.0, 300 mM NaCl, 10% glycerol, 1mM PMSF). Bound r-α-enolase was eluted from the column with 250 mM imidazole-HCl in 50mM Na-phosphate (pH 6.0), 300 mM NaCl a flow rate of 15 ml/h. The eluted samples were analysed by SDS-PAGE and fractions containing a 42 kDa component were pooled and further purified by gel filtration

using a G75 Superdex column (Pharmacia) with column buffer (25 mM Na-phosphate, 2.5 mM $MgSO_4$, 0.05% NaN_3) at a flow rate of 30 ml/h. The samples were analysed by SDS-PAGE, enolase enzyme activity, and plasminogen binding capacity. Fractions containing a 93 kDa or a 42kDa component with plg binding capacity and/or enolase enzyme activity were dialysed overnight against the G75 Superdex column buffer containing 50% glycerol and stored at -20°C until required.

2.2.6 Enolase Glycolytic Activity Assay

Enolase glycolytic activity in each of the G75 Superdex fractions was assayed by measuring the rate of phosphoenolpyruvate (PEP) production as described by Christian and Wold (1942). Briefly, a sample of each fraction (100µl) was incubated with 1 mM 2-phospho-D-glycerate (2-PGA) in 1 ml of reaction buffer (50 mM imidazole-HCl, (pH 6.8), 400 mM KCl, 3 mM $MgSO_4$) at 25°C. The appearance of PEP with time was monitored at 240nm. A standard curve was constructed by measuring the A240nm of 0 to 100 µM PEP.

2.2.7 Denatured purification of r-α-enolase.

Cells were solubilised by incubating with buffer A (10 mM Tris-HCl (pH 8.0), 6M guanidine-HCl, 0.1 M Na-phosphate) for 1 h at room temperature and the lysate centrifuged at 10,000g for 15 min at 4°C. The supernatant was incubated with 4 ml of Ni^{2+}-NTA-agarose previously equilibrated with buffer A at room temperature for 1 h. The column was poured and the resin washed with 10 column volumes of buffer A at a flow rate of 15 ml/h. The column was then washed with 10 column volumes of buffer B (0.01 M Tris-HCl (pH 8.0), 8 M urea, 0.1 M Na-phosphate) and 10 column volumes of buffer C (0.01 M Tris-HCl (pH 6.3), 8 M urea, 0.1 M Na-phosphate). The final wash using buffer D (Tris-HCl (pH 5.9), 8 M urea, 0.1 M NaCl) effectively removed all the contaminating proteins. Elution of r-α-enolase was

performed using buffer E (Tris-HCl (pH 4.5), 8 M urea, 0.1 M Na-phosphate) and 3 ml fractions were collected and analysed by non-reducing 12% SDS-PAGE (under reducing conditions).

2.2.8 Plasminogen ligand blotting.

R-α–enolase was fractionated on 12 % SDS-PAGE gels and transferred to PVDF membranes overnight at 30 V at 48C. The membranes were washed once with TNCM (Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$) and blocked with TNCM/0.05% Tween-20/BLOTTO for 1 h at room temperature followed by three 5 min washes with TNCM/0.05% Tween-20. The blots were then probed with 2μg/ml biotinylated plasminogen in TNCM/0.05% Tween-20/BLOTTO for 1 h at room temperature and washed for 1 h with TNCM/0.05% Tween-20. A 1:2000 dilution of avidin-AP (Calbiochem) in TNCM/0.05% Tween-20/BLOTTO was added and incubated with the membrane for 1 h. This was followed by three 5 min washes with TNCM/0.05% Tween-20 and one wash with TNCM. The blot was developed using the NBT/BCIP colour development system at room temperature.

2.2.9 96-well microtitre plasminogen binding assay.

R-α-enolase (500 pM) was coated onto 96-well microtitre plates in 50 mM $NaHCO_3$ buffer (pH 8.5) overnight at 4°C. Biotinylated plasminogen standards 0-50 nM in 50 mM $NaHCO_3$ (pH 8.5) were also coated onto the same microtitre plates overnight at 4°C, thereby enabling quantification of biotinylated plasminogen bound to r-α-enolase. The plates were then washed twice with PiNT (100 mM Na-phosphate (pH 7.5), 150 mM NaCl 0.05% Tween-80) and blocked with 50 μl of PiNT containing BLOTTO at 37°C for at least 1 h. The plates were washed as above and varying concentrations of biotinylated plasminogen with or without competitors in PiNT/BLOTTO were added and incubated for 1 h at 37°C then rinsed

three times with PiNT. A 1:2000 dilution of avidin-HRP in PiNT/BLOTTO was added and incubated for 1 h at 37°C. This was followed by 4 washes with PiNT. The plates were developed using the OPD colour development system and read at 490 nm.

2.2.10 Carboxpeptidase B digestion of r-α-enolase and plasminogen binding assay.

To examine whether or not the C-terminal lysine residues of r-α-enolase were important for plasminogen binding, r-α-enolase (1 μg/ml) was coated onto microtitre plates overnight at 4°C in 50 mM $NaHCO_3$ (pH 8.5). Once again biotinylated plasminogen standards (0-50 nM) were also coated onto the plates in 50 mM $NaHCO_3$ overnight at 4°C. The plates were then washed with PiNT three times and blocked with PiNT/1% BSA in wash buffer for 1 h at 37°C followed by one wash. Bound r-α-enolase was treated with various concentrations of carboxypeptidase B (CBP) for 10 min at 37°C in PiNT, after which the plates were washed three times and probed with 1μM biotinylated plasminogen in PiNT/1% BSA for 1 h at 37°C. The plates were washed and probed with avidin-HRP conjugate in PiNT/1% BSA and colour developed using OPD substrate as described above.

2.2.11 Protein Analysis.

All protein concentrations were determined using the Bio-Rad DC Protein Assay. The apparent molecular weight of purified r-α-enolase after fractionation by 12% SDS-PAGE was determined using scanning laser densitometry (Bio-Rad) relative to the mobility of protein standards and these averaged between 42 and 54 kDa.

2.3 Results

2.3.1 Plasminogen purification and characterisation

Representative data showing the purification of plasminogen using lysine sepharose chromatography are shown. Plasminogen was eluted from the lysine-sepharose column (figure 2.1A) with 200 mM ε-ACA as illustrated by 12% non-reducing SDS-PAGE (figure 2.1B). This band had an apparent molecular weight of 92 kDa and was immunologically reactive against both anti-NTP (#3641) and anti-K1-3 (#3642) monoclonal antibodies. This suggested that glu-plasminogen was successfully purified from plasma. The lysine-dependent activation of purified glu-plasminogen was examined by incubating 10μg of glu-plasminogen with a 25:1 molar ratio of tc-uPA for 10 min at 37°C in the presence of various concentrations of ε-ACA. The reactions were terminated with reducing sample buffer, boiled and the samples fractionated by 12% SDS-PAGE (figure 2.1C). Incubation of glu-plasminogen and tc-uPA with 5 mM ε-ACA resulted in a 2.4 fold increase in the amount of plasmin A-chain formed compared to glu-plasminogen that was not treated with ε-ACA (figure 2.1C). This suggested that glu-plasminogen was isolated in a closed conformation that was capable of an ε-ACA-dependent conformational change which facilitated an increase in the activation rate of the zymogen.

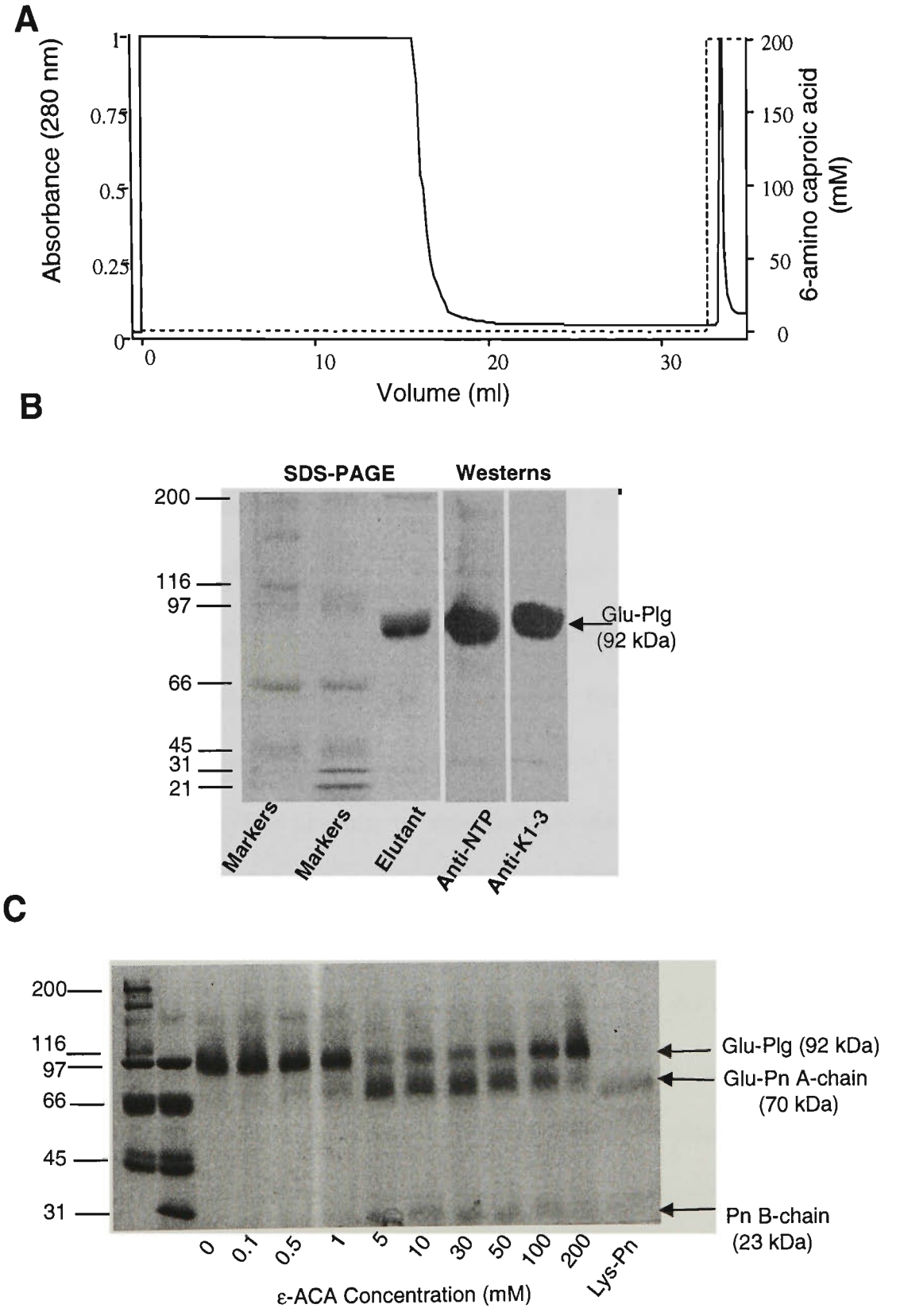

Figure 2.1: The purification of glu-plasminogen from human plasma.

A typical chromatogram of the purification of glu-plasminogen from plasma. (B) Non-reducing 12% SDS-PAGE and western blots of the elutant from the lysine-sepharose column. The western blots were probed with anti-plasminogen NTP (#3641) and anti-K1-3 (#3642) plasminogen monoclonal antibodies. (C) Reducing 12% SDS-PAGE of a uPA-dependent activation of glu-plasminogen in the presence of increasing concentrations of ε-ACA.

2.3.2 Modifications of glu-plasminogen

Glu-plasminogen was either biotinylated or FITC conjugated for use as probes in various glu-plasminogen binding assays (chapters 2 and 5). However, the modification of glu-plasminogen with either NHS-X-biotin or FITC may ablate the ability of the protein to interact in a lysine-dependent manner with a potential receptor. Thus, the lysine binding capacity of modified glu-plasminogen was assessed by batch affinity chromatography using lysine-sepharose.

After either biotinylation or FITC conjugation, a sample of the modified glu-plasminogen was loaded onto lysine-sepharose at 4°C for 30 min, the resin was washed, and the bound modified glu-plasminogen was batch eluted with 200 mM ε-ACA. The fractions from the batch lysine-sepharose chromatography were fractionated by 12% non-reducing SDS-PAGE and either stained with Coomassie blue (figure 2.2A; biotinylated glu-plasminogen) or exposed to UV light and photographed (figure 2.2B; FITC conjugated glu-plasminogen). The absence of modified glu-plasminogen in the flow throughs and wash buffers suggested that both biotinylated and FITC conjugated glu-plasminogen were able to interact with lysine-sepharose. Moreover, the elution of biotinylated and FITC conjugated glu-plasminogen with ε-ACA demonstrated that the interactions of the modified glu-plasminogen with lysine-sepharose was both lysine-dependent and reversible. This suggested that both biotinylated and FITC conjugated glu-plasminogen had retained their lysine-dependent binding characteristics and thus may be used as *in vitro* probes in glu-plasminogen binding assays.

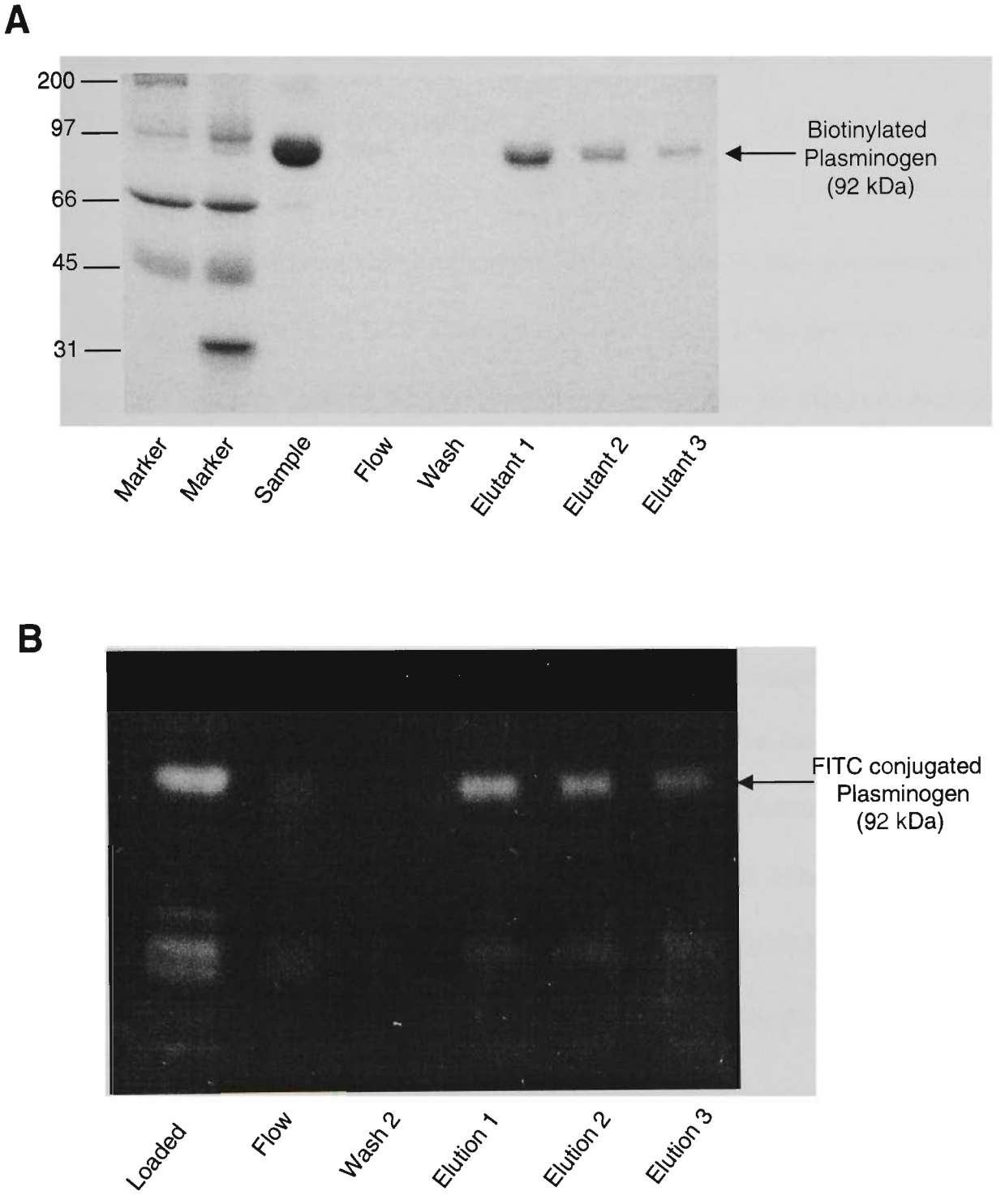

Figure 2.2: Reversible lysine-dependent binding of biotinylated and FITC conjugated glu-plasminogen to lysine sepharose.

Non-reducing 12% SDS-PAGE of biotinylated glu-plasminogen after lysine Sepharose-4B batch chromatography. Biotinylated glu-plasminogen (10 μg; lane 1) was incubated with lysine Sepharose-4B for 30 min. The resin was washed with PBS (lanes 2 and 3) and incubated with PBS containing 200 mM ε-ACA (lanes 4-6). (B) Non-reducing 12% SDS-PAGE of FITC conjugated glu-plasminogen after lysine Sepharose-4B batch chromatography. FITC conjugated glu-plasminogen (20 μg; lane 1) was incubated with lysine Sepharose-4B for 30 min. The resin was washed with PBS (lanes 2 and 3) and incubated with PBS containing 200 mM ε-ACA (lanes 4-6).

2.3.3 Recombinant α-enolase purification.

Purification of r-α-enolase by Ni^{2+}-NTA chromatography under non-denaturing conditions yielded a preparation containing dimeric (94 kDa), monomeric (52 kDa) and proteolytic fragments of r-α-enolase (figure 2.3A). Ligand blot analysis indicated that the full-length monomeric and dimeric forms of r-α-enolase were able to bind plasminogen (figure 2.3B). After gel filtration on a G-75 Superdex column (figure 2.4A) the crude r-α-enolase preparation was separated into an SDS-stable dimeric r-α-enolase (93 kDa) enriched fraction and a monomeric r-α-enolase (42 kDa) enriched fraction as shown by non-reducing SDS-PAGE (figure 2.4B). Enolase glycolytic activity corresponded to the dimeric r-α-enolase enriched fraction (fractions 14-16) isolated from the gel filtration column (figure 2.4A). When r-α-enolase was purified using Ni^{2+}-NTA chromatography under denaturing conditions (figure 2.5A) no glycolytic activity was present. SDS gel electrophoresis of the denatured purified r-α-enolase showed a single band at 48 kDa (figure 2.5B). Ligand blotting analysis of purified r-α-enolase using biotinylated plasminogen revealed a single 48 kDa band (figure 2.5B). Taken together, these data suggested that plasminogen could only bind full length r-α-enolase and that the tertiary structure of r-α-enolase was not essential for its plasminogen binding activity.

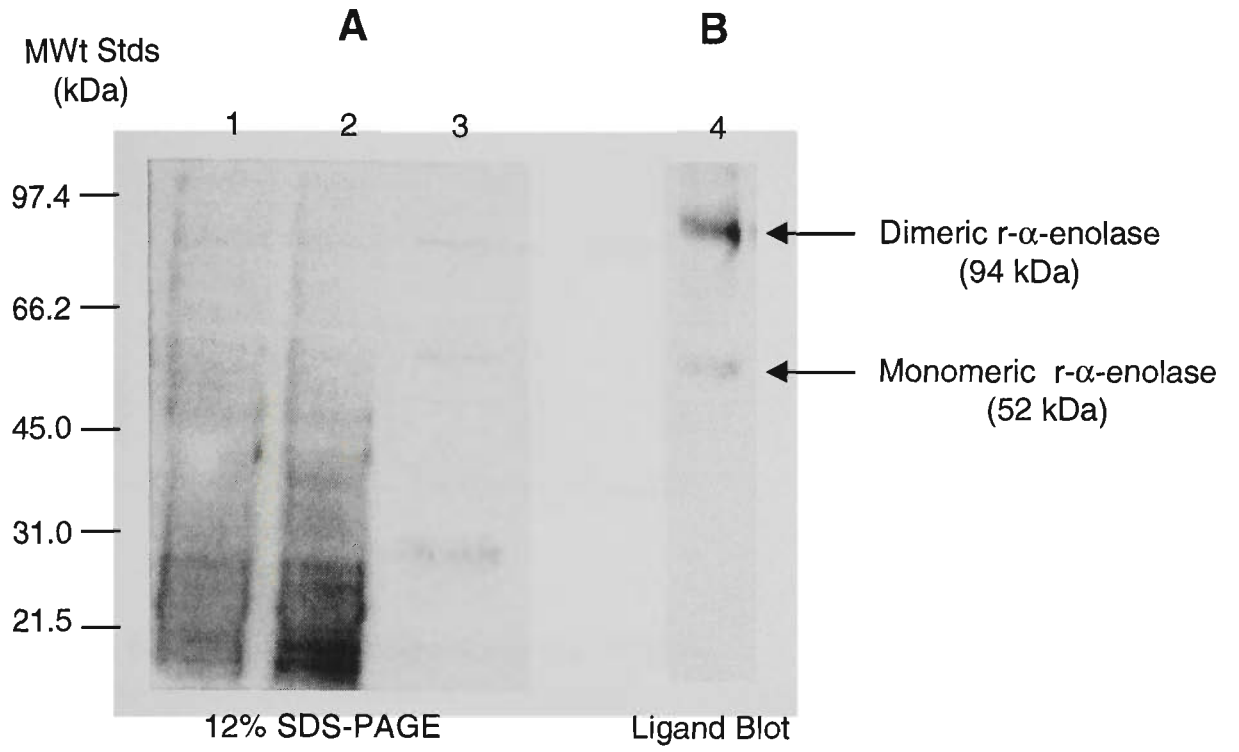

Figure 2.3: Non-reducing 12% SDS-PAGE and ligand blot of non-denatured, partially purified r-α-enolase.

LANE 1: *E.coli*JM109pQE30-ENO1 whole cell lysate. LANE 2: Ni^{2+}-NTA column flow through (wash buffer). LANE 3: Proteins eluted from the Ni^{2+}-NTA column with 250 mM imidazole-HCl. LANE 4: Ligand blot: elutant from lane 3 was probed with 2 µg/ml biotinylated plasminogen and avidin-AP (1:2000 dilution).

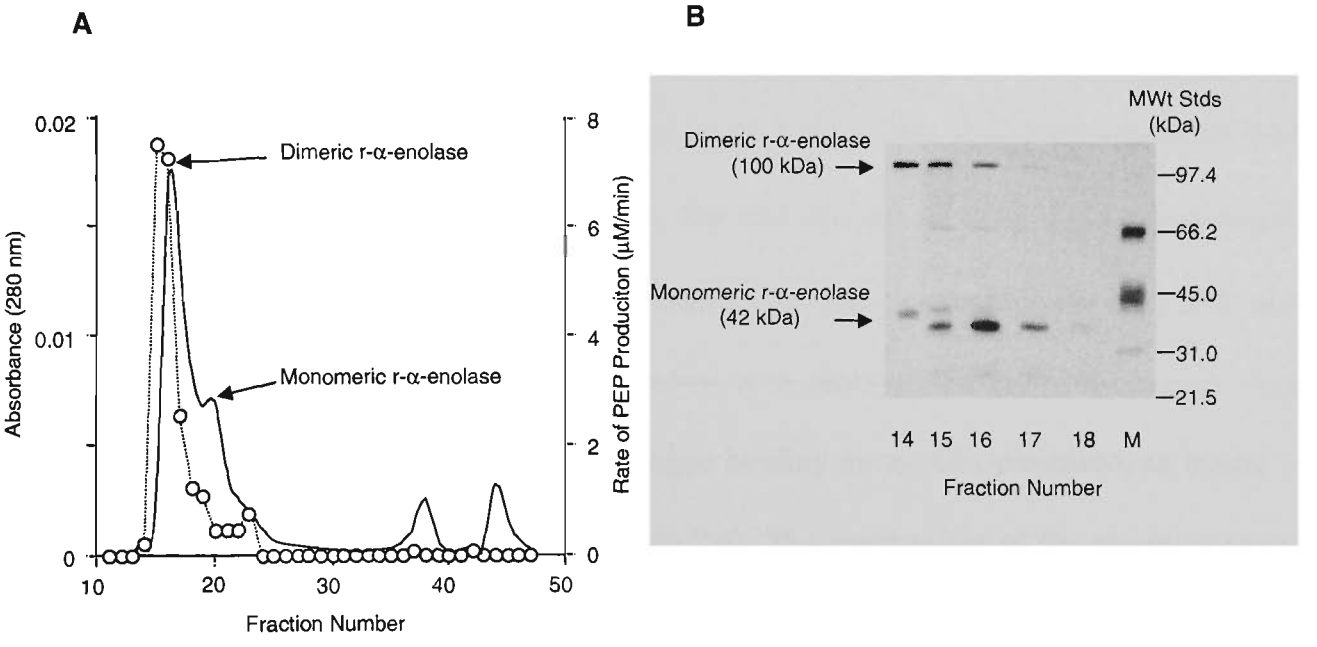

Figure 2.4: Purification of crude non-denatured r-α-enolase by G75 Superdex gel filtration.

(A) Gel filtration chromatograph (-) and corresponding enolase glycolytic activity assay profile (O). (B) Silver stained 12% SDS-PAGE (non-reducing) of the fractions eluted from the gel filtration column.

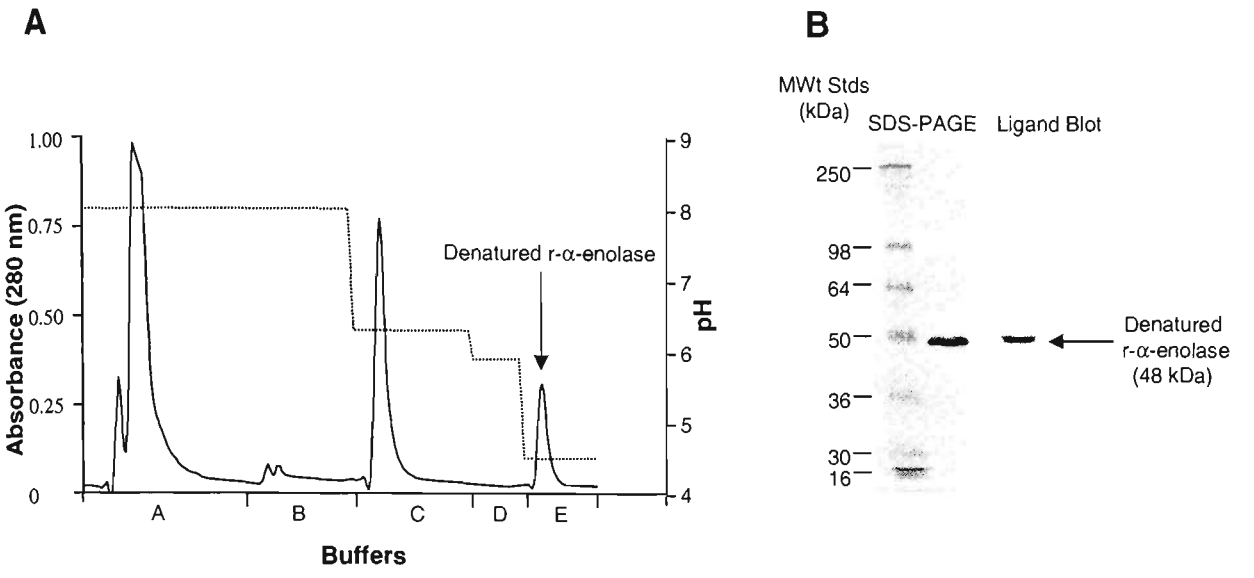

Figure 2.5: R-α-enolase purified under denaturing conditions.

(A) Ni^{2+}-NTA agarose chromatograph of the purification of denatured r-α-enolase (refer to material and methods (Section 2.2.6) for explanation of buffers A to E). (B) Silver stained 12% SDS-PAGE (reducing conditions) and ligand blot of the buffer E elution peak probed with 2 μg/ml biotinylated plasminogen.

2.3.4 Plasminogen binding properties of r-α-enolase.

Non-denatured dimeric enriched fraction of r-α-enolase (fraction 14) was immobilised onto microtitre plates and incubated with varying concentrations of biotinylated glu-plasminogen (0-20 μM). Glu-plasminogen binding to r-α-enolase was dose-dependent and approached saturation above 10 μM (figure 2.6). The non-specific binding of glu-plasminogen to BLOTTO was low, typically 0.07% at a plasminogen input concentration of 2 μM, and thus wells coated with only BLOTTO and probed with biotinylated glu-plasminogen were used as controls in the microtitre plate plasminogen binding assay. Glu-plasminogen bound r-α-enolase with an apparent K_d of 1.9 μM (figure 2.6). The mechanism of the plasminogen:r-α-enolase binding was characterised using plasminogen concentrations that corresponded to the linear portion of the saturation curve (figure 2.6) in the absence or presence of lysine and arginine analogues (figure 2.7). The lysine analogues were the best

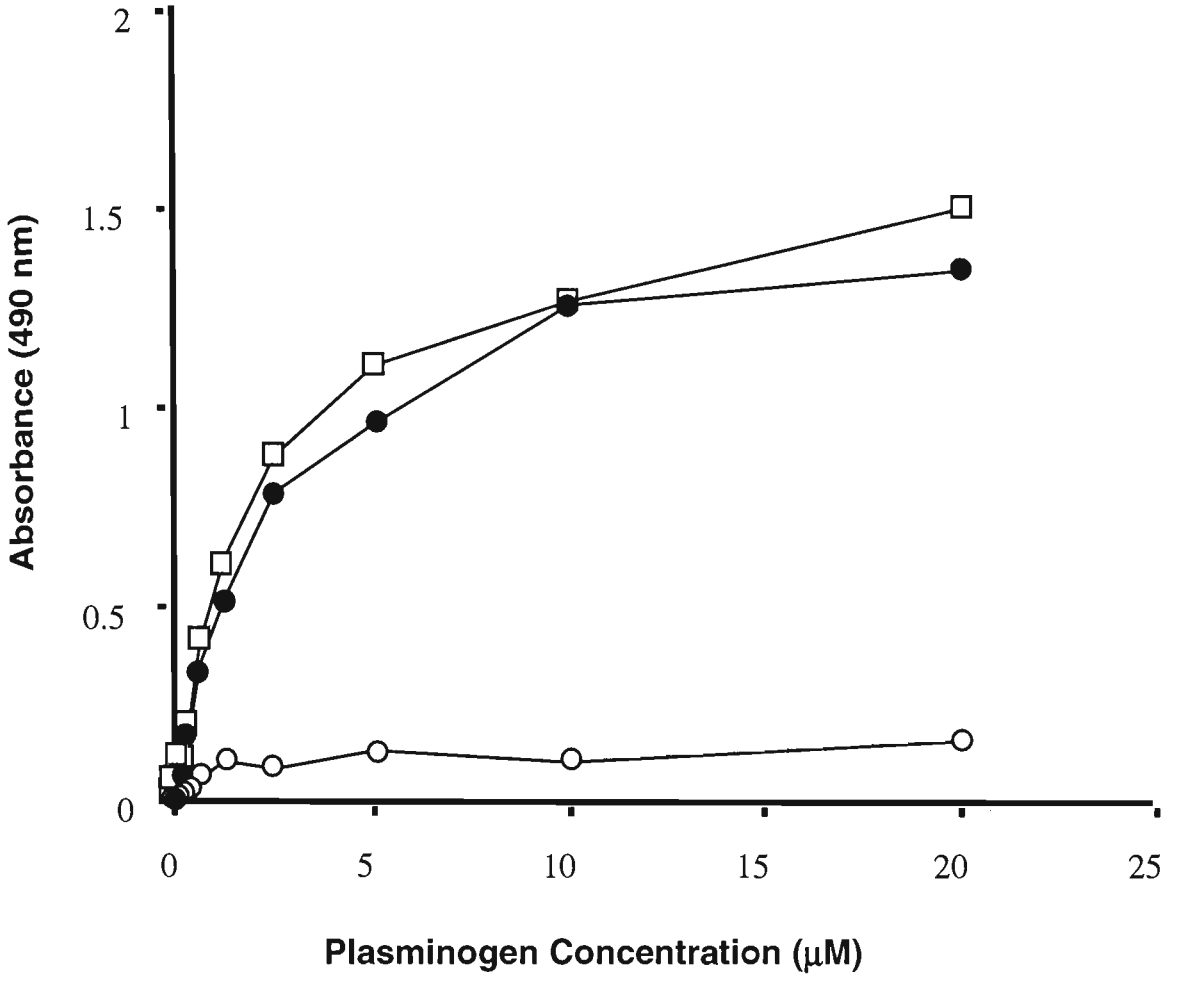

Figure 2.6: The r-α-enolase:biotinylated plasminogen saturation binding curve.

R-α-enolase (500 pM) was coated onto a 96 well microtitre plate and probed with biotinylated plasminogen (□). Non-specific binding was determined by probing with biotinylated plasminogen in the presence of a 50 fold excess of non-biotinylated plasminogen (○). Specific binding was calculated by subtracting total binding from non-specific binding (●).

inhibitors of glu-plasminogen binding (ε-ACA (94%) > TA (80%)), whilst benzamidine had minimal inhibitory capacity on glu-plasminogen binding (14%).

2.3.5 Carboxypeptidase digestion of r-α-enolase.

To examine whether the C-terminal lysine residue of r-α-enolase mediated its glu-plasminogen binding capacity, r-α-enolase was immobilised onto the wells of the microtitre plate and incubated with CPB to remove the its C-terminal lysine residue. Digestion of

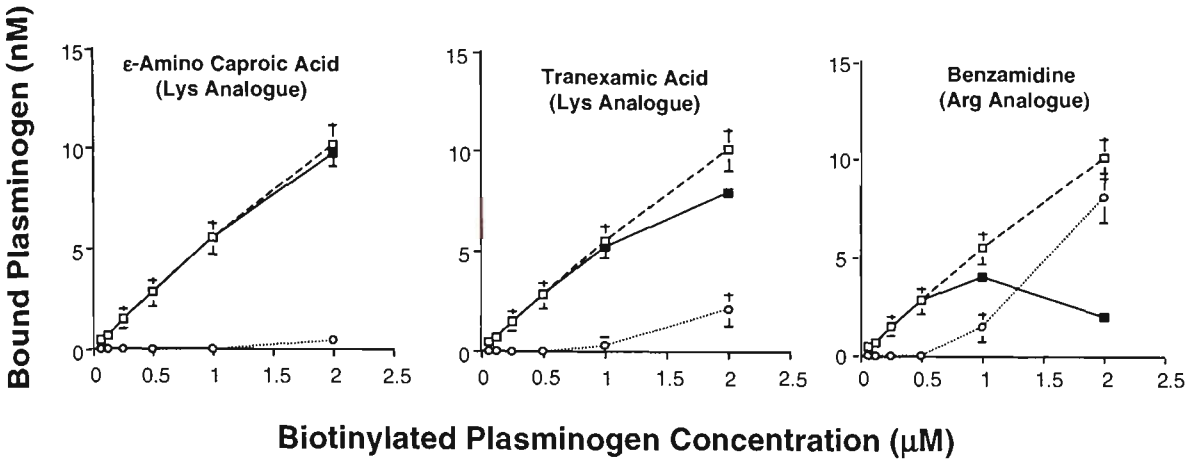

Figure 2.7: Glu-plasminogen binding inhibition curves.

R-α-enolase (500 pM) was coated to the plates and probed with varying concentrations of biotinylated glu-plasminogen (0-2μM) in the absence ( ) or presence (O) of 100 mM inhibitors. Inhibitor-dependent glu-plasminogen binding was calculated as the difference between the inhibitor + glu-plasminogen binding data and the total glu-plasminogen binding data (■).

r-α-enolase with CPB (0-20 IU) inhibited plasminogen binding to the non-denatured dimeric enriched fraction of r-α-enolase in a concentration dependent manner. This inverse relationship suggested that the presence of the C-terminal lysine residue from r-α-enolase was important for glu-plasminogen binding (figure 2.8A). The non-specific binding of biotinylated glu-plasminogen to BSA or CPB was negligible (data not shown). Similarly, removal of the C-terminal lysine residue from denatured r-α-enolase (figure 2.8B) by treatment with CPB (20 IU) neutralised its glu-plasminogen binding capacity, indicating that the presence of a C-terminal lysine residue was critical for the glu-plasminogen binding capacities of both the non-denatured and denatured forms of r-α-enolase. Both the pharmacological inhibitor results (figure 2.7) and CPB digestion data suggested that the mechanism of glu-plasminogen binding to r-α-enolase was a lysine-dependent process mediated in part by the C-terminal lysine residue of r-α-enolase.

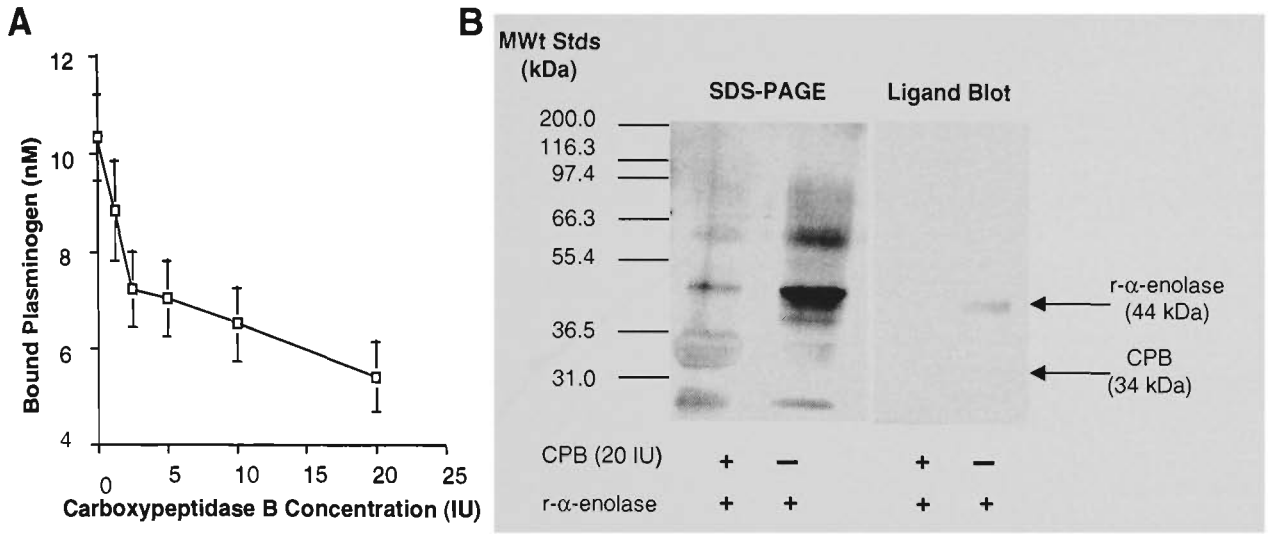

Figure 2.8: The effect of carboxypeptidase B digestion on the plasminogen binding capacity of r-α-enolase.

(A) R-α-enolase (1 μg/ml) was coated onto the microtitre plate and digested with increasing concentrations of carboxypeptidase B (0-20 IU) for 10 min at 37°C. The carboxypeptidase was removed and the wells probed with 1 μM biotinylated plasminogen. (B) Non-reducing 12% SDS-PAGE and ligand blot showing the effect of carboxypepidase B digestion of denatured r-α-enolase and the subsequent ability of biotinylated plasminogen to bind digested r-α-enolase. Denatured r-α-enolase was incubated with either carboxypeptidase B (20 IU) or with PBS buffer for 15 min at 37°C. The samples were then fractionated under non-reducing conditions by 12% SDS-PAGE.

2.3.6 Plasminogen Activation Assay.

The efficiency of r-α-enolase-bound glu-plasminogen activation by uPA was compared between r-α-enolase bound glu-plasminogen and unbound glu-plasminogen using the Spectrozyme-PL assay. As illustrated by figure 2.9 the activation rate for glu-plasminogen bound to immobilised r-α-enolase was 36-times greater than that for unbound glu-plasminogen after 20 minutes.

2.3.7 α2-Antiplasmin Competition Assay

The ability of r-α-enolase to compete with α2-antiplasmin for the lysine binding sites of glu-plasminogen was examined by coating microtitre plates with α2-antiplasmin (10 pM) and probing with biotinylated glu-plasminogen (100 nM) in the presence or absence of varying concentrations of r-α-enolase (0-3μM). R-α-enolase was able to specifically inhibit glu-plasminogen binding to α2-antiplasmin in a concentration-dependent manner (figure 2.10),

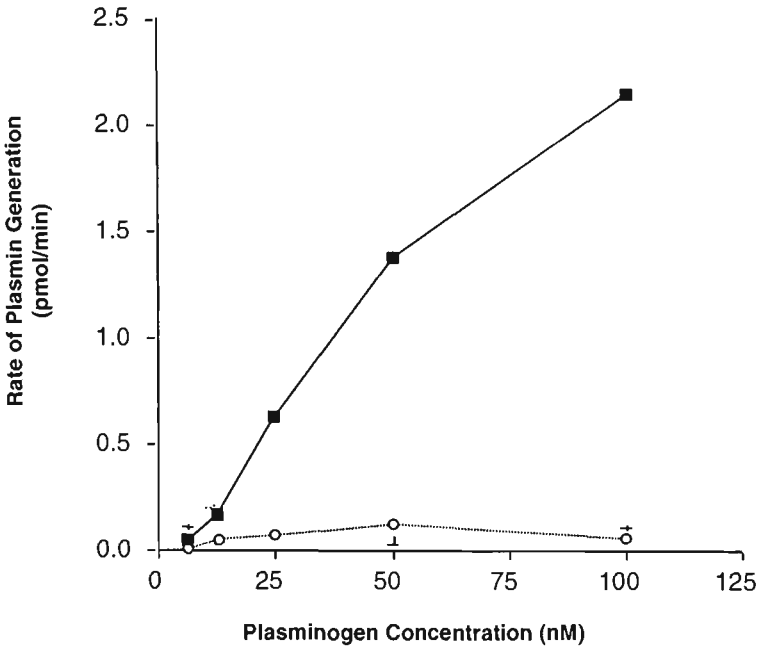

Figure 2.9: Activation of r-α-enolase bound plasminogen by uPA.

R-α-enolase (500 pM) was coated onto 96-well microtitre plates and incubated with varying concentrations of glu-plasminogen (0-100 nM). Active uPA was incubated with r-α-enolase bound plasminogen (■) or unbound (solution phase) glu-plasminogen (O) in the presence of Spectrozyme-PL substrate.

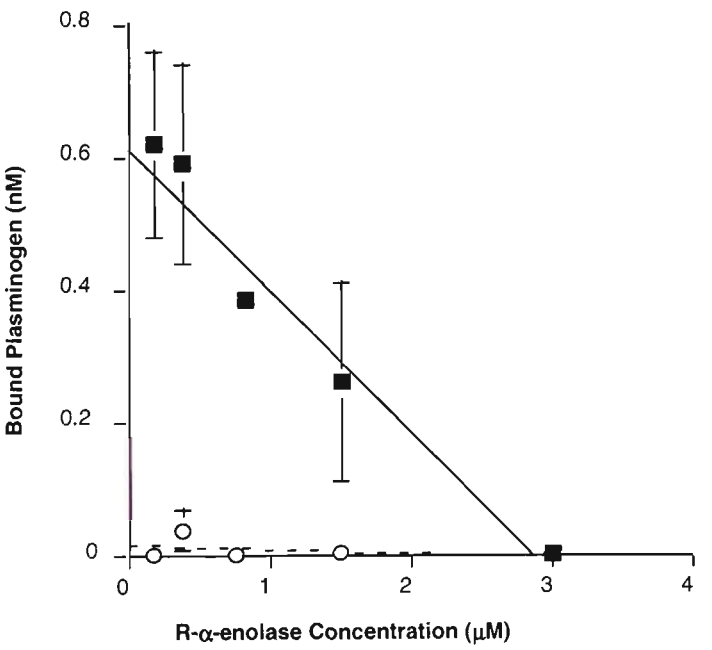

Figure 2.10: R-α-enolase/α2-antiplasmin plasminogen binding competition assay.

α2-antiplasmin (10 pM) was coated to the microtitre plate and then probed with 100 nM biotinylated glu-plasminogen in the presence of increasing concentrations of r-α-enolase (0-3 μM) plus (O) or minus (■) of 100 mM ε-ACA for 1 h at 37°C. The wells were subsequently incubated with avidin-HRP (1:2000 dilution) for 1 h at 37°C and colour developed.

with an IC_{50} = 1.4 μM. At a concentration of 3 μM, r-α-enolase completely inhibited glu-plasminogen from binding α2-antiplasmin. Moreover, ε-ACA (100 mM) was able to completely inhibit glu-plasminogen binding α2-antiplasmin, suggesting that the interaction of α2-antiplasmin with glu-plasminogen was mediated by the lysine binding sites of glu-plasminogen. Thus, r-α-enolase prevents the lysine-dependent binding of α2-antiplasmin of binding to glu-plasminogen.

2.3.8 Refolding r-α-enolase

R-α-enolase was purified under denaturing conditions as previously described was refolded on the Ni-NTA column using an FPLC-mediated binary gradient to slowly dilute the concentration of urea in solution (figure 2.11A). The most prominent band that was eluted from the Ni-NTA column was a 42 kDa band that corresponded with the predicted molecular weight of r-α-enolase. The refolded r-α-enolase that eluted from the column was devoid of glycolytic activity however, it did retain its glu-plasminogen binding capacity (figure 2.11B).

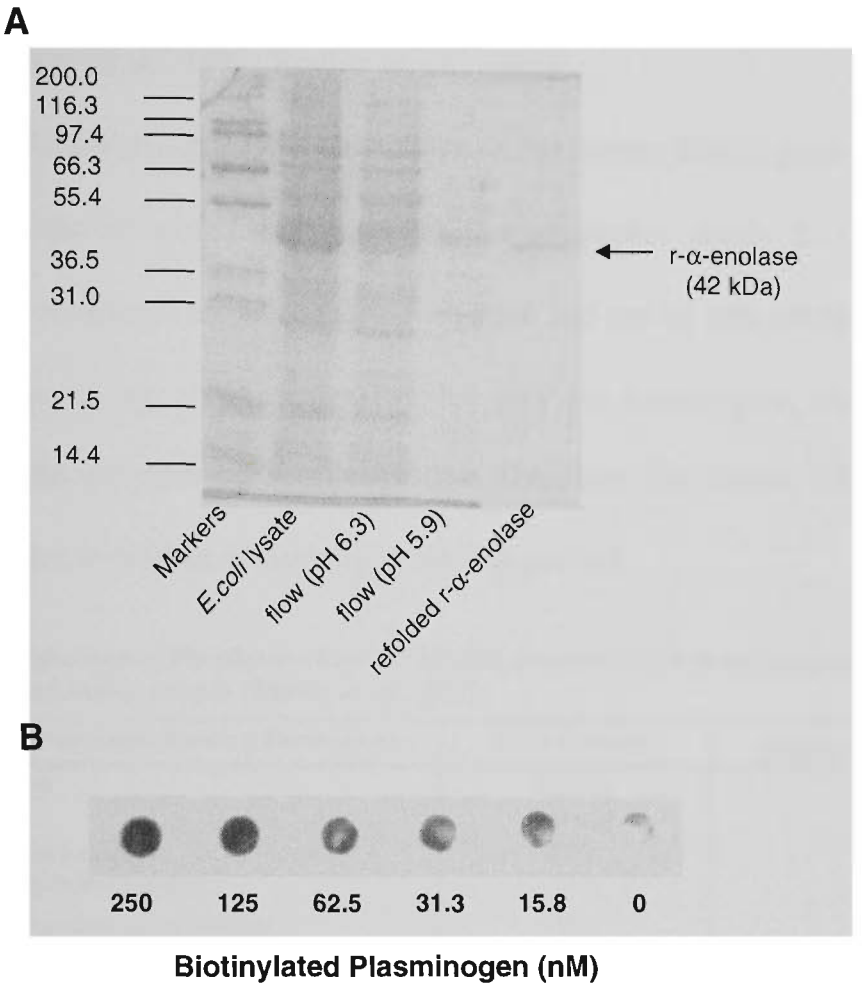

Figure 2.11: Purification of refolded r-α-enolase.

(A) 12% SDS-PAGE of r-α-enolase purified under denaturing conditions and refolded while remaining bound to the Ni-NTA column. R-α-enolase was refolded on the column by gradually decreasing the urea concentration from 6 M down to 1 M urea. The r-α-enolase was eluted from the column with PBS containing 250 mM imidazole-HCl. (B) Biotinylated glu-plasminogen ligand dot blot. Refolded r-α-enolase (5 μg/ml) was adsorbed to PVDF membrane for 1 hour. The membrane was washed three times and probed with various concentrations of biotinylated glu-plasminogen for 1 hour, washed, probed with avidin-HRP for 1 hour and washed. The membrane was finally developed by ECL.

2.4 Discussion

Several key components of the plasminogen activation cascade, including urokinase plasminogen activator (uPA) have been localised to the surface of numerous non-malignant and malignant cell types. Urokinase binds to its cell-surface receptor (uPAR) with high affinity (Ellis and Dano, 1991). In contrast, glu-plasminogen binds with low affinity to the cell-surface, although there are large numbers of plasminogen binding sites per cell. Several

cell-surface plasminogen binding protein candidates have been identified, one of which is a 54 kDa ERM (Miles *et. al.,* 1991; Redlitz *et. al.,* 1995).

This chapter describes the expression of the human ENO1 gene product (r-α-enolase) and characterises its glu-plasminogen binding properties (table 2.1). Comparison of the relevant physiochemical properties of r-α-enolase and the 54 kDa ERM plasminogen binding protein (Redlitz *et. al.,* 1995) suggested that they are homologous with respect to their C-terminal glu-plasminogen binding properties. Therefore, the human ENO1 gene encoded an authentic human α-enolase plasminogen binding protein.

Table 2.1: A comparison of the physio-chemical binding properties of r-α-enolase and the 54 kDa α-enolase-like plasminogen binding protein (Redlitz et. al., 1995).

α-Enolase:Plasminogen Binding Parameters	R-α-Enolase	Enolase-like molecule[1]
Molecular Weight	42 kDa	54 kDa
$K_{d(plasminogen)}$	1.9 μM	1.4 μM
ε-ACA-dependent binding	94%	95%
Benzamidine-dependent binding	14%	N/D[3]
C-Terminal Lysine-dependent binding	Yes	Yes
Increased Activation Rate	Yes (uPA)	Yes (tPA)
α-Enolase IC_{50} of α2-antiplasmin Plg Binding	1.4 μM	1.2-2.4 μM

[1]Taken from Redlitz *et.al.* 1995. [2] N/D Not Determined.

R-α-enolase had a predicted molecular weight of 42 kDa, whereas the molecular weight of wild-type human α-enolase is 46 kDa (Shimizu et. al., 1983). The difference of 4 kDa was due to the N-terminal truncation of the human ENO1 cDNA during construction of the pQE-30ENO1 expression plasmid. This was done so as to increase the efficiency of ENO1 expression by the QIAexpress system. The absence of this N-terminal fragment of α-enolase did not significantly affect the plasminogen binding capacity of r-α-enolase, suggesting that amino acid sequence Ser_1 to Glu_{47} was not involved in plasminogen binding interaction with α-enolase. The glycolytic activity of r-α-enolase was poor. One possible reason for the poor glycolytic activity of r-α-enolase is that the sequence Ser_1 to Glu_{47} is involved with the

glycolytic activity of the α-enolase enzyme, or alternatively, the r-α-enolase may be folded incorrectly around its active site.

Dimeric, non-denatured r-α-enolase was SDS stable in contrast to wild-type α-enolase. The 6xHis N-terminal Ni^{2+} affinity tag may have induced the SDS-stable dimerisation of r-α-enolase. Alternatively, N-terminal truncation of r-α-enolase may have altered the tertiary structure of r-α-enolase and hence the dimerisation properties of the molecule, thereby inducing an SDS-stable complex. Finally, the *E.coli* may have folded r-α-enolase incorrectly thereby inducing an SDS-stable dimer. Nevertheless, both dimeric and monomeric non-denatured r-α-enolase were able to bind glu-plasminogen. Moreover, 6M guanidine-HCl and 8M urea denatured r-α-enolase (monomeric only) were able to bind glu-plasminogen. These data suggest that neither the quaternary nor tertiary structures of r-α-enolase were important for the glu-plasminogen binding capacity of the molecule. Glu-plasminogen binds to monomeric wild-type α-enolase as determined by SDS-PAGE (Redlitz *et. al.*, 1995) hence, the glu-plasminogen binding capacity of wild-type α-enolase may also be independent of the tertiary structure of wild-type α-enolase. Removal of the C-terminal lysine residue from both r-α-enolase and wild-type α-enolase reduced the glu-plasminogen binding capacities of these molecules. Based on these structural data the glu-plasminogen binding capacity of α-enolase is dependent upon the primary structure of α-enolase. That is, while the presence of lysine at the C-terminal end of the molecule is important the tertiary and quaternary structures of the molecule is not. Finally the fact that r-α-enolase, expressed in bacteria, had glu-plasminogen binding properties comparable to wild-type α-enolase suggests that eukaryotic cell post-translational modification of α-enolase is not required for its glu-plasminogen binding activity.

In summary, this chapter demonstrated that human ENO1 gene encoded an authentic human α-enolase plasminogen binding protein. A major advantage of utilising r-α-enolase in the further characterisation of α-enolase as a cellular plasminogen binding protein is that large quantities of purified r-α-enolase can be produced in a reproducible and cost effective manner. Furthermore, r-α-enolase has an N-terminal 6-His affinity tag and thus directed binding assays could be performed in which the orientation of r-α-enolase is known.

Chapter 3

Deconstructing the interaction of glu-plasminogen with its receptor α-enolase.

3.1 Introduction

As reviewed in sections 1.1.1.2 and 1.1.1.5 glu-plasminogen exhibits a lysine-dependent, closed, right-handed, spiral conformation (Tranqui *et. al.,* 1979). Removal of NTP from glu-plasminogen results in the formation of lys-plasminogen (86 kDa) which has a more open, U-shaped conformation than glu-plasminogen (Marshall *et. al.,* 1994; Weisel *et. al.,* 1994). Site-directed mutagenesis studies, which removed the lysine binding function of individual kringle domains, have demonstrated that the closed conformation of glu-plasminogen is maintained by the binding of multiple kringle LBS motifs with internal lysine residues (M^{C}Cance *et. al.,* 1995; Cockell *et. al.,* 1998) such as Lys_{50} and Lys_{62} located in the NTP of glu-plasminogen (Cockell *et. al.,* 1998). The presence of this intramolecular lysine binding network suggests the LBS motifs of glu-plasminogen are occupied by the internal lysine residues of the protein. Disruptions to the intramolecular lysine binding network of glu-plasminogen by exogenous lysine analogues induces an open conformation of glu-plasminogen. The interactions of lysine analogues at multiple sites within glu-plasminogen change the conformation of the protein from its closed to open state (Markus *et. al.,* 1978; Marcus *et. al.,* 1979). Marshall *et al.* (1994) suggested that glu-plasminogen can actually exist in three distinct conformations *in vitro*; α (closed), β (partially open), and γ (fully open).

End-point assays have been used (e.g. Chapter 2 and 5) to study the interaction of glu-plasminogen with both cells and cellular plasminogen receptor molecules and have determined binding parameters such as low affinity and high binding capacity (in the case of

intact cells). However, detailed kinetic analysis of the binding of glu-plasminogen to its receptor has not been determined. Thus, BIACORE technology was used to characterise, in real-time, the relationship between binding and conformational switching of glu-plasminogen using the r-α-enolase as a plasminogen receptor model. Furthermore, fluorescence spectroscopy was used to confirm α-enolase-induced conformational changes in glu-plasminogen observed by BIACORE analysis. Kinetic analysis of the binding of lys-plasminogen to α-enolase was also examined since lys-plasminogen has a more open conformation than glu-plasminogen (Marshall *et. al.*, 1994; Weisel *et. al.*, 1994). Thus, by comparing the binding kinetics between the two plasminogen forms additional insights may be gained into the binding mechanism of glu-plasminogen.

The results presented in this chapter provide for the first time direct evidence that the binding of glu-plasminogen to α-enolase involves a multivalent, competition binding reaction that is associated with a conformational change in glu-plasminogen. The implications of these data will be discussed in terms of the activation of glu-plasminogen.

3.2 Material and Methods

3.2.1 BIACORE Analysis

All BIACORE experiments were performed by M. Lackmann at the Ludwig Institute for medical research (Melbourne). The binding of glu-plasminogen to r-α-enolase was analysed on the BIACORE optical biosensor (Pharmacia Biosensor, Sweden) using either α-enolase-$(His)_6$-derivatised CM 5 or NTA-modified sensor chips. Covalent immobilisation of α-enolase onto N-hydroxysuccinimide (NHS, 0.05 M)/N-hydroxysuccinimide-N-ethyl-N'-(diethylaminopropyl)carbodiimide (0.2 M) - activated sensor chips. Prior to analysis, plasminogen was buffer exchanged (Phast-desalting column, 100 x 3.2 mM, Amersham-

Pharmacia) into BIACORE running buffer (10 mM Hepes, 150 mM NaCl, 1.4 mM EDTA, 0.005% Tween-20, pH 7.4) and adjusted to a concentration of 10 μM. From this stock solution dilutions were prepared for BIACORE experiments. The affinity surface was regenerated between subsequent sample injections of plasminogen with 35μl of a desorption buffer (3M $MgCl_2$, 0.075 M Hepes/NaOH, 25% ethylene glycol, pH 7.2) followed by two washes with BIACORE running buffer. Alpha-enolase $(His)_6$ (15 μl, 200 nM) was also bound onto a NTA-modified sensor chip (BIACORE AB, Sweden) which had been charged with an injection of 25 μl $NiCl_2$ (0.5 mM) in modified running buffer containing 50 μM EDTA. In these experiments the chip surface was regenerated after sample application by sequential 15 μl-injections of 6M guanidine-hydrochloride, 50 mM EDTA, 20 mM Tris, pH 8.6 and of 0.35 M EDTA, 0.15M NaCl, 0.005% Tween 20, pH 8.2. To minimise differences in the α-enolase concentration on the chip surface, injection of both $NiCl_2$ and α-enolase $(His)_6$ from the same $NiCl_2$ and α-enolase stock solutions were used for all experiments.

3.2.2 Analysis of BIACORE sensorgram data.

A minimum of six data sets corresponding to glu-plasminogen binding reactions at concentrations between 4 μM and 63.5 nM were analysed. Fitting the sensorgram data to the algorithms of reaction schemes 3.1 and 3.2 involved analysing the individual association or dissociation phases of each binding reaction using the BIAevaluation software, version 3.0. The global fit modeling of reaction schemes 3.3-3.5 involved the simultaneous fitting of the association and dissociation phases of the individual binding reactions.

The χ^2-statistics test was used to determine whether a particular reaction scheme (i.e. expected outcome) was a good mathematical representation of the plasminogen binding sensorgram data (observed binding data).

Models that displayed small χ^2 values and which did not vary by a large magnitude over the range of plasminogen concentrations examined, were deemed to be a good mathematical representation of the sensorgram data. Furthermore, deviations of the sensorgram data from the expected reaction scheme model had to be small and random since

Reaction scheme 3.1: $A+B \leftrightarrow AB$: a simple 1:1 Langmuir kinetic.

Reaction scheme 3.2: $A_i+B_i \leftrightarrow A_iB_i$: a two or more component kinetic, without knowledge of the concentrations of individual components, only dissociation rates can be estimated

Reaction scheme 3.3: $A+B \leftrightarrow AB \leftrightarrow A^*B$: the two-state reaction/conformational change model: Analyte (A = plasminogen) binds to ligand (B = α-enolase). Complex AB changes to A*B which cannot dissociate directly to A+B.

Reaction scheme 3.4: $A+B1 \leftrightarrow AB1$;

$A+B2 \leftrightarrow AB2$: heterogeneous ligand (B = α-enolase), parallel interactions: one analyte (A = plasminogen) binds independently to two binding sites (B1, B2). Two independent sets of rate constants, k_{a1}, k_{d1} and k_{a2}, k_{d2}

Reaction scheme 3.5: $A+B \leftrightarrow AB$;

$AB+B \leftrightarrow AB2$: Bivalent analyte A (plasminogen) binds to a monovalent ligand B (α-enolase)

the magnitude of these residuals demonstrates the quality of the sensorgram data and are another measure of how well the expected reaction scheme fits the observed binding data (O'Shannessy *et. al.*, 1993).

3.2.3 Intrinsic Fluorescence Spectroscopy

All fluorescence experiments were performed using a F4500 Fluorimeter (Hitachi) with a slit width of 5 nm at room temperature. Plasminogen species (1 μM) in 50 mM Tris-HCl (pH 7.0), 100 mM NaCl were excited at 280 nm and the intrinsic fluorescence emission spectra were measured from 300-450 nm.

3.3 Results

3.3.1 Dissociation kinetics of glu- and lys-plasminogen from immobilised α-enolase.

This study describes for the first time the dissociation kinetics of glu- and lys-plasminogen from sensor chip-immobilised α-enolase. The BIACORE sensorgrams (binding curves) obtained from the binding of increasing concentrations of glu- and lys-plasminogen to NHS-immobilised α-enolase are shown in figure 3.1. A qualitative assessment of the

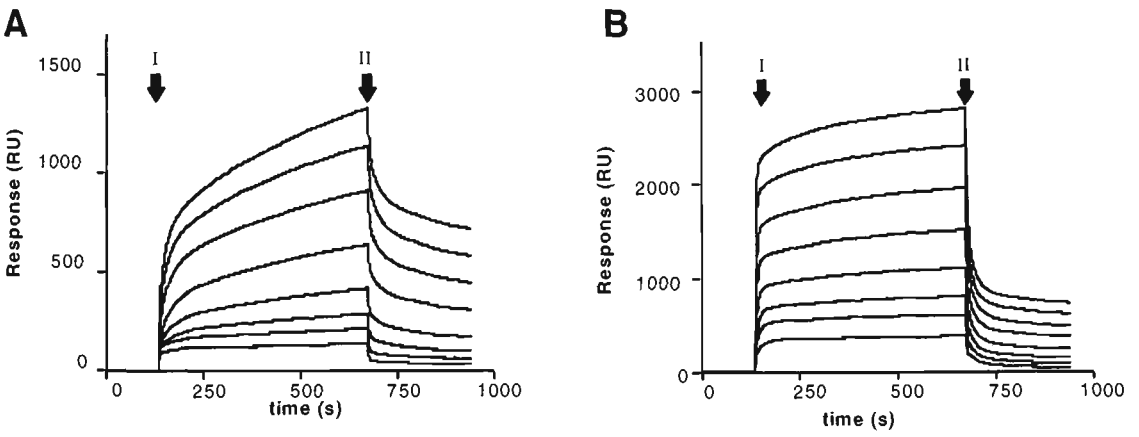

Figure 3.1: BIACORE analysis of glu- and lys-plasminogen binding to NHS-immobilised α-enolase.

(A) Samples containing increasing concentrations of glu-plasminogen in BIACORE running buffer were injected onto a sensor chip which had been derivatised with immobilised α-enolase using NHS/EDC chemistry. The decreasing responses reflect samples containing 4, 3, 2, 1, 0.5, 0.25, 0.125, 0.0625 μM glu-plasminogen. Arrows marked with I and II indicate the start and end of the sample injection (start of association and dissociation phases), respectively. **(B)** The response curves for the interaction of varying concentrations of lys-plasminogen with the sensorchip-immobilised α-enolase are shown. Coupling of α-enolase and concentration of lys-plasminogen were according to the conditions described in A.

sensorgrams reveals obvious differences in the interaction of glu-plasminogen (figure 3.1A) and lys-plasminogen (figure 3.1B) binding with α- enolase. This was most evident in the contours of their steady-state phases (between arrow I and II) which did not result in equilibrium binding under these experimental conditions. Importantly, the contour of the sensorgrams suggested a heterogeneous interaction for both glu-and lys-plasminogen with α-enolase.

By comparing the fit of the experimental data to different kinetic algorithms available in the BIAevaluation software we set out to establish a suitable kinetic model for the observed binding data (Table 1). The linear one-to-one pseudo-first order model (Reaction scheme 3.1) did not fit the data, as indicated by a pronounced decrease in the "goodness of fit", χ^2 (increase of χ^2 above the "signal noise" of 3 – 6 RU) with increasing analyte concentrations (Table 3.1) and by a marked deviation of the "offset values" (deviation of the fitted response at $t = \infty$) from zero (not shown). Similarly, the concentration-dependent increase of χ^2 values in the fit of association data to the single component model confirms that the interaction cannot be described adequately by this model. In contrast, the multiple component dissociation model (Reaction scheme 3.2), which does not require knowledge of the analyte concentration, produced an accurate fit to the dissociation data and yielded χ^2 values close to zero at all concentrations of glu- and lys-plasminogen analysed (Table 3.1). Statistical comparison of the fits (χ^2 values) to the two models by F-test confirmed that the multiple component model is a better mathematical representation of our data, since F-test values of 1.0 were obtained for both glu- and lys-plasminogen binding. Both glu- and lys-plasminogen had similar, apparently rapid (low affinity)

Table 3.1: Kinetic analysis of the interaction of glu-plasminogen with α-enolase from the BIACORE sensorgrams .

Experiment	Kinetic Constants Single Component $(A+B\leftrightarrow AB)^{1}$.		Kinetic Constants Two Component $(A_i+B_i\leftrightarrow A_iB_i)^{2}$.	
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	k_{d1} (s^{-1})	k_{d2}(s^{-1})
Glu-Plasminogen (NHS^{3}·)	**$2.8x\ 10^{4}$**	**$3.2x10^{-3}$**	**$4.7x10^{-2}$**	**$1.6x10^{-3}$**
(goodness of fit (χ2))	(0.054 - 7.51)	(0.073 - 88.2)	(0.0203 - 0.060)	
Lys-Plasminogen (NHS^{3}·)	**$3.4x10^{5}$**	**$4.0x10^{-3}$**	**$4.0x10^{-2}$**	**$6.3x10^{-4}$**
(goodness of fit (χ2))	(0.088 - 7.35)	(3.29 - 83.2)	(0.0389 - 0.112)	
Glu-Plasminogen (NTA^{4}·)	**$4.1x10^{4}$**	**$1.4x10^{-3}$**	**$6.1x10^{-2}$**	**$1.0x10^{-3}$**
(goodness of fit (χ2))	(0.05 - 15.8)	(0.102 - 13.7)	(0.015 - 0.061)	

1. Association and dissociation rate constants derived from progress data of the BIACORE sensorgrams using linear kinetic models included in the BIAevaluation software, $R = R_{eq}(1-e^{-(k_aC+k_d)(t-t_0)})$, were R_{eq} is the steady state response (R) which is not necessarily reached in the sensorgram, C = molar concentration, t_0 = start time of sensorgram. The time interval for the analysis of the association rate constants were 138 s - 165 s (figures 3.1 and 2). The time interval for the analysis of the dissociation rate constants were 695 s - 75 0 s (figures 3.1 and 3.2).

2. Dissociation rate constants for two parallel dissociation reactions were estimated according to $R = R_1e^{-k_{d1}(t-t_0)}+(R_0-R_1)e^{-k_{d2}(t-t_0)}$, where R_1 and R_0-R_1 are the contributions to R_0 from component 1 and 2, respectively (figures 3.1 and 3.2).

3. Experiments on a CM-5 sensor chip derivatised with enolase using NHS/EDC chemistry to yield an increase in the baseline response of 3900 RU. Following each cycle the chip surface was regenerated by desorption of non-covalently bound protein with a $MgCl_2$/ethylene glycol buffer (see methods) (figure 3.1).

4. Experiments on a Ni-NTA-derivatised sensor chip carrying α-enolase coupled by its N-terminal 6-his affinity tag to yield an increase of 1800 RU above baseline. Following each cycle α-enolase together with the Ni^{2+} was stripped from the NTA-chip with GnHCl/EDTA (see methods) and the chip re-charged with Ni^{2+} and loaded with α-enolase (figure 3.2).

dissociation rates (k_{d1} = 4.7×10^{-2} s^{-1} and 4.0×10^{-2} s^{-1}, respectively) from NHS-immobilised α-enolase (Table 3.1). While the slow dissociation rate (high-affinity) component of lys-plasminogen (k_{d2}= 6.3×10^{-4} s^{-1}) was two-fold lower compared to that of glu-plasminogen (k_{d2}= 1.6×10^{-3} s^{-1}) (Table 3.1), it was clear that both glu- and lys-plasminogen interacted with α-enolase via at least two distinct binding sites. In addition, ε-ACA competitively disrupted the binding of glu- and lys-plasminogen to NHS-immobilised α-enolase (M. Lackmann *pers. communication*), confirming the role of lysine in these interactions (Chapter 2; Redlitz *et. al.*, 1995). Similar binding experiments were also performed using a sensor chip with substantially less NHS-immobilised α-enolase (1.3 ng/mm^2 versus 4.3 ng/mm^2 immobilised). Whilst the overall responses were reduced, very similar binding characteristics were observed (M. Lackmann *pers. communication*). This indicated that the kinetics were not effected by mass-transfer limitations during the interactions of glu- and lys-plasminogen on the more densely derivatised sensor chip. Furthermore, to confirm that the heterogeneous kinetics were characteristic of the interaction between glu- and lys-plasminogen and α-enolase and/or its components, and not due to the random orientation of α-enolase on the chip, α-enolase was also immobilised in a defined orientation via its N-terminal 6-Histidine tag. Under these experimental conditions, very similar binding characteristics were observed for both glu- and lys-plasminogen binding, and a representative set of glu-plasminogen sensorgrams are shown in figure 3.2. Again, the multiple component reaction model best approximated the dissociation phase of this interaction (Table 3.1) and the estimated dissociation rate constants (k_{d1} = 6.1×10^{-2} s^{-1} and k_{d2} = 1×10^{-3} s^{-1}) were comparable to those obtained for the binding of glu-plasminogen to NHS-immobilised α-enolase (Table 3.1).

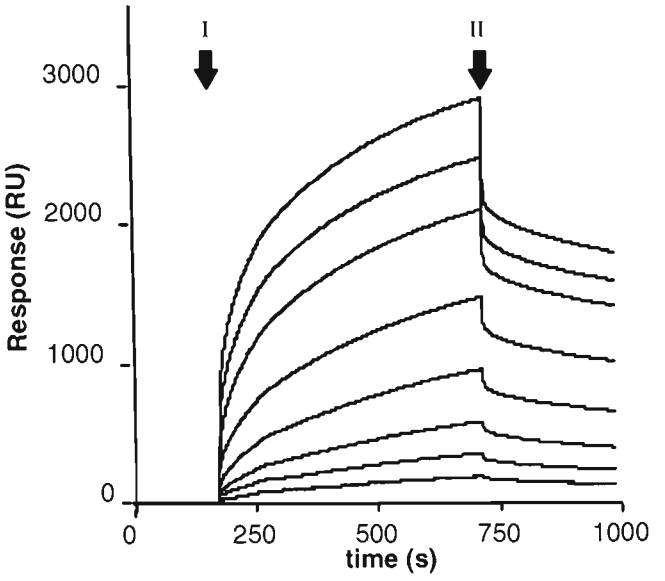

Figure 3.2: BIACORE analysis of glu-plasminogen binding to Ni^{2+}-NTA immobilised α-enolase.

α-Enolase was immobilised onto a Ni^{2+}-NTA chip by its N-terminal 6-his affinity tag. The decreasing responses reflect samples containing 4, 3, 2, 1, 0.5, 0.25, 0.125 and 0.0625 μM glu-plasminogen. Following each injection of glu-plasminogen, bound ligand, Ni^{2+} and α-enolase were stripped from the NI^{2+}-NTA-chip and the affinity surface regenerated by the injection of Ni^{2+} and α-enolase prior to the injection of glu-plasminogen (see methods). The sensorgrams presented only show the progress data for the injection of glu-plasminogen (arrow I) and its dissociation (arrow II) from the α-enolase surface.

3.3.2 Two-state conformational change reaction model

We performed global fitting of the BIACORE binding data to further define the interaction mechanisms. Firstly, a global fitting program was used to identify a suitable kinetic model which would provide a convincing representation of the BIACORE data. In this process, the entire binding data describing the plasminogen/α-enolase interaction at different plasminogen concentrations were fitted to a number of kinetic algorithms, including linear one-to-one interaction and several complex interaction models. The selected kinetic model(s) was then used to simultaneously analyse the kinetic parameters of the association and dissociation phases of the individual plasminogen/α-enolase binding curves (Tables 3.2 and 3.3). The probability of an appropriate fit of the experimental data to the selected model are also shown in Tables 3.2 and 3.3. The two-state reaction/conformational change model

(Reaction scheme 3.3) yielded a good fit within the range of glu-plasminogen concentrations examined (χ^2 : 0.9 – 2.2; Table 3.2). As suggested by the contour of the binding curves, the interaction of glu-plasminogen with α-enolase was characterised by an initial apparent fast dissociation rate ($k_{d1} = 2.64 \pm 0.6 \times 10^{-2} s^{-1}$) and an association rate which was increasing with decreasing glu-plasminogen concentrations ($k_{a1} = 4.3 - 19.3 \times 10^{3} M^{-1} s^{-1}$). In contrast, the second phase of the interaction appeared to proceed in a largely concentration-independent manner with markedly lower on and off rates ($k_{a2} = 3.8 \times 10^{-3} M^{-1} s^{-1}$ and $k_{d2} = 7.4 \pm 0.27 \times 10^{-4} s^{-1}$). Overall, the dissociation rate constants derived by fitting the binding data to this model are within close range of the rate constants estimated by the non-linear regression analysis discussed earlier (Table 3.1). An alternative model, assuming a heterogeneous population of sensor chip-immobilised α-enolase binding glu-plasminogen in parallel reactions (Reaction scheme 3.4) yielded a markedly decreased fit with χ^2 values between 1.9–141 (Table 3.2) and does not adequately represent the data. Similarly, global analysis with an algorithm which adjusts for mass transfer limitations of the analyte (plasminogen) binding to a sensor surface of high ligand (i.e. α-enolase) concentration gave a poor fit to the data (data not shown), confirming that these effects did not influence the kinetics of the interaction. Analysis indicates that the binding of glu-plasminogen to α-enolase involves a conformational change in glu-plasminogen, which in itself effects the binding in a second phase of the interaction. In contrast, the generally large χ^2 values obtained for the lys-plasminogen binding data indicated that none of the global fitting models currently available fitted the data over the entire lys-plasminogen concentration range used (Table 3.3). As stated above, the two-component model is determined independently of the association

phases and provided a strong fit of the lys-plasminogen dissociation data (Table 3.1). Since the dissociation rate constants shown in Table 3.3

Table 3.2: Kinetic parameters of the glu-plasminogen/α-enolase interaction.

Concentration μM	K_d μM	k_{a1} $M^{-1}s^{-1}$ 10^3	k_{d1} s^{-1} 10^{-2}	k_{a2} $M^{-1}s^{-1}$ 10^{-3}	k_{d2} s^{-1} 10^{-4}	χ^2 Two-State-Reaction[1]	χ^2 Heterogeneous Ligand[1]
4	1.5	4.3	3.5	4.5	8.0	2.0	141
3	1.4	5.0	3.26	4.3	9.3	2.2	22.1
2	1.05	6.3	2.8	4.1	9.7	2.0	26.5
1	0.6	8.6	2.2	4.2	9.6	1.1	23.9
0.5	1.5	11.9	2.0	2.8	2.6	0.9	4.94
0.25	0.2	19.3	2.1	2.6	5	0.9	1.9
Mean[2]	1.0	-	2.64	3.8	7.4		

1) The binding data were fitted to the two kinetic algorithms of the BIAevaluation 3.0 software (see methods) which yielded the closest match of experimental data with the chosen model. The listed kinetic constants are derived in each case only from the candidate model with the closest fit (lowest χ^2 value).
2) Mean values were estimated only for those parameters which did not indicate a concentration-dependent change.

Table 3.3: Kinetic parameters of the lys-plasminogen/α-enolase interaction.

Concentration μM	K_d μM	k_{a1} $M^{-1}s^{-1}$ 10^4	k_{d1} s^{-1} 10^{-2}	k_{a2} $M^{-1}s^{-1}$	k_{d2} s^{-1} 10^{-3}	χ^2 Two-State-Reaction[1]	χ^2 Bivalent Analyte[1]
4	3.2	2.0	7.6	3.3×10^{-3}	2.7	48.3	121
3	2.6	2.4	7.6	3.2×10^{-3}	2.6	24.7	475
2	0.8	2.6	6.6	2.6×10^{-3}	0.9	15.8	112
1	0.9	3.8	5.5	1.8×10^{-3}	1.2	41.1	1240
0.5	K_{D1} :3.5	1.9	6.6	6.6×10^{-7}	1.1	363	8.79
0.25	K_{D1} :1.9	2.7	5.3	7.8×10^{-7}	1.0	320	3.42
0.125	K_{D1} :0.8	5.9	4.5	19.5×10^{-7}	1.2	193	1.96
Mean[2]	1.0	-	6.2	-	1.5		

1) The binding data were fitted to the two kinetic algorithms of the BIAevaluation 3.0 software (see methods) which yielded the closest match of experimental data with the chosen model. The listed kinetic constants are derived in each case only from the candidate model with the closest fit (lowest χ^2 value).
2) Mean values were estimated only for those parameters which did not indicate a concentration-dependent change.

were similar to those obtained by the two-component model for the same range of lys-plasminogen concentrations (Table 3.1), it is possible that the association phase is the component of the binding interaction that cannot be deconvoluted by any of the global fitting algorithms currently available, at least under the physiological-like conditions used in these

experiments. In any case, it is clear that the binding mechanisms between glu- and lys-plasminogen are distinct.

3.3.3 *Intrinsic fluorescence spectroscopy*

To confirm that binding with α-enolase induces a conformational change in glu-plasminogen, the intrinsic fluorescence spectra of glu-plasminogen in the presence and absence of α-enolase were determined (figure 3.3). These were compared to the intrinsic fluorescence spectra of glu-plasminogen in the presence and absence of the lysine analogue ε-ACA, previously shown to "open" glu-plasminogen resulting in an increase in its intrinsic fluorescence Christensen and Molgaard, 1992). In addition, the spectra of plasmin and lys-plasminogen, having a more open conformation than glu-plasminogen (Marshall *et. al.,* 1994; Weisel *et. al.,* 1994), are shown for comparison.

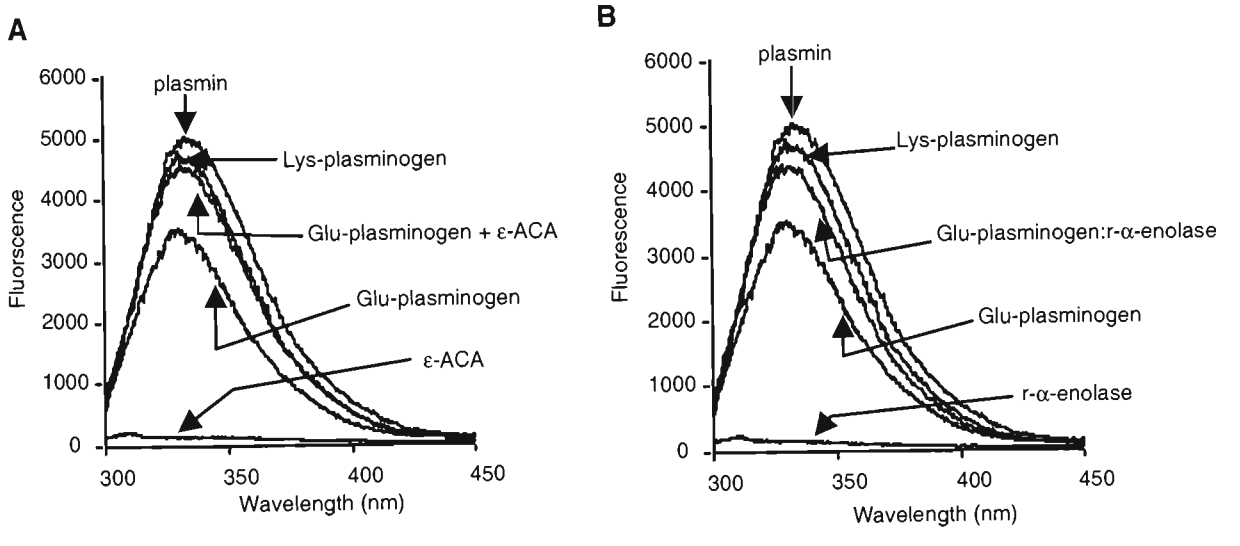

Figure 3.3: Comparison of the intrinsic fluorescence spectra of glu-plasminogen in the absence and presence of ε-ACA or α-enolase to those of plasmin and lys-plasminogen.

(A) The fluorescence spectra of 1 μM plasmin, glu- and lys-plasminogen, glu-plasminogen + 100 mM ε-ACA and 100 mM ε-ACA control were obtained after excitation at 280 nm. **(B)** The fluorescence spectra of 1 μM plasmin, glu- and lys-plasminogen, glu-plasminogen + 190 nM α-enolase and 190 nM α-enolase control were obtained after excitation at 280 nm. The intrinsic fluorescence of both ε-ACA (117) and α-enolase (146) at the concentrations used for these experiments were negligible. Higher concentrations of α-enolase were not be used as this alone was associated with increased intrinsic fluorescence.

Plasmin and lys-plasminogen had comparable relative intrinsic fluorescence emission maxima at 330 nm (4850 and 4694, respectively). In contrast, the relative intrinsic fluorescence maximum for glu-plasminogen was significantly lower (3480). Addition of ε-ACA to glu-plasminogen (figure 3.3A) resulted in a significant increase in the relative intrinsic fluorescence of glu-plasminogen (ΔF=1019) to one which was comparable to the relative fluorescence obtained for the same concentration of either lys-plasminogen or plasmin. The addition of α-enolase to glu-plasminogen (figure 3.3B) also induced a significant increase in the relative intrinsic fluorescence of glu-plasminogen (ΔF= 785) which was suggestive of a conformational change.

3.4 Discussion

This study aimed to define the binding kinetics and associated conformational changes glu-plasminogen undergoes during an interaction with its cellular receptor, α-enolase. Experiments were performed using BIACORE technology under physiological-like conditions and analysed using BIAevaluation software (version 3.0). Identical experiments were performed with lys-plasminogen for direct comparison with glu-plasminogen on the basis that lys-plasminogen has a more open conformation and is more readily activated. This feature of lys-plasminogen that has been exploited by numerous studies that have examined conformational status and activation rate of the different plasminogens, both in solution and in the presence of binding moieties (reviewed in Markus, 1996).

The simplest reaction model that described the lysine-dependent dissociation phases of both the glu- and lys-plasminogen sensorgrams is the non-linear multiple component dissociation model (Reaction scheme 3.2). Glu-plasminogen has also been shown to bind to a lysine-derivitised sensor chip surface with multiple dissociation reactions (Warkentin *et. al.*, 1998). Such multiple dissociation reactions were not surprising since both plasminogen

species have up to four LBS potentially available for binding. The contours of the sensograms, being obviously different, were suggestive of different binding mechanisms, as might also have been expected from the different initial conformations of glu- and lys-plasminogen. While global fitting of the glu-plasminogen binding data could be accurately described by the two state reaction/conformational change model (Reaction scheme 3.3) over the entire concentration range of glu-plasminogen used (which spanned its physiological concentration of 2 μM), the lys-plasminogen binding data could not. In fact, lys-plasminogen could not be adequately described by any of the currently available global fitting algorithms, at least under the physiological conditions we used to compare glu- and lys-plasminogen binding. Since lys-plasminogen is not detectable *in vivo* (Holvoet *et. al.,* 1985; and certainly not at the micromolar concentrations of lys-plasminogen used in these experiments) it is concluded, that under the conditions used in this study, that lys-plasminogen may not be as useful as initially anticipated for comparing to the binding kinetics of glu-plasminogen. For these reasons the remainder of this discussion deals with the mechanism of glu-plasminogen binding.

The two-state reaction/conformational change model that describes the binding of glu-plasminogen to α-enolase suggests that the initial conformation of glu-plasminogen is altered after it has been bound by α-enolase. Furthermore, both α-enolase and ε-ACA produced an increase in the intrinsic fluorescence of glu-plasminogen. This reflected a conformational change to a more open form and validates the two-state reaction/conformational change model as a description of the binding of glu-plasminogen to α-enolase.

The association phase kinetics of glu-plasminogen binding to α-enolase suggested by the two-state conformational change model are complex. The relative magnitudes of k_{a1} and k_{a2} for this interaction differ by approximately 6 orders of magnitude, indicating that the

majority of the observed binding of glu-plasminogen to α-enolase is due to k_{a1}. However, k_{a1}, unlike k_{a2}, is not constant, but increases with decreasing glu-plasminogen concentration. A decreasing concentration of glu-plasminogen can also be viewed as a relative increase in the α-enolase abundance which is paralleled by an increase in k_{a1}. The acceleration in the initial glu-plasminogen to α-enolase binding rate as the relative concentration of α-enolase increases would indicate a competition reaction. This suggests that during the initial interaction an α-enolase lysine residue competes with and displaces a lysine residue of glu-plasminogen from one of its LBS motifs. This would disrupt the closed, lysine-dependent conformation of glu-plasminogen and induce a conformational change in the zymogen revealing a second LBS (figure 3.4). The second binding event (k_{a2}) may stabilise this new conformation of glu-plasminogen. Hence, the interaction of glu-plasminogen with α-enolase would be mediated by at least two binding events; an initial competition reaction that results in a lysine-dependent conformational change to glu-plasminogen, followed by a second binding event that stabilises the new conformation (figure 3.4). In agreement with this notion, the lysine-dependent binding of α2-antiplasmin may also involve two or more LBS motifs of plasmin(ogen) (Christensen *et. al.,* 1995). The actual lysine residues of α-enolase(s) involved in the interaction with glu-plasminogen remain to be determined. There are several possible candidates, of which the C-terminal lysine is likely to play a role (Chapter 2; Redlitz *et. al.,* 1995)

Taken together, these data suggest that at circulating levels of 2 μM, glu-plasminogen will bind to cell surface receptors (such as α-enolase) and be converted to an open

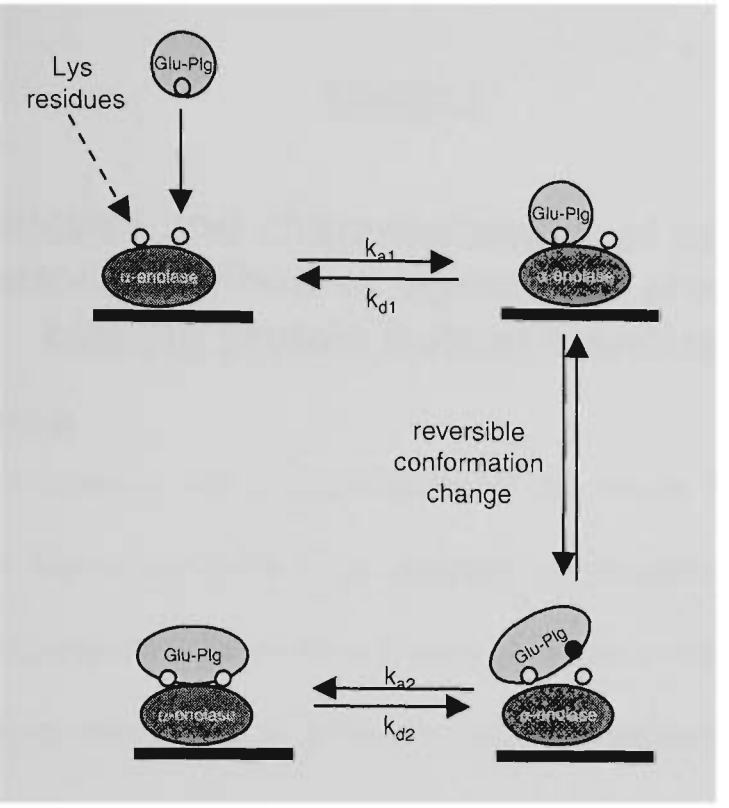

Figure 3.4: A model of glu-plasminogen interaction with α-enolase.

A lysine residue from an α-enolase molecule (O) competes with and dissociates an internal lysine residue of glu-plasminogen that initially occupied a LBS (●) of glu-plasminogen (k_{a1}/k_{d1}). This induces a lysine-dependent conformational change in glu-plasminogen making another LBS (●) available which can interact with a second lysine residue from α-enolase (k_{a2}/k_{d2}). This stabilises an open conformation of glu-plasminogen that may be more susceptible to activation. Note, the second binding interaction of glu-plasminogen may be mediated either by lysine residues within a single α-enolase molecule, or by lysine residues on separate α-enolase molecules. However, for clarity only the former possibility is shown here.

conformation. This finding agrees with the well documented observation that the binding of small lysine analogues to glu-plasminogen changes its conformation which in turn enhances its activation rate by the plasminogen activators (see Section 1.1.1.6). In addition, it has been shown that the lysine-dependent binding of glu-plasminogen to fibrin, cell and isolated receptors including α-enolase (Table 1.2; Chapter 2; Redlitz *et. al.,* 1995), facilitates an increase in the rate of glu-plasminogen activation. Since the interaction between glu-plasminogen and α-enolase is associated with a change to a more open conformation of glu-plasminogen, this suggests that the physiologically important role of cell surfaces plasminogen binding proteins is to present glu-plasminogen in a form that is essential for its efficient activation by the plasminogen activators.

Chapter 4

The production and characterisation of polyclonal and monoclonal antibodies against the plasminogen binding protein human α-enolase.

4.1 Introduction

The expression and characterisation of the human ENO1 gene product confirmed that human α-enolase is an authentic glu-plasminogen binding protein (Chapter 2). In mammals there are three dimeric glycolytic enolase isoforms: neuron-specific γ-enolase, muscle specific β-enolase and the ubiquitous α-enolase (Shimizu *et. al.,* 1983). Since α-enolase is a glycolytic enzyme it is hypothesised that it will be highly conserved. As such human α-enolase may have high sequence similarity to mouse α-enolase. Several groups have successfully generated monospecific rabbit polyclonal antibodies against human α-enolase (Redlitz *et. al.,* 1995; Shimizu *et. al.,* 1983; Suzuki *et. al.,* 1980). However, monoclonal antibodies that have been raised against α-enolase using classical techniques display low reactivity against α-enolase (Redlitz *et. al.,* 1995).

Several human pathological conditions such as discoid lupus erythematosus result in the production of autoantibodies specific for α-enolase (Adamus *et. al.,* 1998; Orth *et. al.,* 1998; Gitlits *et. al.,* 1997; Akisawa *et. al.,* 1997; Moodie *et. al.,* 1993; Walter *et. al.,* 1995) suggesting that these anti-α-enolase autoantibodies may have a broad disease association (Gitlits *et. al.,* 1997).

The New Zealand Black (NZB) mouse strain, which develops a lupus-like pathology, appear to be genetically predisposed to the spontaneous development of a wide range of autoantibodies (Payelle-Brogard *et. al.,* 1998; Theophilopoulos and Dixon, 1985). The reasons for the production of autoantibodies by the mice are

complex and remain ambiguous, however the NZB immune system changes its characteristics over time. For example, B lymphocytes from aging NZB mice replace their normal IgM and IgG cell surface receptors with monoclonal IgM cell surface receptors (Bhoopalam *et. al.,* 1973). Furthermore, NZB mice bone marrow becomes increasing deficient in small pre-B and pro-B/early pre-B cell subpopulations possibly due to the production of anti-IL-7 monoclonal autoantibodies which interfere with the development of the B-cell lineage (Merchant *et. al.,* 1996). Nevertheless, the NZB mouse system has been used for the successful production of monoclonal antibodies against several weak antigens (Frosch *et. al.,* 1985; Adamus *et. al.,* 1991; Jacquemart *et. al.,* 1988; Ballard *et. al.,* 1983; Sanai *et. al.,* 1988; Doberson *et. al.,* 1985) and may represent a suitable system for the production of monoclonal antibodies against human α-enolase.

The aim of this chapter was to generate anti-α-enolase antibodies that could neutralise the plasminogen binding capacity of α-enolase and to determine the fraction of plasminogen binding α-enolase that was due to cell-surface α-enolase. This chapter describes the successful production and limited characterisation of both a rabbit polyclonal antibody sera and a panel of mouse monoclonal antibodies that recognise human α-enolase.

4.2 Materials and Methods

4.2.1 Animals and Materials

New Zealand Black (NZB) mice were purchased from Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia). The Balb/C mice were bred in house at the University of Wollongong. New Zealand White rabbits were purchased from the University of New South Wales (Sydney, Australia).

Polyethylene glycol (4000), Hybrigrow, HAT, HT media and complete protease inhibitor tablets were all purchased from Boehringer Mannheim (Heidleberg, Germany). Iscoves modified Delbeccos medium (IMDM) was purchased from Life Technologies (Sydney, Australia). Monoclonal antibody isotyping (Isostrip) kit, Freunds complete and incomplete adjuvants were purchased from Sigma. Goat anti-mouse alkaline phosphatase (GAM-AP), goat anti-rabbit-HRP (GAR-HRP), goat anti-rabbit alkaline phosphatase (GAR-AP) Goat anti-mouse IgM-HRP conjugate were all purchased from Calbiochem/Novabiochem Sydney, NSW Australia. The DAKO LSAB+ Kit was from DAKO Corporation (CA, USA).

4.2.2 Preparation of Antigen

Human r-α-enolase was expressed and purified as described previously in chapter 2.

4.2.3 Preparation of R-α-enolase inclusion bodies.

The r-α–enolase inclusion bodies were prepared according to the method of Harlow and Lane (1988) and stored at –20°C until required. Briefly, *E.coli*JM109ENO1 cells were centrifuged at 10,000g for 10 min at 4°C. The pellet (10% w/v) was resuspended in ice cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, protease inhibitor cocktail [Boehringer Meinheim]) and incubated with lysozyme (1 mg/ml) for 20 min at room temperature with gentle agitation. The lysate was centrifuged for 10 min at 10,000g at 4°C and the pelleted spheroplasts resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA and 1% Na-deoxycholate for 10 min on ice. The lysate was supplemented with 8 mM $MgCl_2$ incubated with 10 μg/ml DNase1 for 20 min at room temperature with occasional agitation of until the viscosity of the solution decreased. The lysate was pelleted at 7000 g for 10 min at 4°C and washed once with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA and 0.1% nonidet (NP-40). The inclusion bodies were

centrifuged at 14,000 g for 10 min at 4°C and washed twice with ice cold PBS. The purity of r-α-enolase in the inclusion body preparation was determined by SDS-PAGE.

4.2.4 Preparation of Fibroblast Conditioned Medium (FCM).

The mouse fibrosarcoma cell line L929 was cultured for 48 hours in 50 ml IMDM medium supplemented with 10% heat inactivated FCS, 100 μg/ml streptomycin and 100 IU/ml penicillin G at 378C in a 10% CO_2, 85% humidified incubator. At confluence the L929 cell culture supernatant was centrifuged at 200g and sterile filtered. The FCM was stored at –20°C until required.

4.2.5 Preparation of Hybridomas

Both female NZB and BALB/C mice (6-8 weeks old) were injected intraperitoneally with 100 μg of r-α-enolase inclusion bodies emulsified 1:1 with Freunds complete adjuvant (250 μl/mouse). Four weeks later the mice were given a second intraperitoneal injection of 50 μg of r-α-enolase inclusion bodies emulsified with 1:1 Freunds incomplete adjuvant (250 μl/mouse). Sera were examined for r-α-enolase antibodies by ELISA (see below). Two weeks later all mice were hyperimmunised by intraperitoneal injection with 50 μg of r-α-enolase inclusion bodies in sterile PBS (250 μl/mouse) a total of three times in seven days. The sera were examined for r-α-enolase antibodies by ELISA (see below). Four weeks after the final hyperimmunisation injection the best responder were injected i.v. with 50 μg of soluble r-α-enolase in 200 μl of sterile PBS for three consecutive days prior to splenectomy. Splenocytes were fused to the non-Ig producing Sp2/Ag8 mouse myeloma cell line according to the method described in Harlow and Lane (1988). R-α-enolase antibody secreting hybrids were identified using the ELISA assay described

below and cloned out by limiting dilution. The resulting monoclonal antibodies were isotyped using the Isostrip isotyping kit (Sigma).

4.2.6 Antibody Screening ELISA

Hybridomas and subsequent clones were screened for antibody production by ELISA. Briefly, 5 µg/ml of r-α-enolase (100 µl/well) was coated onto 96 well plates in 0.1 M Na-carbonate (pH 9.6) and incubated overnight at 4°C. The antigen solution was removed and the wells washed three times with PiNT wash buffer (50 mM Na-phosphate (pH 7.2), 150 mM NaCl, 0.05% Tween-80). Each well was blocked by incubation with 100 µl of wash buffer supplemented with 1% BSA (PiNT/BSA) for 1 hour at 37°C. The blocking buffer was removed and the wells washed once with PiNT and either 100 µl aliquots of immune (1:100 dilution) or pre-immune (1:100 dilution) sera in PiNT/BSA or 100 µl of hybridoma tissue culture supernatant were added to the wells and incubated for 1 hour at 37°C. The micro-titre plates were washed three times with PiNT and then 100 µl of either GAR-HRP or GAM-HRP solution diluted to 1:5000 with PiNT/BSA was added as the secondary antibody. After incubation for 1 hour at 37°C the wells were washed four times with PiNT and the plates were developed and processed as described above.

4.2.7 Monoclonal IgM storage and Western Blotting.

Hybridoma supernatant was centrifuged at 10,000g for 30 min and filtered through 0.22 µm membrane. The pH was adjusted to 8.2. Glycerol (30% v/v) and thirmersal (0.02% w/v) were added. The supernatant was stored at 4°C until required. Anti-r-α-enolase IgM monoclonal antibodies were concentrated and partially purified by ultrafiltration using centriprep 500 concentrators. These supernatants were also stored at 4°C until required.

Denatured r-α-enolase or whole cell lysates were fractionated by 12% SDS-PAGE under reducing conditions and transferred to PVDF membrane. The transferred membranes containing r-α-enolase were washed for 5 min with TBS and then blocked overnight at 4°C with TBS supplemented with 1% BSA (TBS/BSA). The blots were incubated for 1 hour at room temperature with neat hybridoma supernatants containing 0.05% Tween-20 (pH 7.5; 10 ml). The blots were washed three times for 5 min each with TBS supplemented with 0.05% Tween-20 (TBST) and re-blocked for 15 min with TBST/BSA. The blots were incubated with a 1:2000 dilution of GAM-HRP (IgM-specific), washed twice for 5 min with TBST and once with TBS and developed using ECL.

4.2.8 Anti-human α–enolase rabbit polyclonal antibody production.

A male New Zealand White (NZW) rabbit (14 weeks old) was immunised s.c. with 1 mg of antigen in sterile PBS emulsified 1:1 with complete Freunds adjuvant (1 ml) between the scapulae (100 µl/site). Twenty one days post-immunisation the rabbit was boosted with another subcutaneous injection of 1 mg of antigen in sterile PBS emulsified 1:1 with incomplete Freunds adjuvant (1 ml) (100 µl/site). Immune serum from the rabbit was taken, 28 days after the initial immunisation. The rabbit was boosted with 1 mg of antigen in 1 ml sterile physiological saline 49 days post-immunisation and one week later was bled and the serum collected and processed.

4.2.9 Polyclonal Antibody immunopurification.

A Ni-NTA-sepharose column was loaded with r-α-enolase as described for the non-denaturing purification of r-α-enolase. However, the r-α-enolase was not eluted but rather the column was equilibrated with 10 column volumes of equilibration buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl) and the r-α-enolase anti-serum (2 ml) was applied to the column by gravity flow. The column was then washed with 5

column volumes of equilibration buffer, followed by 5 column volumes of wash buffer (50 mM Tris-HCl (pH 7.4), 2M NaCl). The anti-r-α-enolase polyclonal antibody was eluted by incubating the column with 1 column volume of 4M $MgCl_2$ (pH 7.2) for 15 minutes, and the column was allowed to flow under gravity. The anti-r-α-enolase polyclonal antibody was collected and dialysed against ddH2O for 1 hour and then against PBS for 24 hours with three changes of buffer.

4.2.10 Human cancer cell culture.

The human breast cancer cell lines (MCF-7, MDA-MB-231, T-47D) were gifts from Prof. R. Sutherland (Garvan Institute of Medical Research, Sydney, NSW, Australia). The human colon cancer cell lines (HCT116, KM12-SM) were obtained from the John Curtin School of Medical Research. U937 cells were purchased from ATCC. The human breast and colon cancer cell lines were all routinely cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum. The insulin-dependent T-47D cell line was cultured in the above media containing insulin (0.2 IU/ml). U937 cells were routinely grown and passaged in DMEM supplemented with 10% heat inactivated fetal calf serum. All the cells were incubated in a humidified incubator at 37°C with a 5% CO_2: 95% air atmosphere.

4.2.11 Whole cell lysate preparation

Confluent cells were washed with sterile ice-cold phosphate-buffered saline (PBS) and harvested by scraping with a rubber policeman. The cells were incubated with cell lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM $MgCl_2$, 10 % glycerol, 5 mM EDTA, 1 mM PMSF, 0.1% (v/v) Triton X-100, 0.5% (w/v) Na-deoxycholate] at 4°C for 20 min. The resulting lysates were collected and centrifuged at 14000 g for 15 min at 4°C and the supernatant stored at -20°C until required.

4.2.12 Immunohistochemistry

Cells were grown on glass coverslips for 48 without changing the growth media. The cells were washed three times with PBS at room temperature. The cells were then fixed by incubation with PBS supplemented with 1% gluteraldehyde for 1 hour at room temperature and then washed once with PBS. The fixed cells were permeabilised by incubation with PBS supplemented with 0.2% TX-100 for two minutes and then washed with PBS. Endogenous peroxidases were inactivated with 3% H_2O_2 for 5 minutes. The cells were incubated with 10% human serum for 30 minutes and washed with PBS. Rabbit anti-r-α-enolase polyclonal antibody diluted 1:50 with PBS/10 % human serum (200 µl) for 1 hour at room temperature. The cells were washed twice with PBS and incubated with the secondary reagents from the DAKO LSAB+ Kit according to the manufactures instructions. After rinsing with PBS the cells were viewed using a video camera (National Panasonic) attached to an inverted compound microscope (Leica, Germany). Colour images of the cells (original magnification x400 unless otherwise stated) were captured by a Power PC (Macintosh 8500/20) using Apple Video Player software (Macintosh).

4.2.13 Flow Cytometry

Subconfluent, adherent cells that had been in culture for 48 h without a change of media, were harvested by rinsing flasks twice with cold PBS (pH 7.2) and then detaching with 5 mM EDTA/PBS at 37°C for 5 min. Cells were incubated with various concentrations of either an irrelevant isotype control (i.e. rabbit anti-DNP), or with rabbit anti-α-enolase polyclonal antibodies for 30 min in ice-cold RPMI/0.1% BSA, washed three times with 200 µl ice-cold RPMI/0.1% BSA. The cells were then incubated with FITC-conjugated anti-rabbit IgG (1:50 dilution of stock in ice-cold RPMI/0.1% BSA) for 30 min on ice in the dark. The cells were washed again,

resuspended in 200 µl of binding buffer containing 5 µg/ml PI and the cells immediately analysed by dual-colour flow cytometry

4.3 Results

4.3.1 Antigen Preparation

Recombinant-α-enolase inclusion bodies were used as the antigen for all immunisations. Figure 4.1A the SDS-PAGE gel of the *E.coli*.JM109 inclusion body preparation shows two bands at approximately 45 kDa (full length r-α-enolase) and at approximately 33 kDa. Only the 42 kDa r-α-enolase band possessed glu-plasminogen binding activity as detected by a biotinylated glu-plasminogen ligand blot (figure 4.1B). The 42 kDa r-α-enolase band accounted for approximately 80% of the total protein concentration of the inclusion body preparation. Thus the effective dose r-α-enolase was approximately 40 µg/mouse and 800 µg/rabbit.

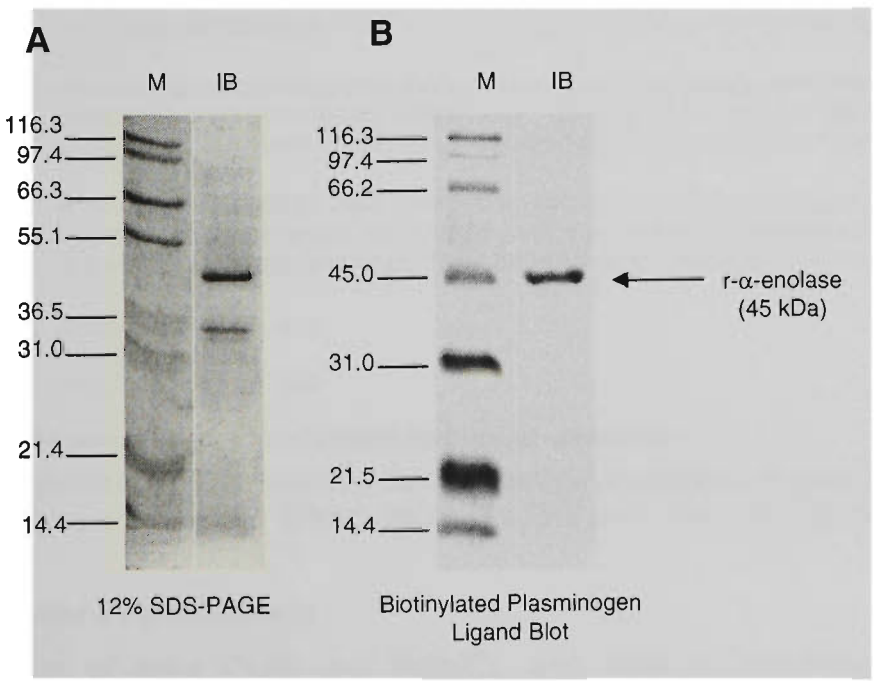

Figure 4.1: α-Enolase inclusion bodies.

(A) 12% reducing SDS-PAGE of r-α-enolase inclusion bodies isolated from *E.coli*.JM109pQE30ENO1. (B) Glu-plasminogen ligand blot of the inclusion body preparation probed with biotinylated glu-plasminogen. M: Molecular weight markers, IB: inclusion bodies.

Sequence alignment of human (SWISS-PROT: P06733) and mouse (SWISS-PROT: P17182) α-enolase demonstrated that these enzymes have 94% amino acid sequence identity and 98% amino acid sequence similarity (figure 4.2). Since human and mouse α-enolase have high sequence identity mice pre-disposed to the production of autoantibodies (i.e. the NZB mouse system) may generate a good humoral immune response after immunisation with recombinant human α-enolase.

```
Human: 1   SILKIHAREIFDSRGNPTVEVDLFTSKGLFRAAVPSGASTGIYEALELRDNDKTRYMGKG 60
           SIL+IHAREIFDSRGNPTVEVDL+T+KGLFRAAVPSGASTGIYEALELRDNDKTR+MGKG
Mouse: 1   SILRIHAREIFDSRGNPTVEVDLYTAKGLFRAAVPSGASTGIYEALELRDNDKTRFMGKG 60

Human: 61  VSKAVEHINKTIAPALVSKKLNVTEQEKIDKLMIEMDGTENKSKFGANAILGVSLAVCKA 120
           VS+AVEHINKTIAPALVSKK+NV EQEKIDKLMIEMDGTENKSKFGANAILGVSLAVCKA
Mouse: 61  VSQAVEHINKTIAPALVSKKVNVVEQEKIDKLMIEMDGTENKSKFGANAILGVSLAVCKA 120

Human: 121 GAVEKGVPLYRHIADLAGNSEVILPVPAFNVINGGSHAGNKLAMQEFMILPVGAANFREA 180
           GAVEKGVPLYRHIADLAGN EVILPVPAFNVINGGSHAGNKLAMQEFMILPVGA++FREA
Mouse: 121 GAVEKGVPLYRHIADLAGNPEVILPVPAFNVINGGSHAGNKLAMQEFMILPVGASSFREA 180

Human: 181 MRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILENKEGLELLKTAIGKAGYTDKVV 240
           MRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILENKE LELLKTAI KAGYTD+VV
Mouse: 181 MRIGAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILENKEALELLKTAIAKAGYTDQVV 240

Human: 241 IGMDVAASEFFRSGKYDLDFKSPDDPSRYISPDQLADLYKSFIKDYPVVSIEDPFDQDDW 300
           IGMDVAASEF+RSGKYDLDFKSPDDPSRYI+PDQLADLYKSF+++YPVVSIEDPFDQDDW
Mouse: 241 IGMDVAASEFYRSGKYDLDFKSPDDPSRYITPDQLADLYKSFVQNYPVVSIEDPFDQDDW 300

Human: 301 GAWQKFTASAGIQVVGDDLTVTNPKRIAKAVNEKSCNCLLLKVNQIGSVTESLQACKLAQ 360
           GAWQKFTASAGIQVVGDDLTVTNPKRIAKA +EKSCNCLLLKVNQIGSVTESLQACK AQ
Mouse: 301 GAWQKFTASAGIQVVGDDLTVTNPKRIAKAASEKSCNCLLLKVNQIGSVTESLQACKPAQ 360

Human: 361 ANGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIEEELGSKA 420
           +NGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQ+LRIEEELGSKA
Mouse: 361 SNGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQILRIEEELGSKA 420

Human: 421 KFAGRNFRNPLAK 433
           KFAGR+FRNPLAK
Mouse: 421 KFAGRSFRNPLAK 433
```

Figure 4.2: Sequence alignment of human and mouse α-enolases.

Sequence alignments were performed by the online automated alignment program BLAST2. (A) The sequence alignment of human (SWISS-PROT: P06733) and mouse (SWISS-PROT: P17182) α-enolase.

4.3.2 Immune Responsiveness

Two species of mice (NZB and Balb/C), with different pre-dispositions for the development autoimmune diseases, were immunised with r-α-enolase inclusion bodies to determine which mouse strain displayed the best immune response against the antigen after hyper-immunisation. As shown by figure 4.3A, the anti-r-α-enolase

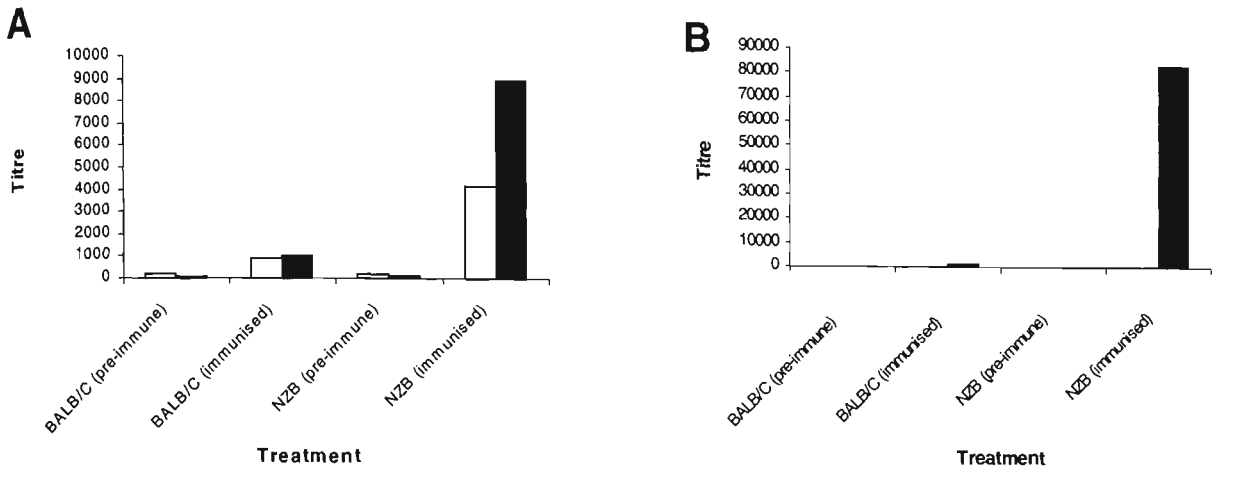

Figure 4.3: The immune response to r-α-enolase in BALB/C and NZB mice.

ELISA assays were used to measure the (A) IgM and (B) IgG immune responses on day 21 (□) and day 49 (■) from the BALB/C and NZB mice after intraperitoneal immunisation with r-α-enolase inclusion bodies. The plates were coated with 5 μg/ml of r-α-enolase and probed with different sera from the different strains of mice.

IgM sera raised in the NZB strain displayed a 4.8 fold higher titre for r-α-enolase than the BALB/C strain on day 21. The NZB anti-r-α-enolase IgM titre was 9 fold greater than the BALB/C titre on day 49. The anti-r-α-enolase IgG response was absent in both the NZB and BALB/C strains on day 21, however on day 49 the anti-r-α-enolase IgG titre from the NZB strain was 83 fold higher than the BALB/C anti-r-α-enolase titre (figure 4.3B). Thus, the monoclonal antibodies described below were produced using the splenocytes from a hyperimmunised NZB mouse.

A panel of three hybridomas that secrete anti-r-α-enolase monoclonal antibodies were derived from the splenocytes of a hyperimmunised NZB mouse. All of these antibodies had an IgM κ, μ isotype. The concentrations of monoclonal antibodies secreted into the supernatant by their respective hybridomas were estimated using a combination of 2-12% SDS-PAGE gradient gels and total protein concentrations of the supernatants and were approximately 100, 220, and 160 μg/ml for #1A6, #1A8 and #1C8 respectively.

4.3.3.Characterisation of the mouse anti-r-α–enolase IgM monoclonal antibodies.

Concentrated hybridoma supernatants containing the monoclonal antibodies #1A6, #1A8 and #1C8 were examined for cross-reactivity to different enolase antigens by ELISA (figure 4.4). All monoclonal antibodies were reactive against denatured r-α-enolase, refolded r-α-enolase and rabbit muscle enolase. However, monoclonal antibodies #1A6 and #1A8 and #1C8 displayed better reactivity against both rabbit muscle and denatured r-α-enolase compared to refolded r-α-enolase.

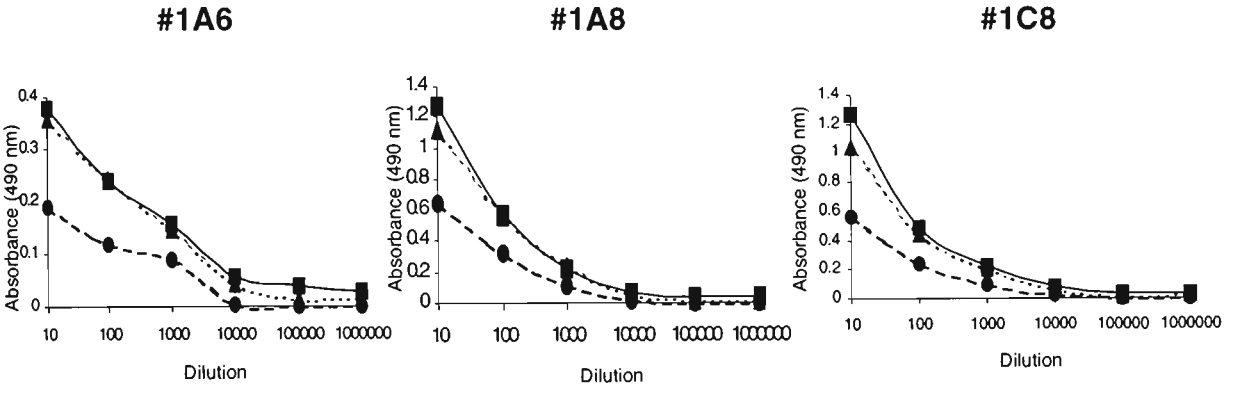

Figure 4.4: Cross reactivity of anti-r-α-enolase monoclonal antibodies against different samples of enolase.

Comparison of the cross reactivity of anti-r-α-enolase hybridoma supernatants containing monoclonal antibodies #1A6, #1A8 and #1C8. Increasing dilutions of supernatants were assayed by ELISA on denatured r-α-enolase (▲), rabbit muscle β-enolase (■) and refold r-α-enolase (●) adsorbed to the wells of microtitre plates.

Hybridoma supernatants were also examined for their reactivity against r-α-enolase by western blot analysis. In all cases the reactivity of the anti-α-enolase monoclonal antibodies against r-α-enolase by western blot was poor (figure 4.5). Nevertheless, neat hybridoma supernatant containing MAb #1A8 was the most efficient at detecting r-α-enolase by western blotting. In contrast to MAb #1A8, MAb #1C8 did not detect r-α-enolase by western blotting. Thus, these anti-α-enolase monoclonal antibodies may not represent sensitive probes for the detection of α-enolase antigen using a western blot format.

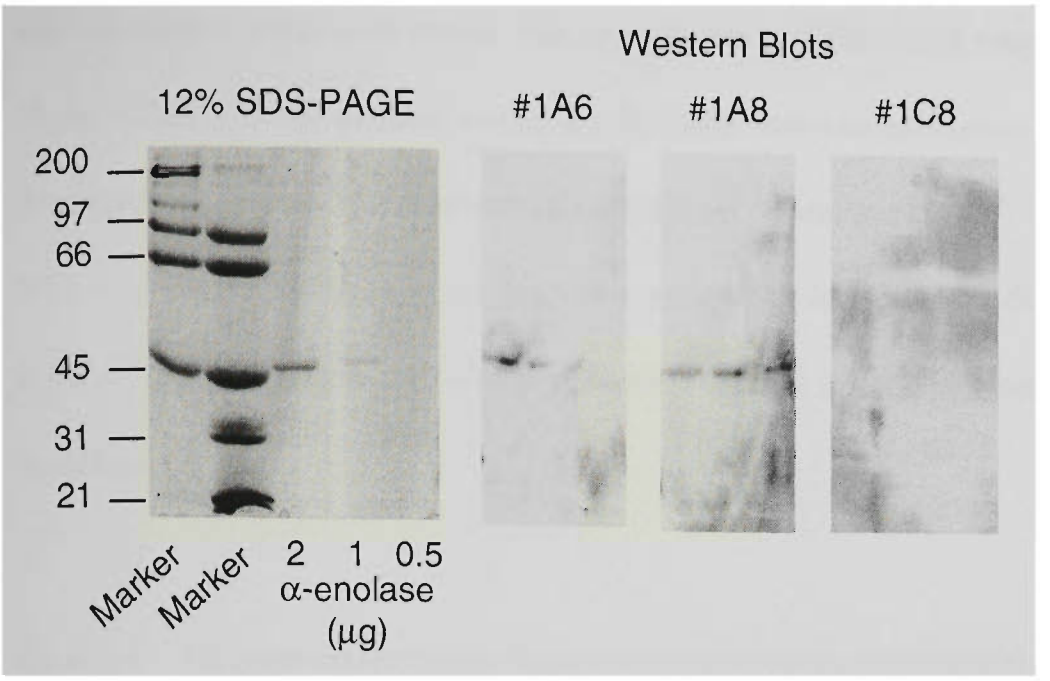

Figure 4.5: Western blot analysis of hybridoma supernatants reactivity against r-α-enolase.

Various amounts of r-α-enolase (2 μg, 1 μg and 0.5 μg) were fractionated by 12% SDS-PAGE (reducing conditions) transferred to PVDF membrane and probed with neat supernatant with the hybridoma supernatants. The blots were developed by ECL and all three blots were exposed simultaneously on the same autoradiography film.

4.3.4 Characterisation of the rabbit anti-r-α–enolase polyclonal antibodies.

Rabbit β-enolase has less sequence identity (85%) and similarity (91%) to human α-enolase than does mouse α-enolase (figure 4.6). Thus, the rabbit may represent a better system for the production of anti-α-enolase antibodies than the mouse.

The cross-reactivity of the anti-r-α-enolase rabbit day 28 post immunisation polyclonal sera raised against r-α-enolase inclusion bodies was examined by ELISA both before and after adsorption of the sera onto a Ni-NTA-r-α-enolase antigen column. The non-adsorbed anti-r-α-enolase rabbit polyclonal serum displayed high reactivity for r-α-enolase whilst not detecting rabbit muscle enolase at dilutions greater than 10^3 (figure 4.7A). For the adsorbed polyclonal serum, microtitre plates wells were coated with either non-denatured r-α-enolase, denatured r-α-enolase, rabbit muscle enolase or BSA (figure 4.7B). At dilutions greater than 10^3 the adsorbed

anti-r-α-enolase polyclonal serum was specific for non-denatured r-α-enolase only (figure 4.7B). The r-α-enolase adsorbed polyclonal sera was unreactive against non-denatured r-α-enolase after dilutions of greater than approximately 10^6. Thus, the Ni-NTA-r-α-enolase adsorbed anti-r-α-enolase polyclonal rabbit serum displayed high reactivity for r-α-enolase, and was also dilution specific for non-denatured human r-α-enolase.

```
Human: 4    KIHAREIFDSRGNPTVEVDLFTSKGLFRAAVPSGASTGIYEALELRDNDKTRYMGKGVSK 63
            KI AREI DSRGNPTVEVDL T+KG FRAAVPSGASTGIYEALELRD DK+RY+GKGV K
Rabbit:4    KIFAREILDSRGNPTVEVDLHTAKGRFRAAVPSGASTGIYEALELRDGDKSRYLGKGVLK 63

Human: 64   AVEHINKTIAPALVSKKLNVTEQEKIDKLMIEMDGTENKSKFGANAILGVSLAVCKAGAV 123
            AVEHINKT+ PAL+ KKL+V +QEK+DK MIE+DGTENKSKFGANAILGVSLAVCKAGA
Rabbit:64   AVEHINKTLGPALLEKKLSVVDQEKVDKFMIELDGTENKSKFGANAILGVSLAVCKAGAA 123

Human: 124  EKGVPLYRHIADLAGNSEVILPVPAFNVINGGSHAGNKLAMQEFMILPVGAANFREAMRI 183
            EKGVPLYRHIADLAGN +++LPVPAFNVINGGSHAGNKLAMQEFMILPVGA++FREAMRI
Rabbit:124  EKGVPLYRHIADLAGNHDLVLPVPAFNVINGGSHAGNKLAMQEFMILPVGASSFREAMRI 183

Human: 184  GAEVYHNLKNVIKEKYGKDATNVGDEGGFAPNILENKEGLELLKTAIGKAGYTDKVVIGM 243
            GAEVYH+LK VIK KYGKDATNVGDEGGFAPNILEN E LELLKTAI  AGY DKVVIGM
Rabbit:184  GAEVYHHLKGVIKAKYGKDATNVGDEGGFAPNILENNEALELLKTAIQAAGYPDKVVIGM 243

Human: 244  DVAASEFFRSGKYDLDFKSPDDPSRYISPDQLADLYKSFIKDYPVVSIEDPFDQDDWGAW 303
            DVAASEF R GKYDLDFKSPDDP+R+I+  +L +LYKSFIK+YPVVSIEDPFDQ DWGAW
Rabbit:244  DVAASEFHRDGKYDLDFKSPDDPARHITGQKLGELYKSFIKNYPVVSIEDPFDQGDWGAW 303

Human: 304  QKFTASAGIQVVGDDLTVTNPKRIAKAVNEKSCNCLLLKVNQIGSVTESLQACKLAQANG 363
             +F A   IQVVGDDLTVTNPKRIA+AV +K+CNCLLLKVNQIGSVTES+QACKLAQ+NG
Rabbit:304  SRFLAGVDIQVVGDDLTVTNPKRIAQAVEKKACNCLLLKVNQIGSVTESIQACKLAQSNG 363

Human: 364  WGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLLRIEEELGSKAKFA 423
            WGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQL+RIEE LG KA FA
Rabbit:364  WGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSERLAKYNQLMRIEEALGDKAVFA 423

Human: 424  GRNFRNPLAK 433
            GR FRNP AK
Rabbit:424  GRKFRNPKAK 433
```

Figure 4.6: Sequence alignment of human α-enolase and rabbit β-enolase.

Sequence alignments were performed by the online automated alignment program BLAST2. The sequence alignment of human α-enolase (SWISS-PROT: P06733) and rabbit β-enolase (SWISS-PROT: P25704).

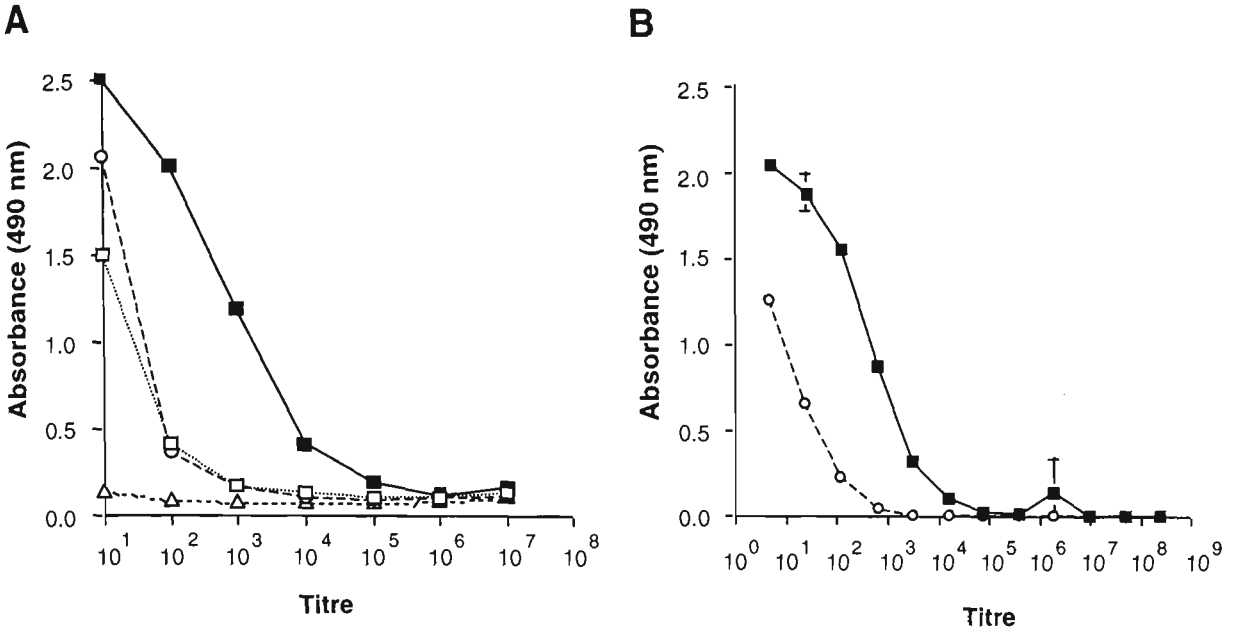

Figure 4.7: Cross reactivity of anti-r-α-enolase polyclonal sera against different samples of enolases.

(A) Comparison of the cross reactivity of rabbit anti-r-α-enolase polyclonal antiserum raised against r-α-enolase inclusion bodies. Increasing dilutions of antiserum were assayed by ELISA on non-denatured (fraction 14) r-α-enolase (■), denatured r-α-enolase (□), rabbit muscle enolase (○) and BSA (Δ) adsorbed to the wells of microtitre plates. (B) Comparison of the cross-reactivity of immunopurified rabbit anti-r-α-enolase polyclonal antibodies against r-α-enolase (■) and rabbit muscle β-enolase (○) adsorbed to the wells of the microtitre plate.

The affinity purified rabbit anti-r-α-enolase polyclonal antibody was able to detect a 47 kDa protein present in the whole cell lysate extracts of several human cancer cell lines that co-migrated with purified denatured r-α-enolase (figure 4.8). Moreover, the affinity purified rabbit anti-r-α-enolase polyclonal detected a single band from the mammalian cell line whole cell lysates, thereby suggesting that the polyclonal antibody was monospecific. All of the cell lines examined expressed similar levels of α-enolase. The higher molecular weight band in the r-α-enolase lane (lane 6) may represent aggregated r-α-enolase, possibly due to long term storage of the recombinant protein.

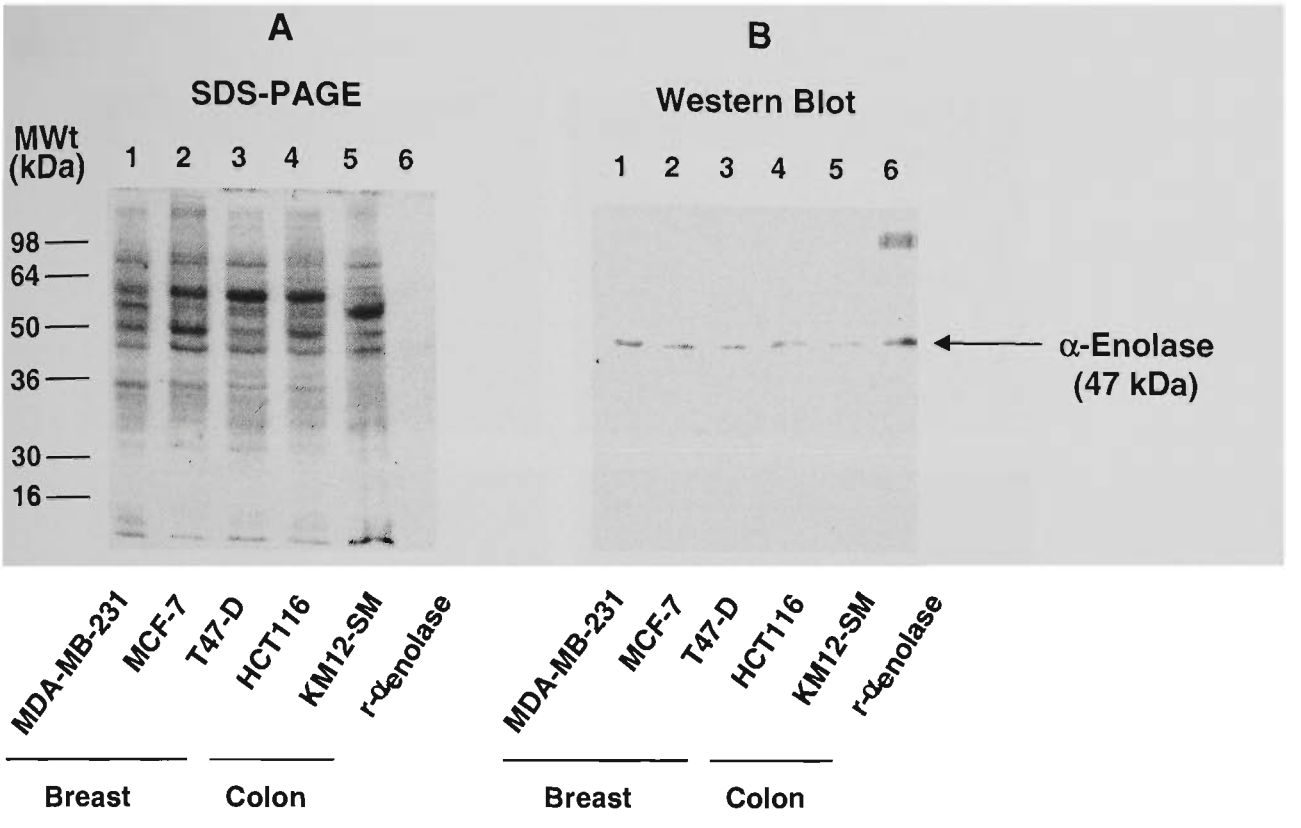

Figure 4.8: Western blot analysis of α-enolase from human breast and colon cancer cell lines.

Whole cell lysates (40 μg/lane) were fractionated by 12% SDS-PAGE under non-reducing conditions and stained with Coomassie brilliant blue R-250. (B) The Western blot was probed with immunopurified anti-α-enolase antibody (0.5 μg/ml) and GAR-HRP (1:2000) for 1 hour each and developed using ECL. Lane 6 contains purified denatured r-α-enolase (276 ng).

Anti-α-enolase polyclonal antibodies were also examined for their reactivity against gluteraldehyde fixed cells that were known to express α-enolase (figure 4.9). Both the permeabilised MDA-MB-231 and MCF-7 cells were stained when incubated with anti-α-enolase polyclonal antibodies. In contrast, the staining of the non-permeabilised cells with anti-α-enolase polyclonal antibodies was comparable to the anti-DNP isotype control antibody (figure 4.9). This suggested that α-enolase was not present on the surfaces of these human breast cancer cell lines where it can bind extracellular glu-plasminogen.

Flow cytometric analysis (figure 4.10) of unfixed MDA-MB-231 cells that had been incubated with either anti-α-enolase or anti-DNP polyclonal antibodies did not

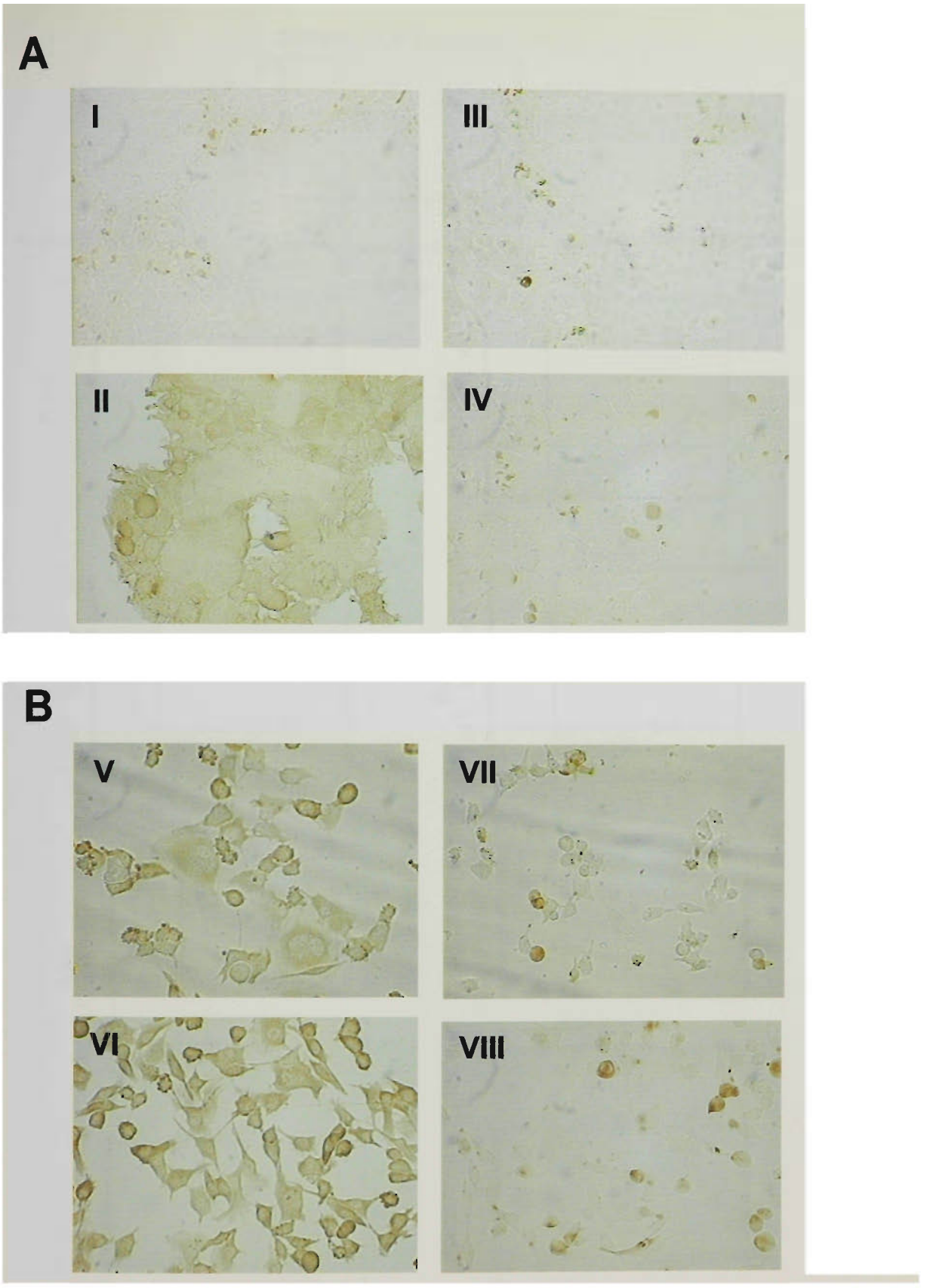

Figure 4.9: α-Enolase immunohistochemistry on permeabilised and non-permeabilised breast cancer cells.

(A) Immunohistochemistry of gluteraldehyde fixed, TX-100 permeabilised MCF-7 cells (I and II) and non-permeabilised MCF-7 cells (III and IV). Panels I and III probed with rabbit anti-DNP (isotype control) antibody and panels II and IV were probed with the rabbit anti-α-enolase polyclonal antibodies. (B) Immunohistochemistry of gluteraldehyde fixed, TX-100 permeabilised MDA-MB-231 cells (V and VI) and non-permeabilised MDA-MB-231 cells (VII and VIII). Panels V and VII probed with rabbit anti-DNP (isotype control) antibody and panels VI and VIII were probed with the rabbit anti-α-enolase polyclonal antibodies.

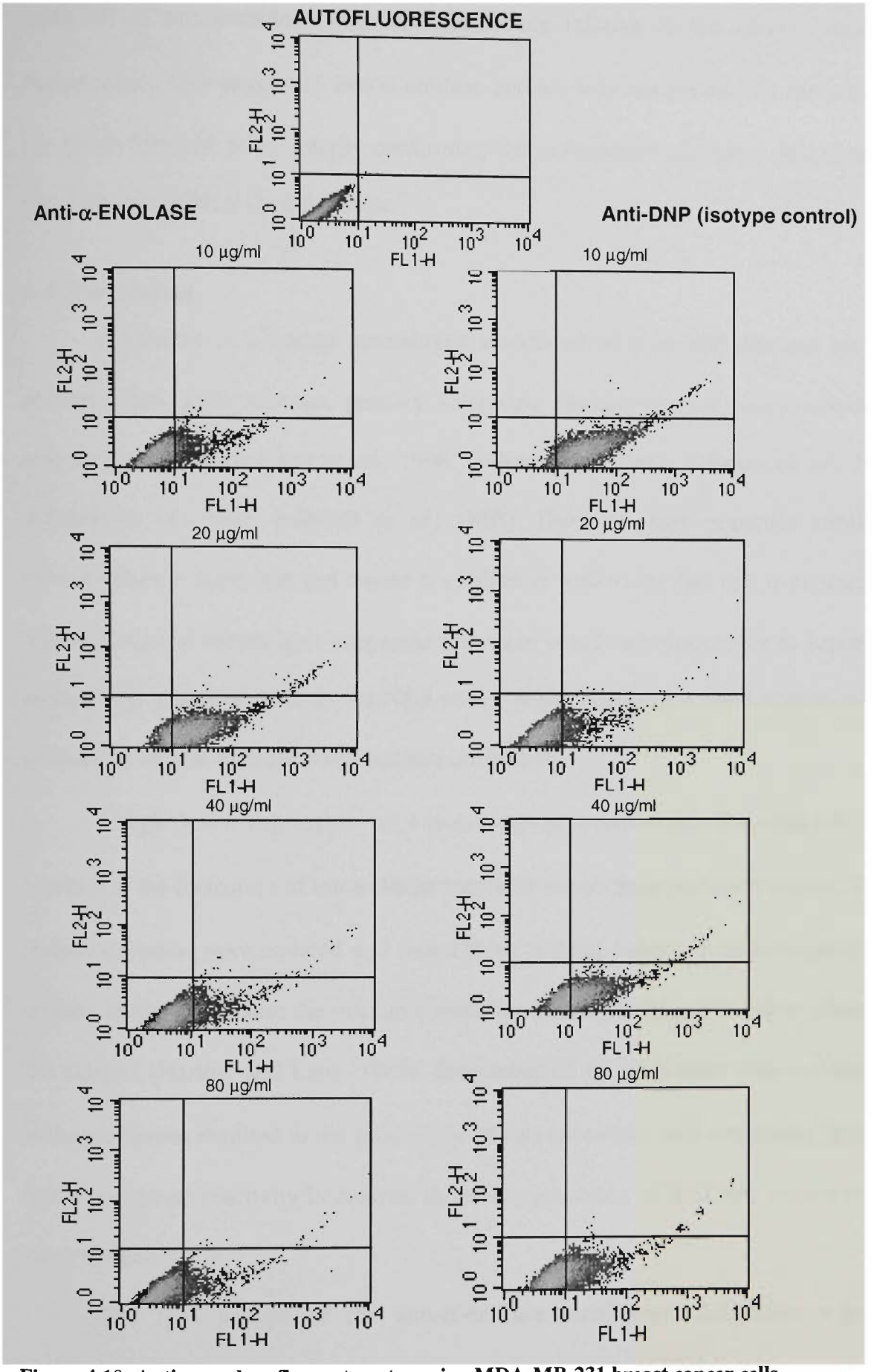

Figure 4.10: Anti-α-enolase flow cytometry using MDA-MB-231 breast cancer cells.

Density plots of MDA-MB-231 cells incubated with various concentrations of either rabbit anti-α-enolase polyclonal or rabbit anti-DNP polyclonal (isotype) antibodies. The cells were incubated just prior to flow cytometry analysis with the non-vital stain propidium iodide (PI).

result in an anti-α-enolase shift in fluorescence relative to the control antibody fluorescence. This suggested that α-enolase antigen was not present on the surfaces the MDA-MB-231 cells, thereby confirming the immunohistochemical data obtained using the gluteraldehyde fixed cells.

4.4 Discussion

α-Enolase is a human autoantigen associated with several diseases such as discoid lupus erythematosus, primary sclerosing cholangitis and cancer-associated retinopathy syndrome (Orth *et. al.,* 1998; Gitlits *et. al.,* 1997; Adamus *et. al.,* 1996; Adamus *et. al.,* 1993; Adamus *et. al.,* 1998). Thus, the high sequence similarity between human α-enolase and mouse α-enolase as well as the fact that α-enolase is a lupus-associated autoantigen suggested that mice which are susceptible to lupus-like autoimmune diseases, such as the NZB strain, might represent a good system for the production of anti-α-enolase monoclonal antibodies.

High level expression of r-α-enolase in transformed *E.coli*JM109 cells resulted in the formation of intracellular insoluble r-α-enolase inclusion bodies. These inclusion bodies were isolated and found to be a good source of immunogenic r-α-enolase probably because the insoluble inclusion bodies facilitated the slow release of the antigen (Harlow and Lane, 1988). Immunisation of NZB mice with r-α-enolase inclusion bodies resulted in the production of anti-α-enolase sera containing IgM and IgG of moderate reactivity in contrast to the immunisation of BALB/C mice with the same antigen.

The IgM isotype of the anti-α-enolase monoclonal antibodies was not unexpected since several studies that have used NZB or NZB×NZW F_1 crosses for the production of monoclonal antibodies have isolated monoclonal antibodies with an IgM isotype (Dobersen *et. al.,* 1985; Sanai *et. al.,* 1988, Ballard *et. al.,* 1983;

Jacquemart *et. al.,* 1988;). However, a study has isolated an IgG monoclonal antibody against a weakly immunogenic bacterial antigen using the NZB mouse system (Frosch *et. al.,* 1985).

The anti-α-enolase monoclonal antibodies were able to recognise both r-α-enolase and rabbit muscle enolase by ELISA, however these antibodies did not efficiently recognise r-α-enolase by western blotting. In contrast, the rabbit anti-α-enolase polyclonal antibody sera displayed a dilution-specific, high reactivity response for r-α-enolase as compared to rabbit muscle enolase. Moreover, this polyclonal sera recognised wild-type human α-enolase as detected by western blotting. Finally, α-enolase antigen was detected in the breast cancer cell lines MDA-MB-231 and MCF-7 cells thereby confirming that this glycolytic enzyme is expressed in breast cancer tissue (Hennipman *et. al.,* 1987). However, α-enolase was not detected on the surfaces of these cells.

In conclusion, anti-α-enolase monoclonal and polyclonal antibodies were successfully produced. The complete characterisation of the anti-α-enolase monoclonal antibodies requires the immunopurification of these antibodies from the hybridoma supernatants using either a r-α-enolase antigen column or antigen-independent chromatography methods. Since the α-enolase polyclonal antibodies did not detect this antigen on the surfaces of these human breast cancer cell lines, it was unnecessary to determine the plasminogen binding inhibitory capacity of the anti-α-enolase monoclonal antibodies in this study. However, future studies using these anti-α-enolase monoclonal antibodies would require the purification and further characterisation of these anti-α-enolase antibodies.

Chapter 5

Increased plasminogen binding is associated with metastatic breast cancer cells: Differential expression of plasminogen binding proteins.

5.1 Introduction

The acquisition of the malignant phenotype requires a multitude of complex processes including a loss of control of cell proliferation (e.g., via oncogene activation), higher metabolic requirements and the ability of cells to invade and metastasise throughout the body (reviewed in Evans, 1991). The processes of tumour cell invasion and metastasis are likely to involve the inappropriate expression of proteinases (reviewed in Mignatti and Rifkin, 1993). The serine proteinases of the plasminogen activation proteolytic cascade appear to play an important role in this process (reviewed in Duffy, 1993; Mignatti and Rifkin, 1993). Overexpression uPA and PAI-1 are associated with poor prognosis, shorter disease-free periods and reduced survival in breast cancer patients (Janicke *et al,* 1991; Christensen *et al,* 1996; Constantini *et al*, 1996; Nielsen *et al*,. 1996). The uPA:uPAR interaction on cancer cell surfaces is a key event that results in increased *in vitro* matrix degradation and migration (Duffy, 1993; Mignatti and Rifkin, 1993). For example, inhibition of uPA and plasmin by specific inhibitors or antibodies, or antisense inhibition of uPAR resulted in decreased plasminogen activation and hence decreased extracellular matrix degradation *in vitro* or decreased metastasis in nude mouse models with various cancer cell lines (Baker *et. al.,* 1990; Kook *et. al.,* 1994), including human breast cancer cell lines (Holst-Hansen *et. al.,* 1996; Stonelake *et. al.,* 1997). *In vitro* invasiveness has been correlated with uPA and plasmin activity as well as high

uPAR and plasminogen activator inhibitor type 1 (PAI-1) protein levels in human breast cancer cell lines (Holst-Hansen *et. al.*, 1996).

Plasminogen antigen has been localised to cell surfaces in sections of human mammary carcinoma tissue (Burtin *et. al*, 1993), and to the invasive front of cutaneous melanoma lesions (De Vries *et. al*, 1996). Cell-surface localisation of plasminogen is advantageous for cell migration since the activation of receptor-bound plasminogen to plasmin is enhanced while cell-bound plasmin is protected from circulating inhibitors (e.g.: α2-antiplasmin) (Plow and Miles, 1990). Glu-plasminogen binds to numerous different cell types in a lysine-dependent manner with low affinity and a high number of receptor sites/cell (see section 1.2.3 ; table 1.5).

Breast cancer is an example of a hormone-dependent cancer. Breast cancer cell lines that are oestrogen receptor (ER) positive and do not express the receptor tyrosine kinase epidermal growth factor receptor (EGFR) have a fundamentally non-metastatic phenotype (Lee *et, al.*, 1990). In contrast to the non-metastatic ER positive phenotype approximately 20-30% of all human breast cancers overexpress two closely related receptor tyrosine kinases, EGFR and/or p185*HER2/neu* (c-*erb*B-2), and this phenotype is associated with more aggressive tumour growth and reduced patient survival (Singleton and Strickler, 1992). The presence of ER is also inversely related to uPA and uPAR levels in breast carcinomas and cell lines (Mignatti and Rifkin, 1993; Long and Rose, 1996). In addition, it is apparent that breast cancer cell lines that readily form tumours and metastasise in nude mouse models and/or are invasive in *in vitro* models of metastases, (e.g.., MDA-MB-231 cells) (Thompson *et. al.*, 1992), tend to be EGFR(+)/*erb*B-2 protein(+) and ER(-) (Lee *et. al.*, 1990), as well as uPA/uPAR(+) (Holst-Hansen *et. al.*, 1996). EGFR stimulation or c-*erb*B-2 overexpression in human breast cancer cells enhances the expression and secretion of uPA and expression of uPAR (Long and Rose, 1996; Connolly and Rose, 1997).

The purpose of this chapter was to characterise the cell surface glu-plasminogen binding events in three human breast cancer cell lines. In the cell lines used in this study, elevated cell-surface lysine-dependent plasminogen binding and plasmin formation is associated with metastatic capacity and other parameters (EGFR/*erb*B-2 status, ER status, uPA/uPAR status) commonly associated with human breast cancer malignancy. Furthermore, non-viable cells breast cancer cells bound 100-fold more glu-plasminogen than viable cells and that the presence of non-viable cells must be considered when determining cell-surface binding parameters. In addition, a distinct plasminogen receptor profile was evident in the plasma membranes of the breast cancer cell lines, suggesting heterogeneity of plasminogen binding proteins in these cell lines.

5.2 Materials and methods

5.2.1 Materials

RPMI 1640, L-glutamine, and Hanks Buffered Salt Solution were purchased from Trace Biosciences (Castle Hill, NSW, Australia). Fetal calf serum was obtained from CSL (Parkville, Vic, Australia). TA, ε-ACA, aprotinin, polyvinyl pyrrolidine (PVP-40), bovine serum albumin (fraction V) (BSA) and fluorescein isothiocyanate (isomer 1) (FITC) were purchased from Sigma Chemical Co (St Louis, MO, USA). Biotin-X-NHS was from Calbiochem (San Diego, CA, USA). Z-lysine thiobenzylester was from Peninsula Laboratories (CA, USA). Enhanced Chemiluminescence (ECL) detection kit was purchased from Amersham International (Buckinghamshire, UK). Molecular weight protein standards were obtained from NOVEX (San Diego, CA, USA). The DAKO LSAB+ Kit was from DAKO Corporation (CA, USA).

5.2.2 Specific proteins and antibodies

Human recombinant α–enolase was prepared as previously described (Chapter 2). Active human uPA was from Serono (Sydney, NSW, Australia). Plasmin, the plasmin specific substrate Spectrozyme-PL, mouse anti-human uPAR monoclonal antibody (#3696) and rabbit anti-human uPAR polyclonal antibody (#399R) were provided by American Diagnostica Inc (Greenwich, CT, USA). Rabbit anti-human plasminogen polyclonal antibody and horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody were purchased from Calbiochem (San Diego, CA, USA). Mouse anti-human EGFR monoclonal antibody (clone LA1) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), while mouse anti-human *c-neu* (9G6) monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-DNP IgG_1 was kindly donated by Dr Mark Wilson and FITC-conjugated anti-mouse IgG was from Silenus (Sydney, NSW, Australia). Aprotinin

(Roche Biochemicals) was FITC conjugated in PBS according to the method described by Goding (1976).

5.2.3 Cell Culture

Routine cell culture for this study was performed as described in chapter 4.

5.2.5 Membrane Preparations

5.2.5.1 Total cellular membrane

Confluent cells were washed and harvested by scraping in ice-cold PBS and subjected to hypotonic shock in a small volume of ice-cold hypotonic buffer (3 mM $NaPO_4$ (pH 7.4), 5 mM EDTA, 1 mM PMSF phenylmethylsulfonylfluoride). The suspensions were then sonicated with several 20 second bursts at high power with a Branson Sonifier 250 (CT, USA), checked under a microscope for effective disruption of the cells, then centrifuged at 500 g for 5 min. The supernatant was centrifuged at 46000 g for 30 min and the resultant supernatant was frozen in aliquots at -70°C. The crude membrane pellet was resuspended in 5 ml ice-cold 5 mM EDTA/PBS, briefly resonicated, diluted to 50 ml with EDTA/PBS and centrifuged at 46000 g for 30 min. The resulting membrane pellet was resuspended in 1 ml EDTA/PBS with brief sonication, then aliquoted and stored at -70°C.

5.2.5.2 Plasma membrane

Confluent cells were washed, harvested, subjected to hypotonic shock as above then homogenised in a Dounce homogeniser. Plasma membranes were then isolated using the aqueous two-phase PEG polymer/dextran system as described by Rana and Majumder (1987).

5.2.6 Western blotting and plasminogen ligand blotting

Whole cell lysates and membrane preparations were boiled in sample buffer, fractionated on 10 or 12 % SDS-PAGE gels and parallel gels were either stained with Coomassie blue or transferred to PVDF membranes at 100 V for 1 h or 30 V

overnight at 4°C. For Western blotting the membranes were washed in TBST [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% (v/v) Tween-20], blocked in 10% milk powder/TBST at room temperature for 1-2 h, rinsed in TBST, then incubated with the primary antibody in 2% milk/TBST for 1 h at room temperature. After extensive washing the membrane was reblocked for 20 min with 6% milk/TBST, and incubated with the appropriate secondary antibody diluted 1:2000 in 2% milk/TBST. After three washes with TBST and one wash with TBS the immune complexes were detected by ECL.

For ligand blotting, membranes were washed once with TNCM [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$] and blocked with TNCM/2% PVP-40 overnight at room temperature. The membranes were then probed with 5 nM glu-plasminogen in the absence or presence of 100 mM EACA in TNCM/PVP-40 containing 0.05% (v/v) Tween 20 (TNCMT) for 45 min and washed for 1 h with three changes of TNCMT. After re-blocking for 30 min, a 1:2000 dilution of rabbit anti-plasminogen polyclonal antibody in TNCMT/PVP-40 was added and incubated with the membrane for 1 h. This was followed by three 5 minute washes, re-blocking for 10 min and probing with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit-polyclonal antibody in TNCMT/PVP-40 for 1 h. This was followed by three washes with TNCMT and one wash with TNCM. The blots were then developed by ECL.

5.2.7 Urokinase plasminogen activator activity and plasmin activity assays

The activity of uPA in the membrane preparations was measured as described by Coleman and Green (1981). Briefly, membrane preparations (10 µg protein/well) were preincubated with glu-plasminogen (1 µM) for 45 min at 37°C, followed by a further 45 min incubation with the chromogenic plasmin substrate, Z-lysine

thiobenzylester (170 μM). Colour development was read at 414 nm. Standard curves were constructed using uPA. Plasmin activity was measured using the Spectrozyme-PL assay (American Diagnostica Inc., conditions are described in the figure legend). Standard curves for the uPA and plasmin assays were constructed using either uPA or plasmin, respectively.

5.2.8 Flow cytometry and fluorimetry

Subconfluent, adherent cells that had been in culture for 48 h without a change of media, were harvested by rinsing flasks twice with cold PBS (pH 7.2) and then detaching with 5 mM EDTA/PBS at 37°C for 5 min. For FITC-glu-plasminogen binding assays cells were washed twice and resuspended in freshly made and chilled binding buffer (Hanks Buffered Salt Solution containing 1mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES (pH 7.4), and 0.1% BSA) at a concentration of 1 x 10^6 cells/ml. A 200 μl aliquot of the cell suspension was pelleted by centrifugation at 200g and resuspended in 200 μl binding buffer containing FITC-glu-plasminogen in the presence or absence of the lysine analogue, TA. After incubation for 1 h on ice in the dark, the cells were washed and resuspended in 250 μl binding buffer containing the non-vital dye propidium iodide (PI; 5 μg/ml). Cell-associated fluorescence was then measured by dual-colour flow cytometry (FACSort, Becton-Dickinson). By using dual-colour flow cytometry it was possible to distinguish between two parameters based on the different fluorochromes; i.e., ligand binding (FITC produces a strong green fluorescence) and cell viability (PI binds to DNA and dsRNA and produces a strong red colour; Chucholowski *et. al.,* 1992; Darzynkiewicz *et. al.,* 1994). This technique was used to establish 'gates' - the exclusion of PI for a viable 'gate', the inclusion of PI for a non-viable 'gate'.

Flow cytometry is a semi-quantitative technique and ligand binding, measured in fluorescence units, cannot be related to input protein concentration. Therefore, in order to analyse specific binding isotherms by Scatchard transformation, the binding experiments described above were analysed by fluorimetry. Briefly, after the final wash step the cells were resuspended in 2 ml binding buffer without PI and the extrinsic fluorescence of FITC-glu-plasminogen measured using a fluorimeter (F-4500, Hitachi) with a slit width of 0.5 mm. Excitation and emission wavelengths were set at 488 nm and 521 nm, respectively. A FITC-glu-plasminogen standard curve was constructed in order to relate fluorescence units to glu-plasminogen concentration taking into account the ratio of FITC per molecule of glu-plasminogen. To determine the proportion of non-viable cells, parallel dual-colour flow cytometry experiments were performed and consistently indicated that 10-15% of the cell samples were non-viable according to PI uptake.

Cell-surface plasmin was detected using dual-colour flow cytometry as described above with the following modifications. Cells were preincubated in the absence or presence of unlabelled glu-plasminogen (0.5 μM) for 30 min at room temperature followed by incubation on ice for 1 h with FITC-aprotinin (1 μM) in the absence or presence of a 50-fold excess of unlabelled aprotinin. The cells were then washed, resuspended in buffer containing PI and measured as described above.

For the detection of cell-surface uPAR, uPA and *c-neu* indirect immunofluorescence staining was performed. Cells were incubated with either an irrelevant isotype control (i.e. anti-DNP), or one the above specific monoclonal antibodies for 30 min on ice (20 μg/ml in ice-cold RPMI/0.1% BSA), washed three times with 200 μl ice-cold RPMI/0.1% BSA, and incubated with FITC-conjugated anti-mouse IgG (1:50 dilution of stock in ice-cold RPMI/0.1% BSA) for 30 min on ice in the dark. The cells were washed again, resuspended in 200 μl of binding buffer

containing 5 μg/ml PI and the cells immediately analysed by dual-colour flow cytometry as described above.

In all the fluorescence based experiments autofluorescence was subtracted. All data was analysed using CELLQuest software (Becton-Dickinson).

5.2.9 Plasminogen ligand histochemistry

Cells were passaged onto sterile glass coverslips in their appropriate media and allowed to adhere and spread for at least 48 h. The cells were then washed three times with PBS and fixed with glutaraldehyde (1% v/v in PBS) for 1 h at room temperature. Fixation was necessary since unfixed cells consistently rounded up and floated off the coverslips with the following procedure. After washing twice with PBS the cells were either permeabilised by incubation with 0.2% v/v Triton X-100 for 2 min at room temperature (to allow ligand to reach intracellular binding moieties in the glutaraldehyde fixed cells; Harlow and Lane, 1988) or left in PBS. Following two washes with PBS the permeabilised and non-permeabilised cells were incubated with 3% H_2O_2 for 5 min, rinsed with PBS, and incubated in PBS/0.1% BSA containing either 0.2 μM biotinylated BSA or glu-plasminogen in the absence or presence of 5 mM tranexamic acid for 30 min at room temperature. The cells were washed three times with PBS, incubated with streptavidin-peroxidase and finally with the diaminobenzidine chromogen substrate solution provided with the DAKO LSAB+ Kit. The cell histochemical images were captured as described in section 4.2.12.

5.2.10 Immunohistochemistry

Immunohistochemistry of breast cancer cells grown on coverslips was performed according to the method described in section 4.2.12.

5.2.11 Invasive and metastatic characteristics of human breast cancer cell lines

Local tissue invasion and metastatic ability of the MDA-MB-231, MCF-7, and T-47D cell lines in nude mice has been characterised in detail by Thompson *et. al.*,

(1992). Only the MDA-MB-231 cell line was found to be invasive and metastatic (Thompson *et. al.*, 1992). To confirm the metastatic characteristics of the MDA-MB-231 cell line 1-2 x 10^6 cells/site were injected into the flank region (s.c.) of male 4-6 week old Swiss *nu/nu* nude mice. The mice were sacrificed and the lymph nodes adjacent to the primary tumours were excised and examined histologically.

5.3 Results

5.3.1 Metastatic phenotypes of human breast cancer cell lines

5.3.1.1 Expression of c-neu oncogenic product, epidermal growth factor and oestrogen receptors in human breast cancer cell lines.

Aggressive/invasive human breast cancer express high levels of EGFR/*c-erb*B2 (Singleton and Strickler, 1992) and have been associated with expression of low levels of oestrogen receptor (Lee et al, 1990). The cell lines used in this study were phenotyped with respect to these parameters. Expression of EGFR by the breast cancer cell lines MDA-MB-231 and MCF-7 were examined by SDS-PAGE and western blot. Figure 5.1A shows a western blot probed with an anti-EGFR monoclonal antibody. MDA-MB-231 cells expressed a band corresponding to the molecular weight of EGFR. This band was absent in the MCF-7 whole cell lysate lane.

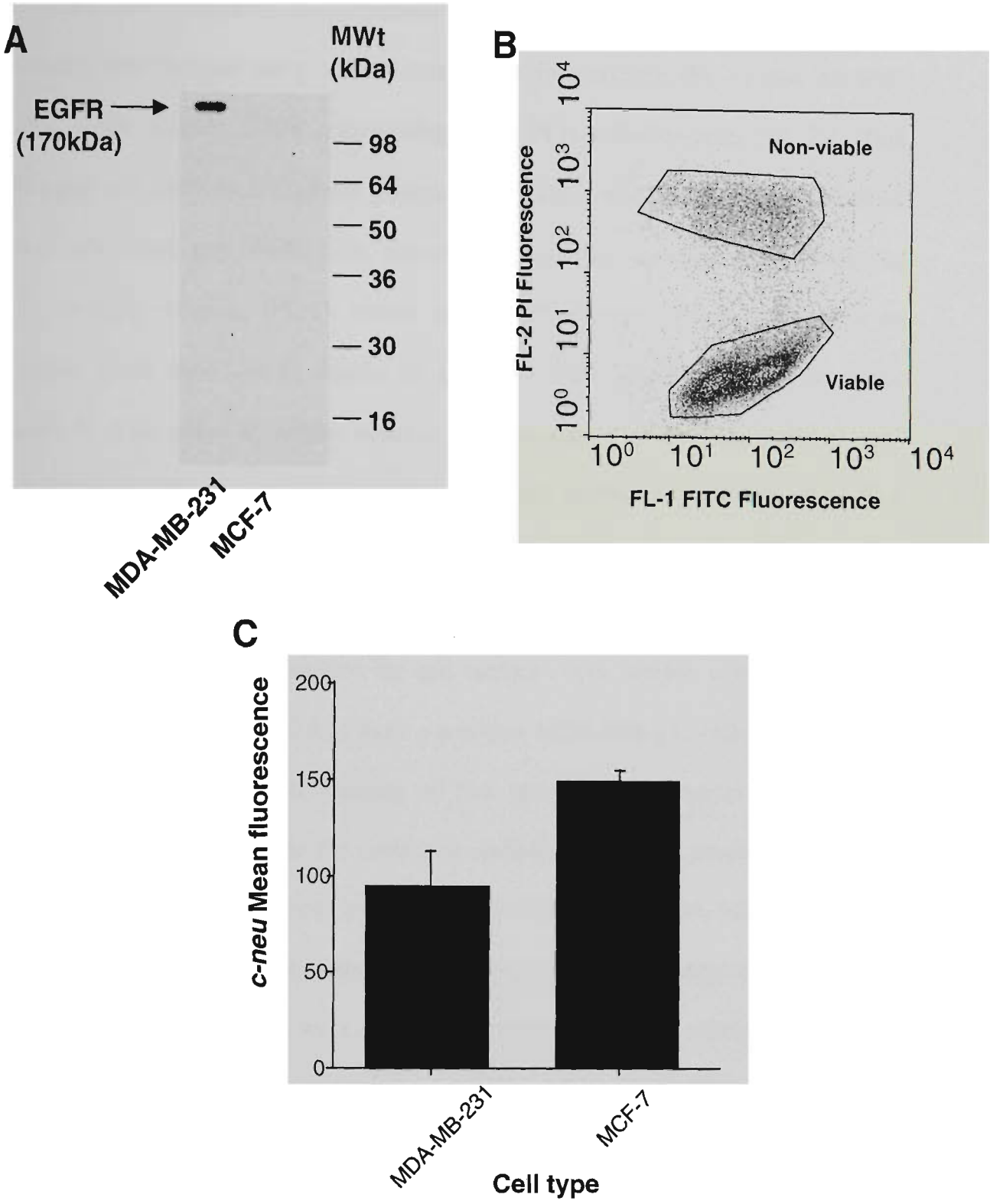

Figure 5.1: The EGFR and *c-neu* status of the human breast cancer cell lines.

Whole cell lysates of MDA-MB-231 and MCF-7 were fractionated by 12% SDS-PAGE under non-reducing conditions and transferred to PVDF membrane. The membranes were probed with anti-human EGFR monoclonal antibody. (B) A representative density plot of fluorescence intensities due to the indirect labeling of MDA-MB-231 cells with an anti-*c-neu* monoclonal antibody (FL-1 FITC) versus PI uptake (FL2-PI). Viable (low PI fluorescence) and non-viable (high PI fluorescence) cell gates were set around populations of cells that either excluded or included PI, respectively. (C) Flow cytometry of viable (PI negative) breast cancer cell lines MDA-MB-231 and MCF-7 ($2x10^5$ cells) probed with 20 µg/ml anti-*c-neu* monoclonal antibody. These data are means (n = 4) ± SEM.

All flow cytometry data presented within this dissertation is dual-colour flow cytometry (cell-surface antigen associated green fluorescence (FL-1) and the non-viable, water soluble, DNA intercalating stain PI-red fluorescence (FL-2)). This technique was able to distinguish antigens or binding moieties on the surfaces of viable cells from non-viable cells. Density plots of fluorescence intensity due to FITC-antibody binding (FL-1) versus nuclear PI staining (FL-2) were used to establish 'gates' based on PI uptake by the cells. Cells which excluded the water soluble PI were gated as viable, whereas cells staining with PI were gated as non-viable (figure 5.1B). The expression of the oncogenic product *c-neu* at the cell surface of viable (i.e. PI negative) breast cancer cell lines were determined by flow cytometry using an anti-*c-neu* monoclonal antibody (figure 5.1C). Both viable MCF-7 and MDA-MB-231 cells were positive for cell surface *c-neu* antigen with MCF-7 cells expressing approximately 1.5 fold more *c-neu* than MDA-MB-231 cells (figure 5.1C).

Immunohistochemical staining of the human breast cancer cells with a monoclonal antibody specific for oestrogen receptor resulted in positive staining of the T-47D and MCF-7 cell lines and negative staining of the MDA-MB-231 cell line compared to the positive control tissue section (figure 5.2). This suggests that both the T-47D and MCF-7 cell lines were oestrogen receptor positive. In contrast, the MDA-MB-231 cell line was oestrogen receptor negative (figure 5.2D). These data confirm the previous observations with respect to the oestrogen receptor status of these cells (deFazio et. al., 1992). Taken together, these data suggest that the MDA-MB-231 cell line is EGFR positive and oestrogen receptor negative, whereas, the MCF-7 cell line is EGFR negative and oestrogen receptor positive. However, both the metastatic and non-metastatic breast cancer cell lines were positive for cell-surface c-neu antigen.

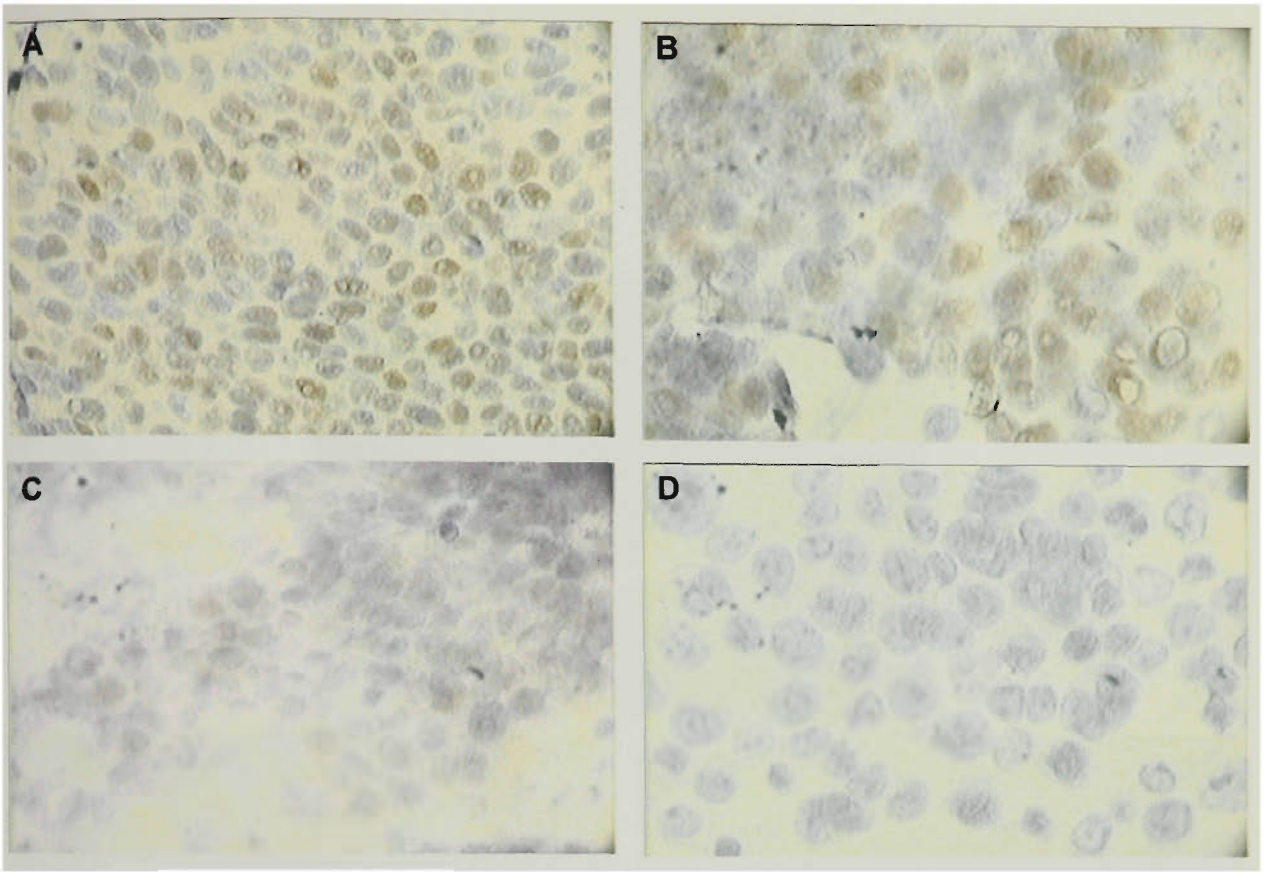

Figure 5.2: The oestrogen receptor status of three human breast cancer cell lines.

Cells were cytocentrifuged onto glass slides at 200g and probed with anti-oestrogen receptor monoclonal antibody (1:30) followed by biotinylated anti-mouse IgG and streptavidin conjugated HRP. The immune complex was detected by diaminobenzidine chromogen precipitate (DAB) and counter-stained with haemotoxylin. (A) malignant breast cancer fine needle biopsy positive control (B) MCF-7, (C) T47-D and (D) MDA-MB-231 breast cancer cell lines. Magnification x400. Performed inhouse by Southern Pathology.

5.3.1.2 Tumourgenecity and metastatic potential of the MDA-MB-231 cell line.

The human breast cancer cell line MDA-MB-231 have previously been characterised as a metastatic cell line (Singleton and Strickler, 1992). In contrast, the human breast cancer cell lines MCF-7 and T-47D have been shown to be only mildly tumourgenic and do not metastasise *in vivo* (Thompson *et. al.,* 1992). The tumourgenicity and metastatic potential of the MDA-MB-231 cell line were confirmed by subcutaneously inoculating the flank of five Swiss *Nu/Nu* mice with $1x10^6$ cells in RPMI. Three out of the five mice (60%) developed subcutaneous primary tumours ranging from 1.1% to 16.5% their total body mass (figure 5.3A). Gross examination of the tumours dissected from these mice revealed the presence of

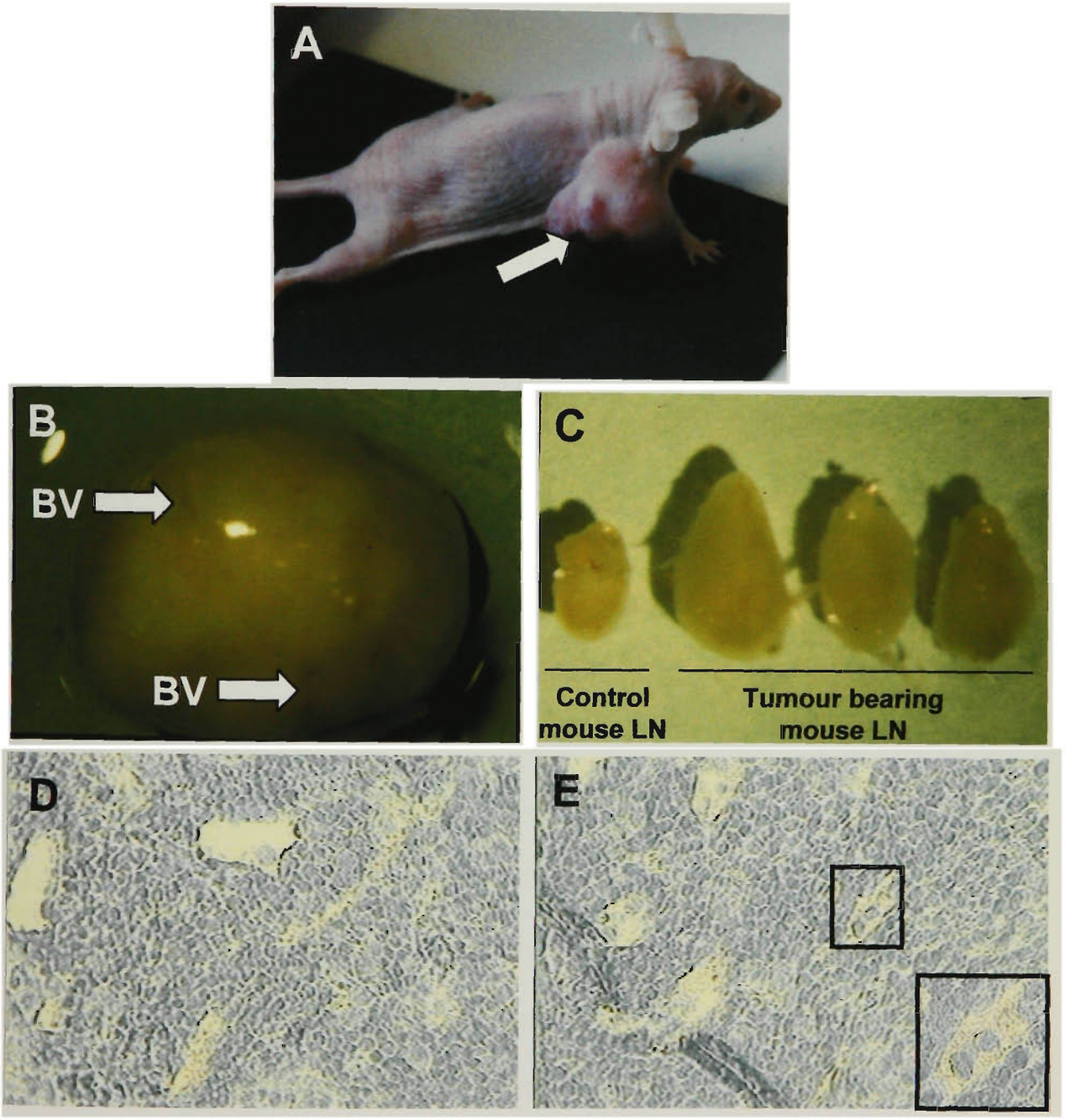

Figure 5.3: Tumourigenesis of MDA-MB-231 human breast cancer cell lines in the swiss *nu/nu* model.

(A) A swiss *nu/nu* mouse with an MDA-MB-231 tumour xenograph. The arrow indicates where the tumour xenograph has invaded the skin of the mouse. (B) An MDA-MB-231 tumour dissected from a swiss *nu/nu* mouse. The arrow indicates a tumour surface-associated blood vessel (BV). (C) Lymph nodes isolated from either a control mouse or a tumour bearing mouse. (D) Lymph node section stained with an isotype control antibody specific for DNP (400× magnification). (E) Lymph node section stained with an anti-human milk fat globulin monoclonal antibody (400× magnification) The inset has a 1000× magnification.

blood vessels associated with the surface of the tumour (figure 5.3B), suggesting that these tumours were vascularised. One of the tumour-bearing mice displayed local invasion of the primary tumour into the skin of the flank (figure 5.3A). The lymph nodes from the tumour-bearing mice were larger than a lymph node from the control (RPMI-inoculated) mouse (figure 5.3C). This may indicate metastasis of the MDA-MB-231 cells from the primary tumour, since breast tumour cells have been shown to metastasis to lymph nodes (Singleton and Strickler, 1992). Immunohistological examination of sections of lymph node material from the tumour bearing mice using a monoclonal antibody specific for the breast carcinogen marker human milk fat globulin (Taylor-Papadimitriou *et. al.,* 1981), shows some slight staining in the lymphatic tissue (figure 5.4). These data suggest that the human breast cancer cell line MDA-MB-231 is tumourgenic and may invade local tissue sites as well as metastasising to the lymph nodes in Swiss *nu/nu* mice. However, the sample size of the study was small. Taken together, the data from figures 5.1-5.3 suggest that the MDA-MB-231 cell line is an aggressive cell line while the MCF-7 and T-47D cell lines may be examples of more benign breast cancer cell lines.

5.3.1.2 uPAR and uPA antigen levels and uPA activity of the MDA-MB-231, MCF-7 and T47-D breast cancer cell lines.

Flow cytometry in conjunction with anti-uPAR and anti-uPA monoclonal antibodies were used to determine the levels of uPAR and uPA expressed on the surfaces of the breast cancer cell lines MDA-MB-231, MCF-7 (figure 5.4A). Viable MDA-MB-231 cells (i.e. cells that were PI negative) expressed approximately 20 fold more uPAR compared to viable MCF-7 cells. Urokinase receptor antigen had a punctate distribution the surfaces of non-permeabilised MDA-MB-231 cells (figure 5.4B II). Immunohistrochemistry of non-permeabilised MCF-7 and T-47D cells demonstrated that low levels of uPAR antigen were present on the surfaces of some

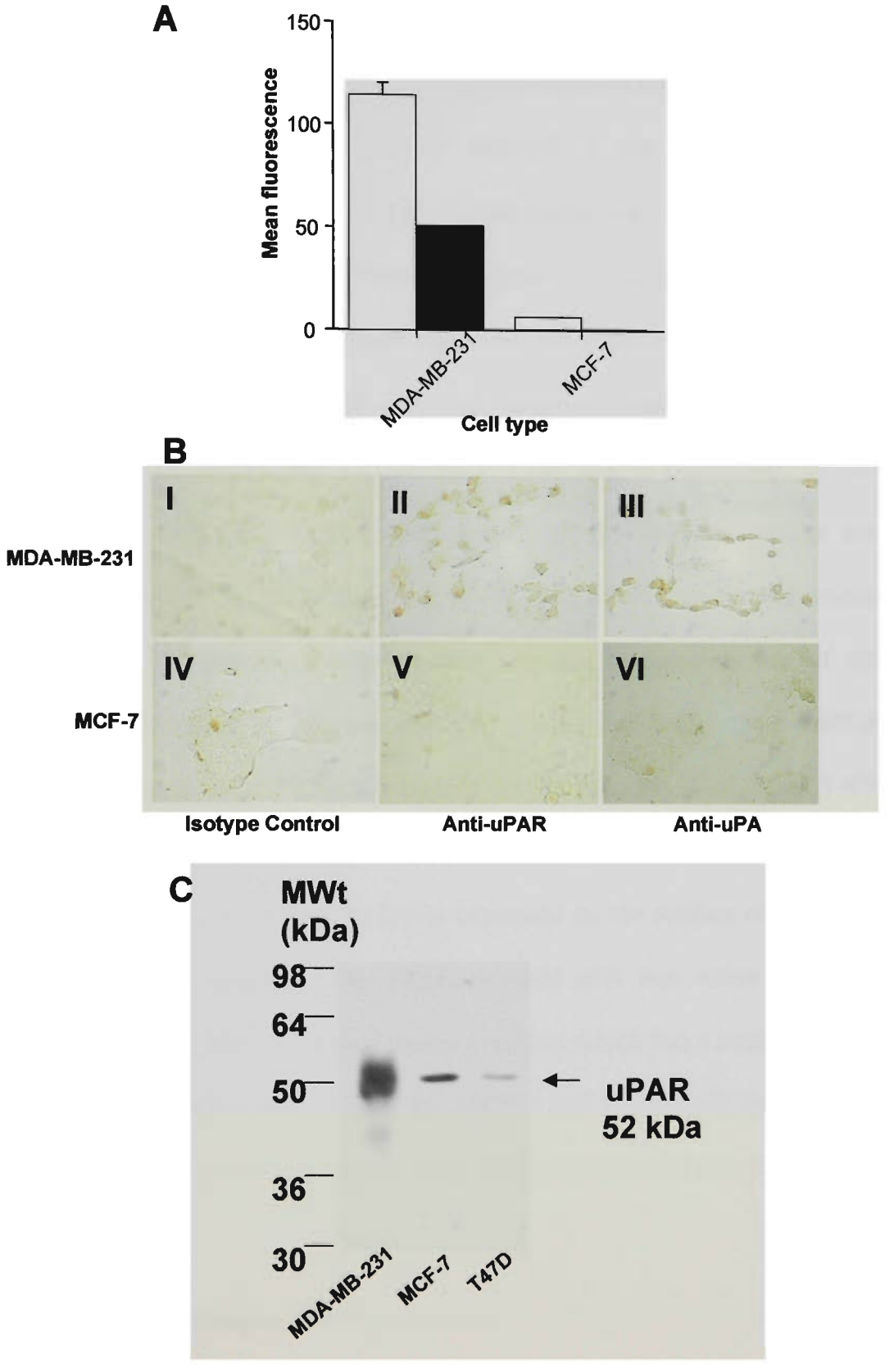

Figure 5.4: uPA and uPAR status of the breast cancer cell lines.

(A) Cells were incubated with a uPA or a uPAR-specific MAb followed by a FITC-anti-mouse IgG and resuspended in buffer containing PI. The labeled cells were analysed by dual-colour flow cytometry. Data presented are mean fluorescences associated with the uPAR (■) and uPA (□) antigen levels on the surfaces of PI negative MDA-MB-231 and MCF-7 cell lines after fluorescence of the isotype controls have been subtracted. (B) Immunohistochemistry depicting the spatial distribution of uPAR (II and V) and uPA (III and VI) on non-permeabilised MDA-MB-231 (I-III), MCF-7 (IV-VI). (C) Western blot of plasma membrane preparations of MDA-MB-231, MCF-7 and T-47D breast cancer cell lines probed with an anti-uPAR antibody and developed using ECL.

cells of a colony (figure 5.4B V and VIII). Finally the levels of plasma membrane and cell surface uPAR protein were markedly reduced in the MCF-7 (4-6 fold respectively) and even more so in T-47D (10-15 fold respectively) compared to levels in MDA-MB-231 cells (figure 5.4C). Thus, the aggressive human breast cancer cell line, MDA-MB-231 used in this study over-expressed cell-surface uPAR.

Similarly the viable MDA-MB-231 cells expressed approximately 50 fold more cell-surface uPA antigen than did the MCF-7 cell line as determined by flow cytometry (figure 5.4A). The punctate distribution of uPA antigen on the non-permeabilised MDA-MB-231 cells (figure 5.4B III) was similar to the distribution pattern of uPAR. The plasma membrane uPA activity, in the presence of glu-plasminogen, as determined by Spectrozyme UK chromagenic assay for the MDA-MB-231 cell line was at least 3 fold and 18 fold greater than the MCF-7 and T-47D cells lines (M. Ranson *pers. communication*). Thus, the level of uPA activity correlated with uPA and uPAR antigen levels expressed on the surface of the MDA-MB-231 cell line. This suggested that receptor-bound uPA was active at the cell surface. Thus, the MDA-MB-231 breast cancer cell line, which has a more malignant phenotype and is less differentiated than the MCF-7 and T-47D cell lines, express more of both uPA and uPAR than these more differentiated and non-invasive cell lines.

5.3.2 Cell-surface plasminogen binding on metastatic versus non-metastatic breast cancer cells.

5.3.2.1 Fluorescence studies

FITC-labeled glu-plasminogen, which retained its lysine-sepharose binding capacity (section 2.3.2), was used in flow cytometric glu-plasminogen binding assays to determine the glu-plasminogen binding capacity of the three viable human breast cancer cell lines. Histogram plots were used to calculate cell-surface, lysine-

dependent glu-plasminogen binding (figure 5.5A) and indicated that while the MCF-7 or T-47D cell lines bound low but detectable amounts of FITC-glu-plasminogen (8.0 ± 0.8 and 9.5 ± 2.3 fluorescence units, respectively), the MDA-MB-231 cell line bound significantly more FITC-glu-plasminogen (21.2 ± 3.6 fluorescence units) than either the MCF-7 or T-47D cell lines.

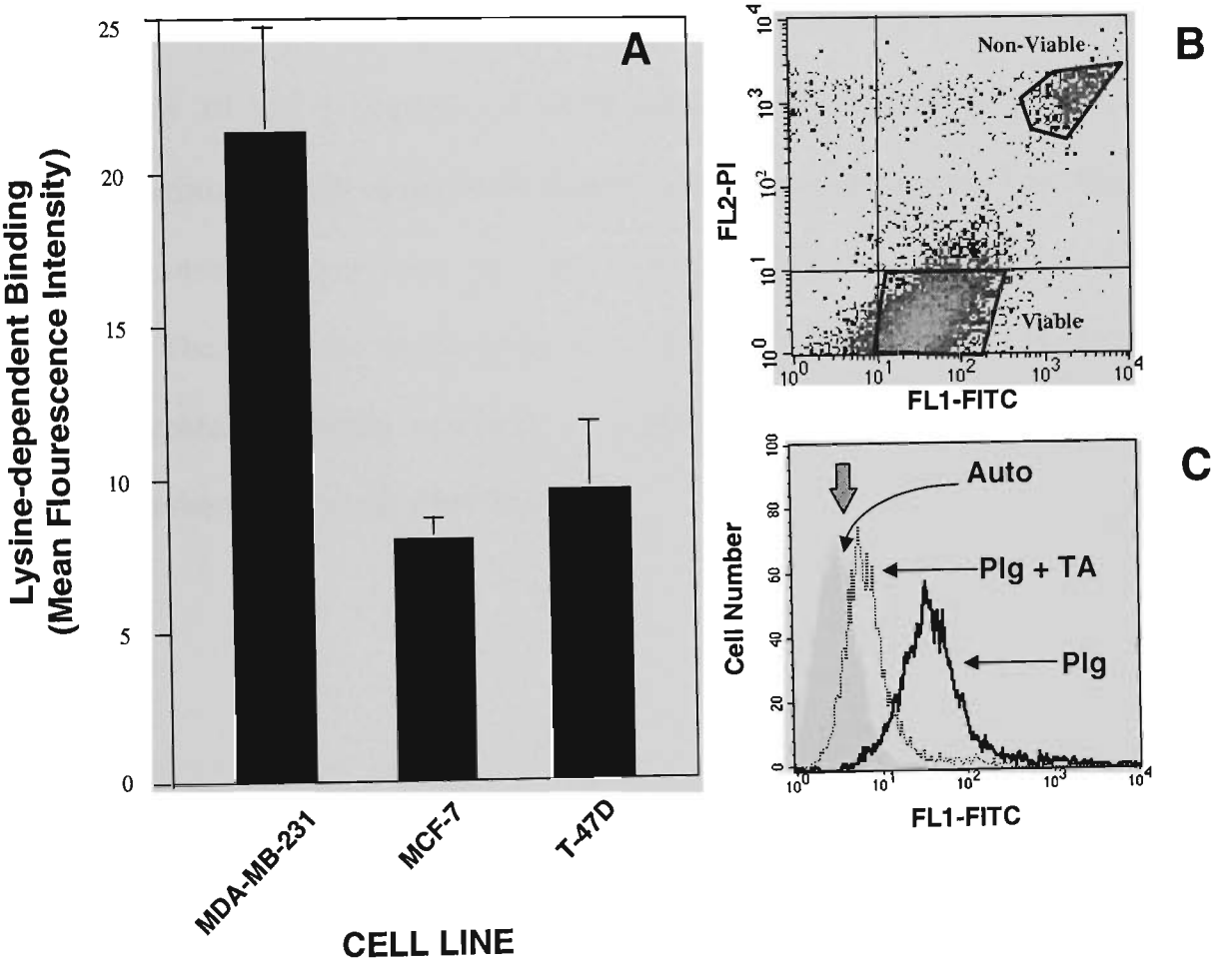

Figure 5.5: Cell-surface lysine-dependent glu-plasminogen binding capacity of breast cancer cell lines.

Cells were incubated with FITC-glu-plasminogen (0.5 μM) in the absence (total binding) or presence (lysine-independent binding) of 1mM tranexamic acid, washed, resuspended in buffer containing PI and analysed by dual-colour flow cytometry. (A) The column graph shows lysine-dependent binding (obtained by subtracting lysine-independent binding from the total binding) calculated from individual histogram plots where the fluorescence intensity values were gated to include viable cells only. Values shown are means ± SD (n = 4). (B) A representative density plot of fluorescence intensities due to FITC-glu-plasminogen binding (FL1-FITC) versus PI uptake (FL2-PI) in MDA-MB-231 cells. Viable and non-viable cell gates were set around cells that excluded or included PI, respectively. (C) Representative histogram plots of FITC-glu-plasminogen binding to viable MDA-MB-231 cells. Plg = FITC-glu-plasminogen; Plg + TA = Plasminogen and tranexamic acid. No shift in fluorescence intensities relative to autofluorescence were observed when cells were incubated with 0.5 μM FITC-BSA (➨).

The amount of lysine-dependent FITC-glu-plasminogen binding to non-viable cells was consistently 2 orders of magnitude higher than to viable cells (refer to figure 5.5A and B). Cell-surface glu-plasminogen binding to MDA-MB-231 cells was lysine- and concentration-dependent indicating a specific interaction (figure 5.5A). The viability of the MDA-MB-231 cells decreased as the FITC-glu-plasminogen concentration increased (figure 5.6). Such that the concentrations of FITC-glu-plasminogen required to saturate binding [e.g.., to saturate binding to r-α-enolase was approximately 10 μM (~1mg/ml) of glu-plasminogen (figure 2.5)] were found to render approximately 75% of the MDA-MB-231 cells non-viable (figure 5.6). Thus, it was not possible to demonstrate saturable FITC-glu-plasminogen binding on these viable cells. The reason for this apparent toxic effect is unclear, but it may be due to a loss of membrane integrity resulting from plasmin proteolysis even though the binding experiments were performed on ice.

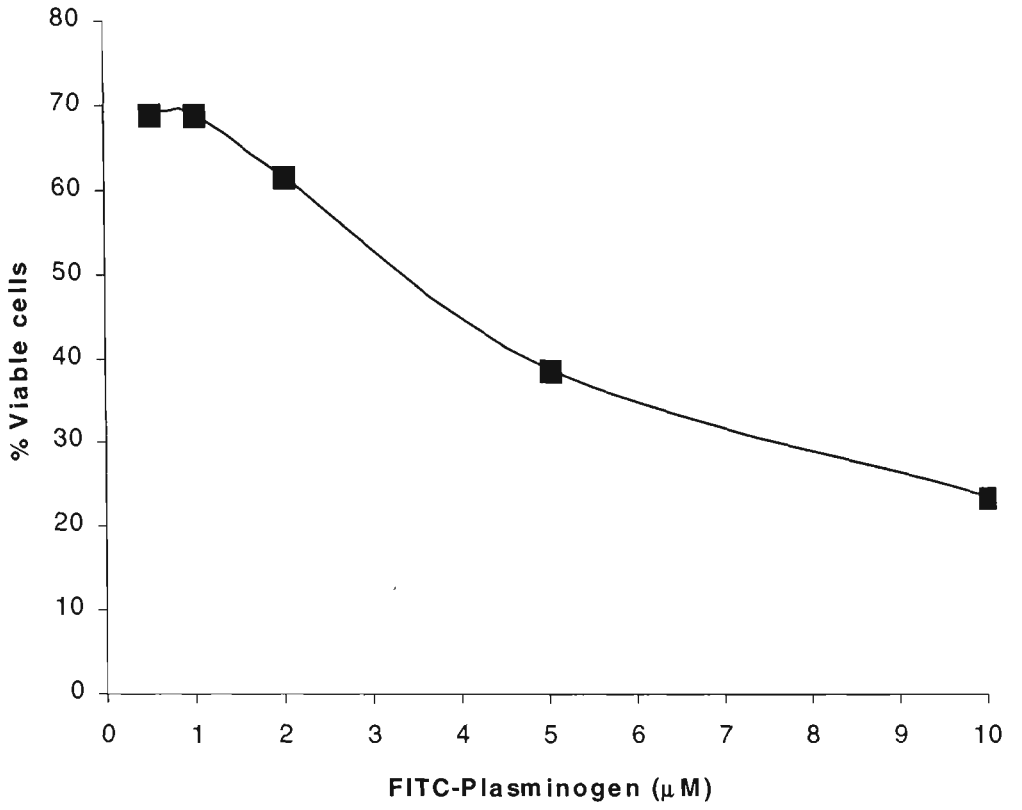

Figure 5.6: Viability status of the MDA-MB-231 cells as the concentration of FITC-glu-plasminogen.

Cells were incubated with various concentrations of FITC-glu-plasminogen for 1 hour on ice, washed, resuspended in buffer containing PI and analysed by dual-colour flow cytometry. The percentage of viable (PI negative) cells were determined and plotted against the concentration of FITC-glu-plasminogen.

Despite the viability limitation, the dose-dependent binding of FITC-glu-plasminogen was determined using fluorimetic analysis in an attempt to determine the K_d of this interaction (figure 5.7). This technique is similar in principle to a radiolabelled glu-plasminogen binding assay and allows the determination of parameters by Scatchard analysis. However, as with all these techniques the viability

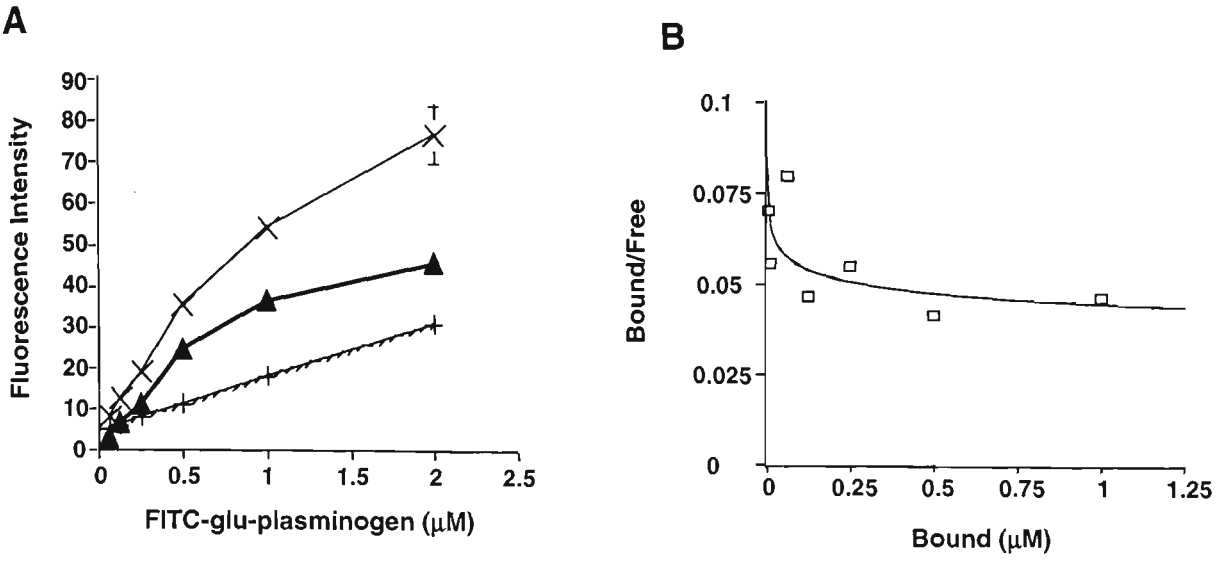

Figure 5.7: Concentration curves and Scatchard analysis of glu-plasminogen binding to MDA-MB-231 cells.

(A) MDA-MB-231 cells were incubated with increasing concentrations of FITC-glu-plasminogen up to 2 μM in the absence (×) or presence of 1 mM tranexamic acid (+) and analysed by dual-colour flow cytometry. The lysine-dependent plasminogen binding of viable cells (▲) was calculated as described in the legend to figure 5.5. The values shown are means ± SD (n = 3) from a representative experiment. (B) Representative Scatchard plot of lysine-dependent FITC-glu-plasminogen binding. This plot was derived from binding data generated by fluorimetry measurements and includes binding due to both viable and non-viable cells.

status of the cells cannot be discriminated. Thus, glu-plasminogen binding to non-viable cells may result in an over-estimation of the magnitude of these binding parameters since a small percentage of non-viable cells were invariably present in most cell preparations, regardless of the care taken to prevent cell damage (figure 5.5). Nevertheless, when lysine-dependent FITC-glu-plasminogen binding curves were transformed into Scatchard plots the best fit for the data was curvilinear (figure. 5.7B) indicating two classes of binding sites. The apparent equilibrium dissociation constants for glu-plasminogen binding to MDA-MB-231 cells were $1.8 \pm 0.6 \times 10^{-6}$ M for the higher affinity site and $2.0 \pm 0.9 \times 10^{-4}$ M for the lower affinity site. The number of binding sites per cell were $5.0 \pm 1.6 \times 10^{7}$ and $3.9 \pm 1.8 \times 10^{9}$ for the higher and lower affinity sites, respectively. This difference in number of binding sites/cell (two orders of magnitude) is comparable to the difference in capacity seen on viable

and non-viable cells by flow cytometry (figure 5.5B) and suggests that the lower affinity sites are attributable to non-viable cells binding FITC-glu-plasminogen.

5.3.2.2 Ligand histochemistry studies

Ligand histochemistry allowed visualisation of the glu-plasminogen binding capacity on viable cells that were attached and spread onto a substrata (figure 5.8 and 5.9). The differences seen by flow cytometry between the cell lines (figures 5.5, 5.7) were reproduced by glu-plasminogen ligand histochemistry with non-permeabilised cells (figure 5.8). The highest amount of positive brown staining was seen in the MDA-MB-231 cell line (figure 5.8A) compared to the MCF-7 (figure 5.8D) or T-47D (figure 5.8G) cell lines. In the presence of 5 mM TA glu-plasminogen binding was greatly reduced in all three cell lines (figures 5.8B, 5.8E, 5.8H), confirming that the cell lines bound glu-plasminogen in a lysine-dependent manner. The ligand histochemistry technique also showed that staining was diffuse over the cell surfaces (figure 5.8).

Deliberate permeabilisation of the cells with detergent resulted in an enhancement of the amount of glu-plasminogen binding which could be substantially reduced in the presence of 5 mM TA in all three breast cancer cell lines (figure 5.9). The distribution of staining in these cells appeared to be diffuse in the cytoplasm and on the cell surface, but was not associated with the nucleus. The results in figure 5.9 suggest that permeabilisation allows glu-plasminogen to bind to intracellular moieties

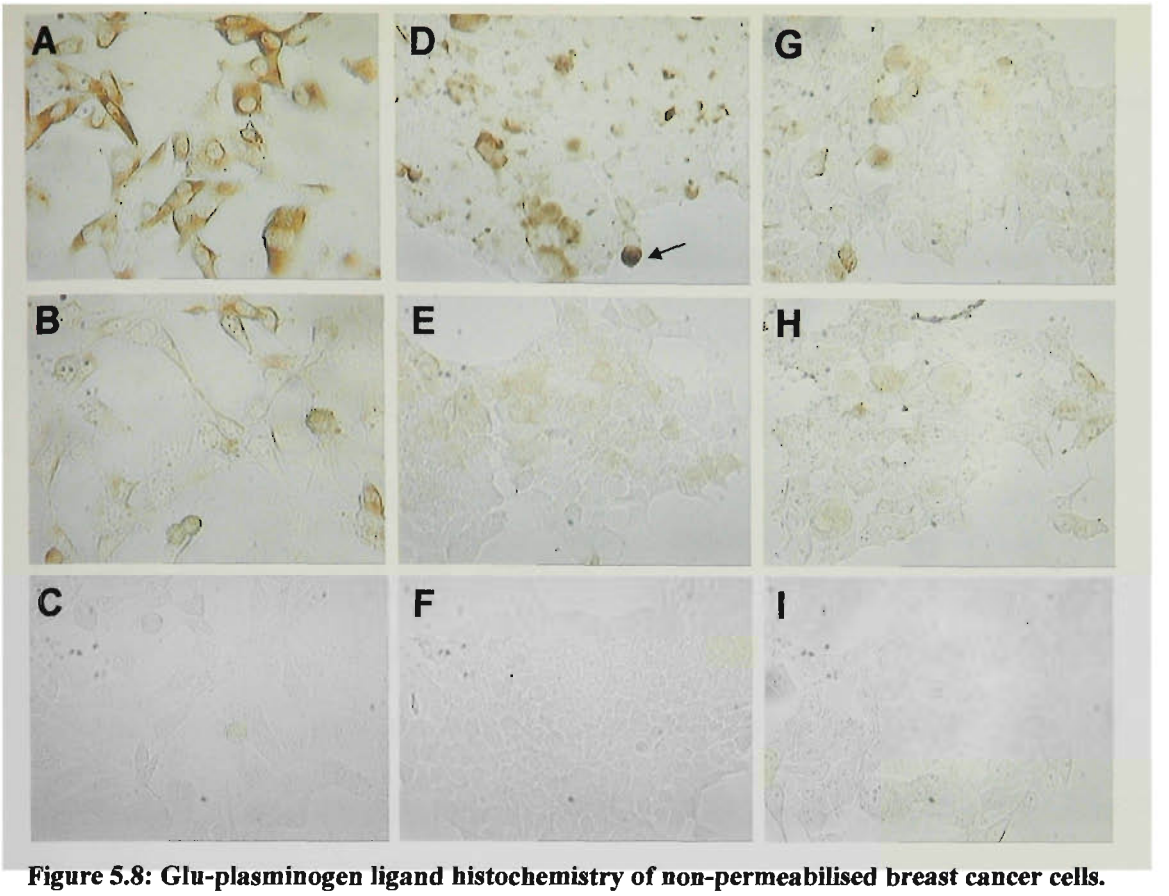

Figure 5.8: Glu-plasminogen ligand histochemistry of non-permeabilised breast cancer cells.

Fixed and non-permeabilised MDA-MB-231 (A-C), MCF-7 (D-F), and T-47D (G-I) cells were incubated with biotinylated glu-plasminogen (0.2 μM) in the absence (A, D, G) or presence (B, E, H) of 5 mM tranexamic acid, followed by incubation with streptavidin-HRP. Lower panels (C, F, I) show a lack of streptavidin-HRP interaction and subsequent colour reaction with cells that had been incubated with biotinylated BSA (0.2 μM). The arrow in panel (D) points to cellular debris.

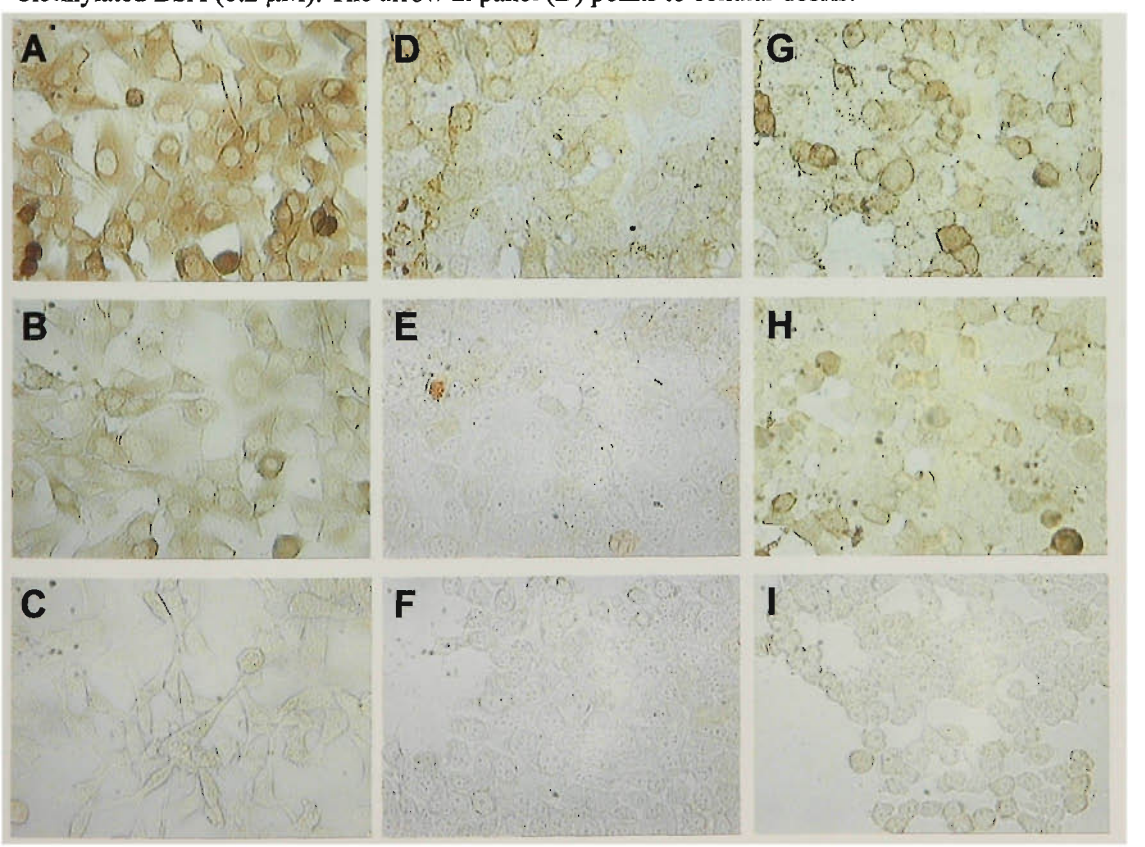

Figure 5.9: Glu-plasminogen ligand histochemistry of permeabilised breast cancer cells.

Same as for legend to figure 5.7 except that after fixation cells were permeabilised with Triton X-100 as described in Materials and Methods (section 5.2.9).

not otherwise available at the cell surface and that there are many more intracellular glu-plasminogen binding moieties than cell surface ones in all these cell lines. This was especially apparent in the MCF-7 and T-47D cell lines where some cells 5.8 D and G). These ligand histochemistry data correlated well with the flow cytometry in which glu-plasminogen binding was substantially greater in non-viable cells compared to viable cells (refer to figure 5.5B). While the permeabilised MDA-MB-231 cells (figure 5.9A) still appeared to bind more glu-plasminogen than the permeabilised MCF-7 (figure 5.9D) and T-47D (figure 5.9F) cell lines, this difference between the three cell lines was not as apparent as in the non-permeabilised cells (figure 5.8). This may be due to the magnitude of glu-plasminogen binding in permeabilised cells which has the effect of diminishing the differences in glu-plasminogen binding capacity between the cell lines.

5.3.3 Total cellular and membrane-associated plasminogen binding proteins in the three breast cancer cells.

Several bands were detected in ligand blots of whole cell lysates from the breast cancer cell lines (figure 5.10). Of these, two were major glu-plasminogen binding bands (apparent molecular masses 50 kDa and 30 kDa) and were present at similar levels in all three cell lines (figure 5.10B). These bands corresponded to glu-plasminogen binding proteins because the lysine analogue, ε-ACA, completely abolished the binding of glu-plasminogen to these proteins (figure 5.10C).

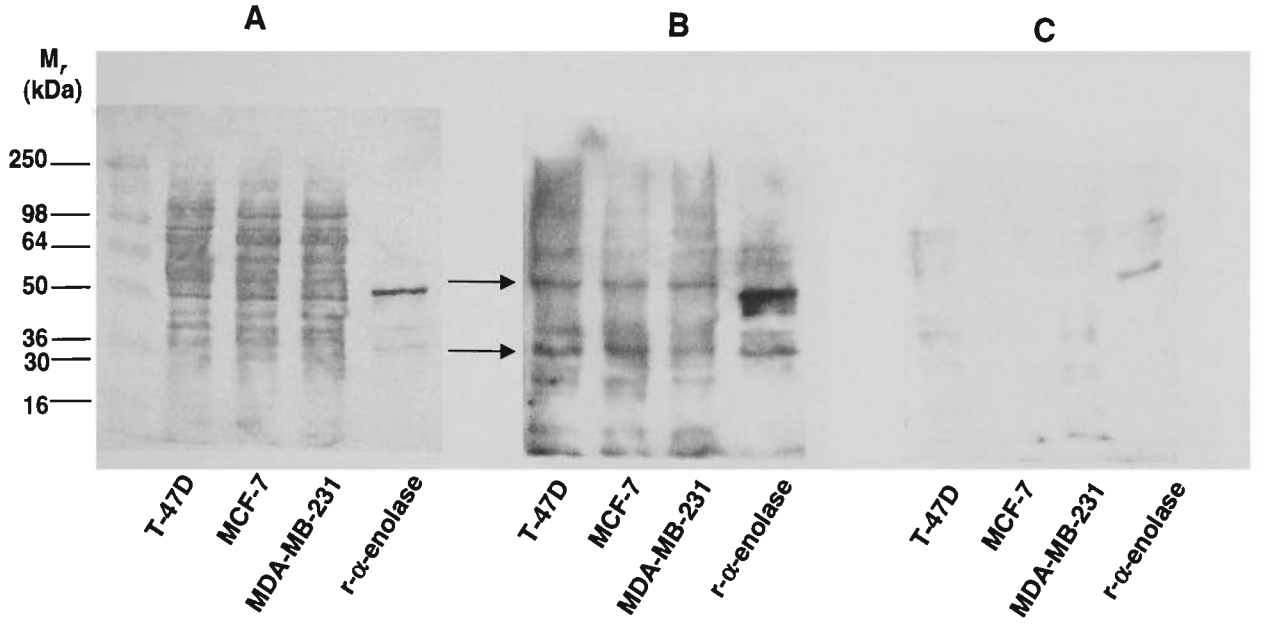

Figure 5.10: Glu-plasminogen ligand blotting of breast cancer cell whole cell lysates.

Whole cell lysates (15 μg/lane) and human recombinant α-enolase (r-α-enolase, 5 μg/lane) were separated by 12% SDS-PAGE under reducing conditions and either stained with Coomassie Blue (A), or transferred and subjected to ligand blotting using 5 nM glu-plasminogen in the absence (B) or presence of 100 mM ε-ACA (C). Blots (B) and (C) were derived from gels run, transferred and probed in parallel and were exposed onto the same piece of autoradiograph film so that a direct comparison could be made between them. Glu-plasminogen binding proteins were detected using an anti-plasminogen polyclonal antibody. The arrows point to bands with apparent molecular masses of 50 kDa and 30 kDa.

Ligand blots of plasma membrane proteins indicated a distinct glu-plasminogen binding protein profile on each of the three cell lines (figure 5.11). The MCF-7 plasma membranes contained three major bands with apparent molecular masses of 57 kDa, 47 kDa, and 33 kDa, as well as two minor bands of 40 kDa and 36 kDa (figure 5.11B). The 47 kDa and the 33 kDa bands in the MCF-7 plasma membranes (figure 5.11B) corresponded to the 50 kDa and 30 kDa bands respectively, in the MCF-7 whole cell lysate (figure 5.10). While the MDA-MB-231 and T-47D plasma membranes did not contain detectable amounts of the 57 kDa or 47 - 50 kDa bands seen in the MCF-7 plasma membranes, the 30 - 33 kDa band was

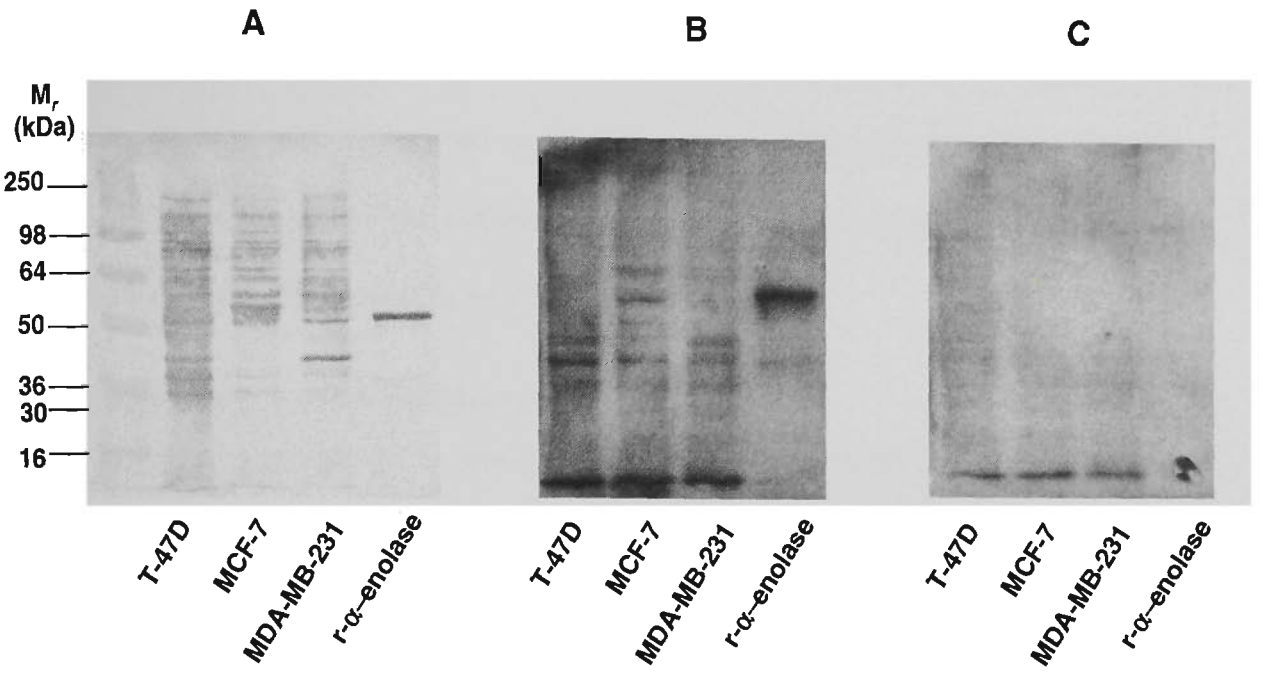

Figure 5.11: Glu-plasminogen ligand blotting of breast cancer cell plasma membranes.

Same as for legend to figure 5.9 except that plasma membrane fractions (15 μg/lane) were used instead of whole cell lysates.

present in all plasma membranes as a major band (figure 5.11B). The MDA-MB-231 plasma membranes contained two other major bands with apparent molecular masses of 36 kDa and 26 kDa (figure 5.10B). The T-47D plasma membranes also contained the 36 kDa band but at a lower concentration than in the MDA-MB-231 plasma membranes (figure 5.11B). In all ligand blots the glu-plasminogen binding protein r-α-enolase (chapter 2) was used as a positive control. In each case the presence of ε-ACA inhibited glu-plasminogen binding to this protein (figures 5.10C and 5.11C).

5.3.4 Expression of identified plasminogen receptor candidates at the cell surface.

Commercially available antibodies against proteins identified as plasminogen receptor molecules (table 1.4) were used in combination with immunohistochemistry to determine if these proteins were present on the surfaces of selected breast cancer cells lines. If present, the proteins may be responsible for localising glu-plasminogen to these cells. Non-permeabilised MDA-MB-231 cells were positive for both annexin II and actin expression but were negative for cytokeratin 8 and tetranectin expression

(figure 5.12). Similarly, a proportion of non-permeabilised MCF-7 cells were positive for annexin II, actin and cytokeratin 8 however, the MCF-7 cells were negative for tetranectin (figure 5.13). These data suggest that some of the proteins responsible for binding glu-plasminogen on the surfaces of the MDA-MB-231 cells were members of the group 2 or latent plasminogen receptor candidates.

5.3.5 Plasminogen receptor purification.

Attempts were made to purify the proteins responsible for localising glu-plasminogen to the surface of the MDA-MB-231 cell line using glu-plasminogen affinity chromatography. Plasma membrane preparations, that contained plasminogen binding activity (figure 5.14A), were solubilised in RIPA buffer and applied to the glu-plasminogen affinity column. Two major glu-plasminogen binding proteins were eluted from the column with apparent molecular weights of 40 and 33 kDa (figure 5.14 C). The molecular weights of these bands are similar to the molecular weights of the proteins that were detected by the glu-plasminogen ligand blot of the MDA-MB-231 plasma membrane preparation (figure 5.11). Furthermore, numerous less abundant proteins were also eluted from the glu-plasminogen affinity column (figure 5.14C). The amount of protein isolated was not sufficient for N-terminal sequencing, thus these plasminogen binding proteins remained unidentified.

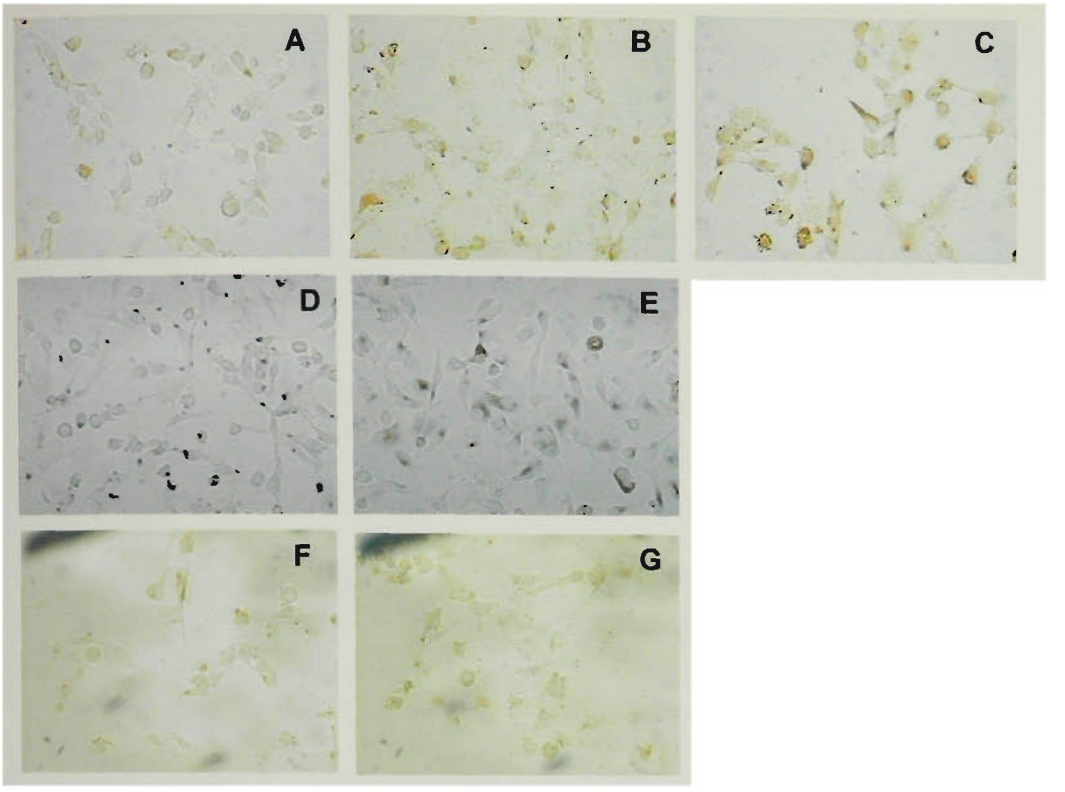

Figure 5.12: Non-permeabilised MDA-MB-231 cell plasminogen receptor immunohistochemistry

Fixed and non-permeabilised MDA-MB-231cells were incubated with either control antibody preparations (A, normal goat serum; D, mouse anti-DNP antibody; F, rabbit anti-DNP antibody) or antigen-specific polyclonal antibodies (B, goat anti-actin; C, goat anti-annexin; E, mouse anti-cytokeratin 8; G rabbit anti-tetranectin).

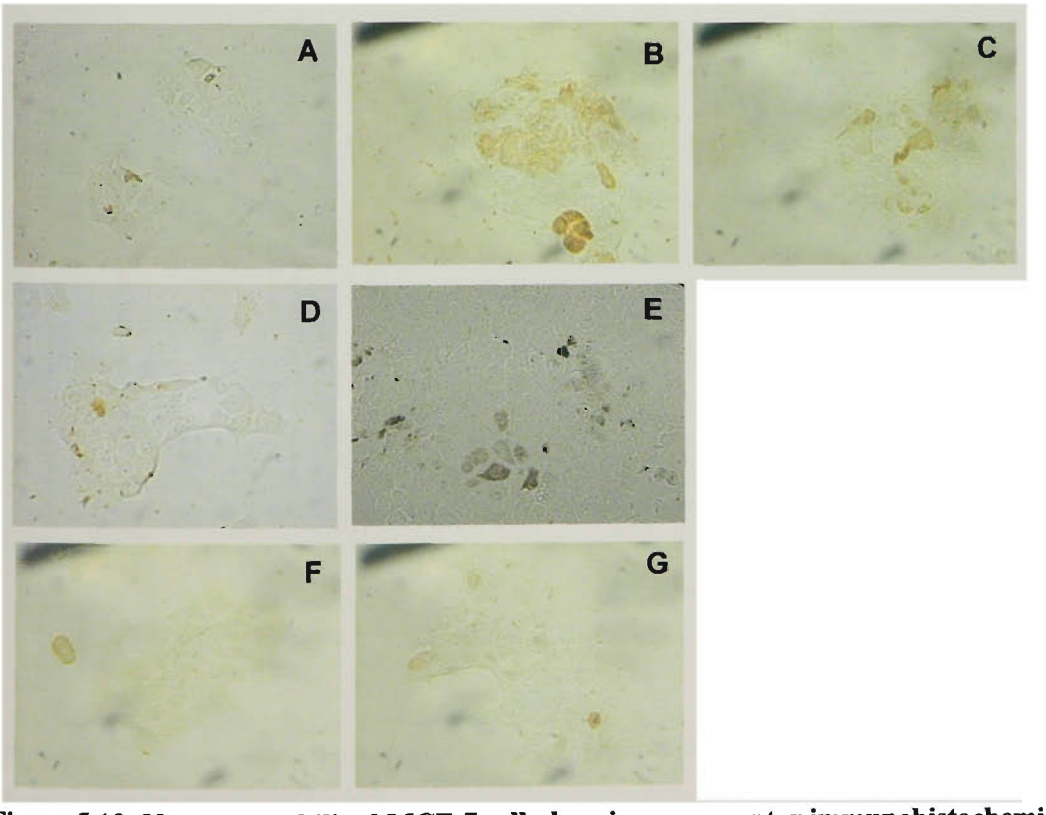

Figure 5.13: Non-permeabilised MCF-7 cell plasminogen receptor immunohistochemistry.

Same as for legend to figure 5.9 except that MCF-7 cells were used instead of MDA-MB-231 cells.

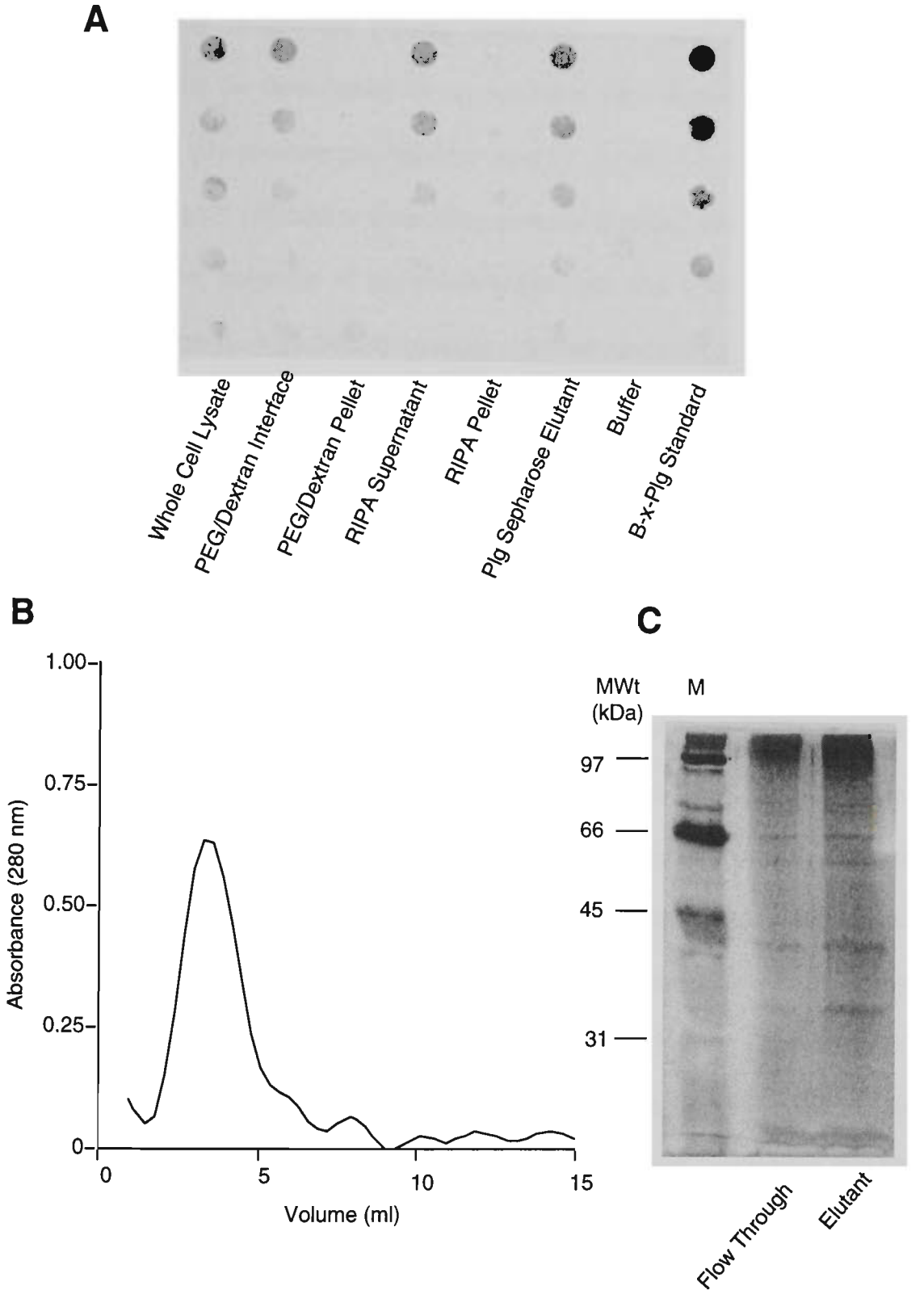

Figure 5.14: Plasminogen receptor purification from MDA-MB-231 cell line.

Plasma membranes of MDA-MB-231 cells were prepared using the two-phase polymer system. The membranes were solubilised and the plasminogen binding proteins isolated using plasminogen affinity chromatography. (A) Dot blot showing the plasminogen binding activity of the sample during all the steps of the isolation process (B) Elution profile of the plasminogen affinity chromatography column. (C) Silver stained, non-reducing 12% SDS-PAGE of the flow through and elution fractions from the plasminogen affinity column.

5.3.6 Plasmin generation is associated with high glu-plasminogen binding capacity.

The ability to generate plasma membrane-associated plasmin activity was compared between the three breast cancer cell lines. The MDA-MB-231 cells, which have the highest glu-plasminogen binding capacity (figure 5.6) and endogenous uPA activity, were highly efficient at generating plasmin (figure 5.15A). This activity was dependent on the presence of glu-plasminogen and was completely inhibited by preincubation with the high affinity plasmin inhibitor aprotinin. At a glu-plasminogen concentration of 0.5 μM, there was no detectable plasmin specific activity in either the MCF-7 or T-47D plasma membranes compared with 110 pmol/min/mg in the MDA-MB-231 plasma membranes.

In order to demonstrate that plasmin was generated on the surfaces of viable MDA-MB-231 cells, dual-colour flow cytometry was used in conjunction with FITC conjugated aprotinin (FITC-aprotinin) as the ligand. The ability of FITC-aprotinin to inhibit plasmin-meditated proteolysis was compared to the plasmin inhibitory capacity of unlabelled aprotinin. Figure 5.15B demonstrates that both FITC-aprotinin and unlabelled aprotinin significantly reduced the plasmin-mediated degradation of fibrinogen compared to the control (fibrinogen plus plasmin). Thus, FITC-aprotinin was used as a specific detector of plasmin activity has been previously described (Ellis *et. al.*, 1987). The inset to figure 5.15A shows that FITC-aprotinin bound to viable cells but only when preincubated with glu-plasminogen, suggesting that MDA-MB-231 cells were capable of activating receptor-bound glu-plasminogen on the cell surface.

MDA-MB-231 cells, grown on coverslips, were preincubated with 0.5 μM glu-plasminogen for 30 min at room temperature followed by incubation on ice for 1

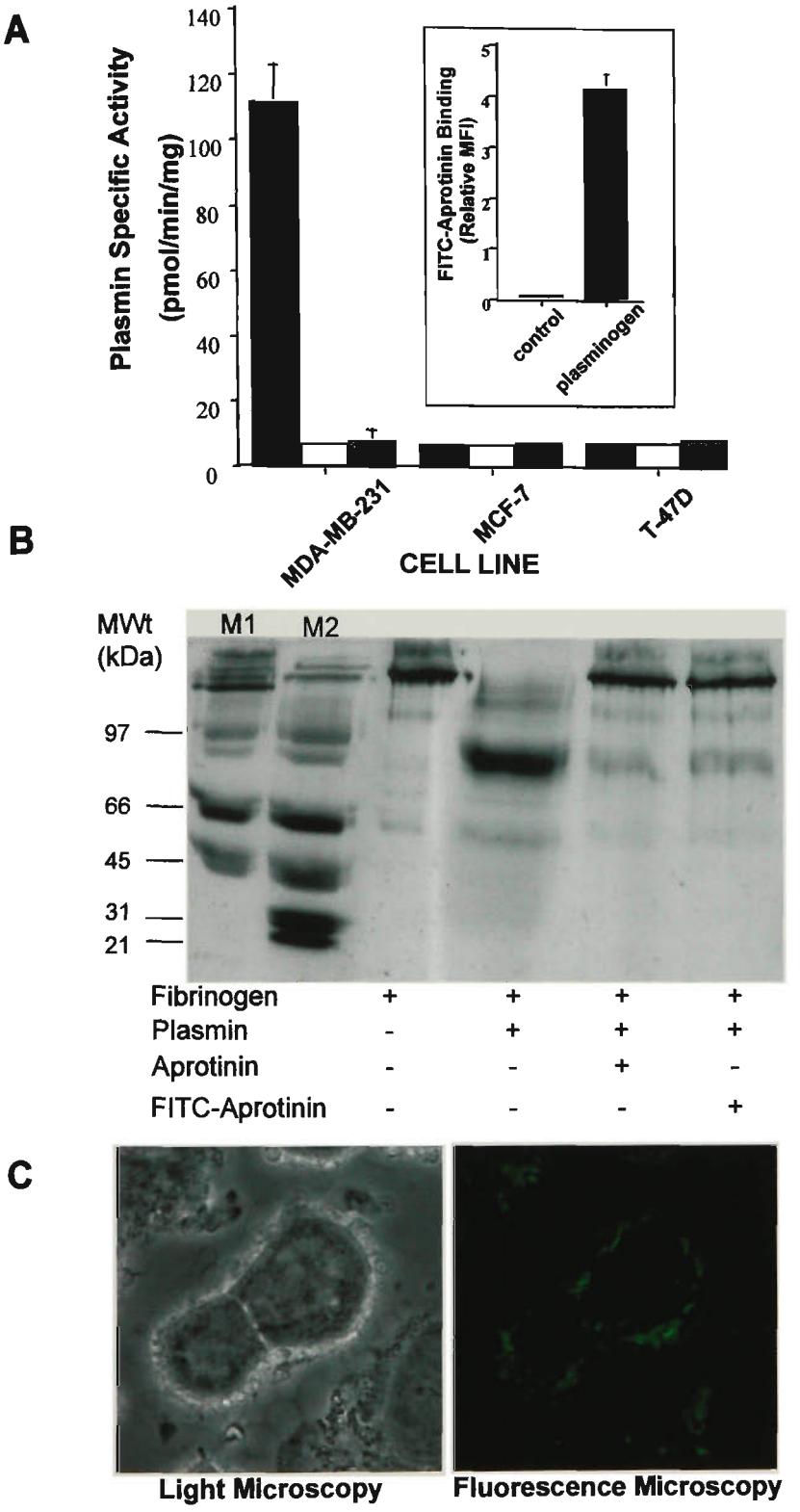

Figure 5.15: Plasmin formation by breast cancer cells.

(A) Breast cancer cell plasma membrane preparations (2 μg) were preincubated with 0.5 μM glu-plasminogen for 30 min at room temperature, followed by a 5 min incubation in the absence (■) or presence (□) of 1 mM aprotinin. Plasmin activity was then measured at 37°C over 20 min using the Spectrozyme PL substrate (0.25 mM final concentration). Plasmin activity in the absence of glu-plasminogen and aprotinin was also measured (▩). The inset shows specific FITC-aprotinin binding to viable MDA-MB-231 cells as assessed by dual-colour flow cytometry (refer to figure 5.5B). The specific binding was calculated by subtracting binding in the presence of a 50-fold excess of unlabelled aprotinin from the total binding. The values shown are means ± SD (n = 3) from representative experiments. (B) A 12% non-reducing SDS-PAGE of the reaction of 1 mg/ml of fibrinogen with 25 μg/ml plasmin in the absence or presence of either 50 μg/ml aprotinin or 50 μg/ml FITC-aprotinin for 30 min at 37°C. (C) Fluorescence microscopy of 1 μM FITC-aprotinin binding after the MDA-MB-231 cells were incubated with 0.5 μM glu-plasminogen for 30 min at room temperature.

h with 1 μM FITC-aprotinin (green) and examined using fluorescence microscopy. As illustrated by figure 5.15C, FITC-aprotinin was localised to the surface of these cells, thereby confirming that glu-plasminogen was activated to plasmin at the surfaces of the MDA-MB-231 cells. It is also noteworthy that the majority of these cells had a rounded morphology compared to the fixed and attached MDA-MB-231 cells in figures 5.4, 5.8, 5.9 and 5.12. This suggests the presence of plasmin proteolytic activity at the cell surface may be responsible for the change in morphology of the MDA-MB-231 cells by inducing the detachment of the cells from the substrata..

5.4 Discussion

This chapter demonstrates that the spindle-shaped, EGFR(+)/*neu*(+), ER(-), and metastatic MDA-MB-231 cells were not only associated with high uPA antigen and activity levels as well as uPAR overexpression but that they also had an increased capacity to bind and activate cell-surface glu-plasminogen compared to the more differentiated, non-metastatic cell lines, MCF-7 and T-47D. These results suggest that expression of cell surface plasminogen receptors play a part in regulating the plasminogen activation cascade, and may contribute to the metastatic phenotype of the MDA-MB-231 cell line.

Dual-colour flow cytometry was initially used as a means to identify cell-surface specific glu-plasminogen binding. However, this technique also revealed that both viable and non-viable cells could bind FITC-glu-plasminogen in a lysine-dependent manner. Moreover, non-viable cells bound two orders of magnitude more glu-plasminogen than viable cells. This was confirmed by ligand histochemistry which showed that biotinylated glu-plasminogen binding capacity was substantially increased in all cell lines after detergent permeabilisation. This is an important finding

since it is very difficult to harvest adherent cells and maintain them throughout experimental procedures at 100% viability (it was consistently found that between 10% and 30%, of the cells were non-viable as assessed by PI uptake by the time cells were analysed by flow cytometry) and suggests that even a small proportion of non-viable cells could affect evaluation of authentic cell surface glu-plasminogen binding capacity if viability status is not considered.

Scatchard plots of glu-plasminogen binding data to MDA-MB-231 cells using a single-colour fluorescence technique resulted in curvilinear plots which are suggestive of two classes of binding sites. Curvilinear Scatchard plots of glu-plasminogen binding have been previously reported for endothelial cells using a radiolabelled glu-plasminogen binding technique (Ganz *et. al.,* 1991). The higher affinity binding site in the MDA-MB-231 cells had an average K_d of 1.8 μM and 5.0×10^7 binding sites per cell while the lower affinity one had an average K_d of 200 μM and 3.9×10^9 binding sites per cell. The affinity and capacity of the higher affinity site are similar to that reported for many cell types determined by radiolabelled glu-plasminogen techniques (Plow and Miles, 1990; Hembrough *et. al.,* 1995) and are within physiological limits since glu-plasminogen is found in the circulation at approximately 2 μM. The capacity of the lower affinity site is two orders of magnitude higher than the higher affinity site - exactly the difference seen in binding capacity magnitude between viable and non-viable cells by dual-colour flow cytometry. Since non-viable cells were present in all fluorimetry experiments, it is likely that the lower affinity, very high capacity binding sites are due to non-viable cells. The affinity and capacity of these non-viable cells implies that it would be hard to saturate binding. Nevertheless, while binding to non-viable cells may not be physiologically relevant, taken together, these results suggest that glu-plasminogen

binding to non-viable cells is so large that subtle or even significant differences in cell-surface specific glu-plasminogen binding capacity between cells can be overestimated when measured by techniques that cannot distinguish between viable and non-viable populations of cells.

The results also suggest that the abundance of intracellular glu-plasminogen binding moieties is greater than the cell surface glu-plasminogen binding moieties regardless of the cell-surface binding capacity. The 50 kDa plasminogen binding protein present in the whole cell lysates of MDA-MB-231, MCF-7 and T-47D cells may account for at least some of the increase in glu-plasminogen binding capacity of both the MDA-MB-231 and T-47D cell lines upon permeabilisation. While all of the cell lines expressed plasma-membrane associated glu-plasminogen binding proteins, the glu-plasminogen ligand blots gave no information about the orientation of the plasminogen binding proteins within the plasma membranes of intact cells. Therefore, the presence of these proteins in the plasma membrane fraction does not necessarily mean that they would be oriented on the cell surface in such a way that they could bind pericellular glu-plasminogen. Since viable or non-permeabilised MCF-7 and T-47D cells had minimal cell-surface glu-plasminogen binding capacity compared to viable or non-permeabilised MDA-MB-231 cells, one possible explanation is that only a small proportion of the MCF-7 and T-47D plasma-membrane associated proteins are physiologically available to bind extracellular glu-plasminogen. Upon permeabilisation a greater proportion of these proteins may also become available for glu-plasminogen binding in the MCF-7 and T-47D cells. It is also possible that non-proteinaceous binding moieties such as gangliosides (Miles *et. al.,* 1989) may account for a significant proportion of the differences in cell-surface glu-plasminogen binding between the cell lines.

Nevertheless, the ligand blot data indicate that more than one protein moiety contributes to total cellular glu-plasminogen binding capacity in these cell lines. The intermediate filament protein cytokeratin 8 and the glycolytic enzyme α-enolase are two potentially important receptors, which have a C-terminal lysine residue, and have been isolated from the plasma membranes of rat hepatocytes (Hembrough *et. al.*, 1995), the lymphoid monocytic cell line U-937 (Miles *et. al.*, 1991) and embryonic rat neurons (Nakajima *et. al.*, 1994). Cytokeratin 8 has been localised to the cell surface of human breast cancer cells (Hembrough *et. al.*, 1995, 1996), and an α-enolase-like protein with glu-plasminogen binding ability was isolated from the whole cell lysates of two human breast cancer cell lines (Lopez-Alemany *et. al.*, 1994). The glycolytic enzyme, α-enolase (encoded by ENO1), is an authentic plasminogen binding protein (Chapter 2) and it is expressed as a 47 kDa protein in whole cell lysates of these human cancer cell lines (figure 4.8). Furthermore α-enolase antigen was detected when the cells were permeabilised by TX-100 (figure 4.9).

Using a ^{125}I-plasminogen overlay assay, Hembrough *et. al.*, (1996) showed the presence of several plasminogen binding proteins in the cytoplasm and plasma membrane fractions of various breast cancer cells. Moreover, they showed the presence of a major 55 kDa plasma membrane-associated glu-plasminogen binding protein in MCF-7 cells which they identified as cytokeratin 8. In this study cytokeratin 8 antigen was not detected on the surfaces of non-permeabilised MDA-MB-231 cells by immunohistochemistry, however a small proportion of non-permeabilised MCF-7 cells were positive for cytokeratin 8. Thus, it is possible that the 57 kDa plasminogen binding proteins detected by the glu-plasminogen ligand blot may be cytokeratin 8, which may contribute to the low levels of glu-plasminogen binding observed on the MCF-7 cells. Neither α-enolase nor cytokeratin 8 antigen

were not present on the surfaces MDA-MB-231 cells, suggesting that these group 1 plasminogen binding proteins do not contribute to the high glu-plasminogen binding capacity of the MDA-MB-231 cells.

In contrast to cytokeratin 8 and α-enolase expression, the latent or group 2 plasminogen binding proteins actin and annexin II were expressed at the cell surface of both the MDA-MB-231 and MCF-7 cell lines and thus may contribute the glu-plasminogen binding capacity of these cells. However, if the presence of a C-terminal lysine residue is important for plasminogen binding, then plasmin proteolysis of these latent receptors might be critical for the glu-plasminogen binding capacity of these cells. The MCF-7 cells only express low levels of uPA at their cell surface, whilst MDA-MB-231 cells express relatively high levels of cell surface uPA. Thus, the high plasminogen binding capacity of the MDA-MB-231 cell line may be due to creation new cell surface C-terminal lysine residues via the activation glu-plasminogen which is bound to the latent plasminogen receptors. In contrast, cells that express low levels of uPA (e.g. MCF-7 cells) would need to express group 1 plasminogen receptors such as cytokeratin 8 to bind glu-plasminogen.

It is clear from both the literature and this study that a number of proteinaceous and possibly non-proteinaceous moieties are likely to contribute to cell-surface glu-plasminogen binding. It is therefore difficult to design specific experiments aimed at establishing a direct relationship between glu-plasminogen binding capacity and metastatic potential without identifying all of the glu-plasminogen binding moieties on the breast cancer cell lines. However, Stonelake *et. al.*, (1997) recently demonstrated that in the presence of glu-plasminogen, MDA-MB-231 and other metastatic breast cancer cell lines, unlike non-metastatic cell lines such as MCF-7 and T-47D, had the ability to degrade human endothelial basement membrane and that this activity was significantly inhibited by specific uPA or

plasmin inhibitors. These authors also demonstrated a similar inhibitory effect by lysine analogues and attributed this result to an inhibition of glu-plasminogen binding. These studies strongly corroborate the data presented which indicates that the glu-plasminogen binding capacity of breast cancer cells modulates plasmin activity in the presence of uPA, and taken together, establishes a relationship between the glu-plasminogen binding capacity of breast cancer cells and their migration and metastatic potential *in vitro* and *in vivo.*

Another explanation for the apparent differences in cell-surface glu-plasminogen binding capacity of the metastatic MDA-MB-231 cells versus the non-metastatic MCF-7 and T-47D cells is possibly related to the ability of cells to target some or all of their plasminogen binding proteins to the cell surface. One possible mechanism may be linked to the difference in uPA/uPAR status between the cell lines. Incubation of the WISH epithelial cell line, which expresses high levels of uPAR but undetectable levels of uPA, with inactive uPA resulted in the phosphorylation and redistribution of the cytoskeletal components cytokeratin 8 and 18 (Busso *et. al.,* 1994). From this it was suggested that signal transduction pathways via the uPAR GPI anchor are involved in cell migration (Busso *et. al.,* 1994). Since cytokeratin 8 has been shown to be a plasminogen receptor associated with the external surfaces of human breast cancer cells (Hembrough *et. al.,* 1995, 1996), signal transduction events associated with uPAR expression may be one mechanism that leads to an increased capacity of breast cancer cells, such as the MDA-MB-231 cells, to bind cell-surface glu-plasminogen.

It is conceivable that variations in the activity of intracellular signaling proteins due to EGFR and *erb*B-2/*neu* expression may initiate events that lead to the translocation of proteins within the cell (Milligan *et. al.,* 1995), and may in some way affect the localisation of proteins that can act as plasminogen receptors if placed in the

correct orientation at the cell surface. Amino- or carboxy-terminal lysines appear to be the only feature common and necessary to all candidate plasminogen binding proteins. Any intracellular protein with an amino- or carboxy-terminal lysine that is also subject to the above modifications could be translocated to the outer surface of the plasma membrane and act as a plasminogen receptor.

Other markers whose cellular expression correlates with metastatic potential have been identified in human breast cancer cell lines. These include matrix metalloproteinase-2 (Azzam *et. al.,* 1993), vimentin (Thompson *et. al.,* 1992), and surface glycoproteins such as CD44 (Culty *et. al.,* 1994). However, a review of the literature indicates that overexpression of components of the plasminogen activation cascade play an important role in breast cancer invasion and metastasis (section 1.3). While stromal cells adjacent to cancer cells in breast cancer tissue may contribute to invasion, by expressing uPA and/or uPAR for example (Christensen *et. al.,* 1996; Costantini *et. al.,* 1996; Nielsen *et. al.,* 1996), data presented here and that of others (Holst-Hansen *et. al.,* 1996; Stonelake *et. al.*, 1997), clearly shows that breast epithelial cells with a metastatic phenotype have the capacity to efficiently convert glu-plasminogen to plasmin. A combination of studies suggest that the concentration and spatial distribution of the combination of uPA, uPAR, PAI-1, and PAI-2 expression in human breast carcinomas allows a better indication of degree of malignancy (Foekens *et. al.,* 1995; Christensen *et. al.,* 1996; Costantini *et. al.,* 1996). The results presented in this chapter strongly suggest that another component of the plasminogen activation cascade, the plasminogen receptors, are also important.

Chapter 6

General discussion, summary and future directions.

The roles of most of the plasminogen activation cascade components in breast cancer metastasis have been well documented (Foekens *et. al.,* 1995; Christensen *et. al,* 1996; Costantini *et. al.,* 1996). Overexpression of many components of this cascade have been associated with aggressive breast cancer and probably play an important role in metastasis (Duffy, 1993). Glu-plasminogen has been demonstrated on the cell-surface of human mammary carcinoma tissue (Burtin *et. al.,* 1993). However, data regarding the class of proteins that are responsible for localising glu-plasminogen to the cell-surface and the mechanism of binding remains rudimentary. The objective of this dissertation was to develop a physiologically relevant model for the interaction of glu-plasminogen with the surface of metastatic human breast cancer cells. Hence, an *in vitro* glu-plasminogen binding model was developed which may be used to predict the mechanism of glu-plasminogen binding to the surfaces of cells such as highly metastatic breast cancer cells which have a high glu-plasminogen binding capacity.

6.1 α-Enolase as a plasminogen receptor molecule

A plasminogen receptor molecule has been isolated from the surface of U-937 monocytoid cells and identified by N-terminal amino acid sequencing as a human α-enolase related molecule (ERM; Miles *et. al.,* 1989). The apparent mass of ERM was 54 kDa, whereas cytoplasmic α-enolase has a molecular mass of 46 kDa. The difference in mass between ERM and α-enolase suggested that these two proteins may not be identical. The increased mass of ERM is not due to the differential

splicing of the ENO1 gene (Redlitz *et. al.*, 1995). However, if ERM and α-enolase are the same molecule then the apparent mass discrepancy may be due to a post-translational modification of cytoplasmic α-enolase necessary for its translocation to the cell surface. R-α-enolase, expressed in *E.coli*, had glu-plasminogen binding activity that was homologous to the ERM (table 2.1; Redlitz *et. al.*, 1995). This suggested that the human ENO1 gene product did encode an authentic glu-plasminogen binding protein and that post-translational modification of α-enolase was not necessary for its glu-plasminogen binding activity. Furthermore, differential gene splicing of α-enolase was not necessary for the glu-plasminogen binding activity of the protein, since the gene product of ENO1 possesses a C-terminal lysine residue. These data suggest that the amino acid sequence of α-enolase is sufficient for glu-plasminogen binding *in vitro*.

6.2 A model describing the interaction of glu-plasminogen with cell surface receptors.

As reviewed in section 1.1.1.2, glu-plasminogen exists in a closed, lysine-dependent, right-handed, spiral structure with an apparent maximum intramolecular distance of 9.1 nm. Multiple LBS motifs of glu-plasminogen may be responsible for interacting with internal lysine residues of the zymogen forming an intramolecular lysine binding network which maintains the closed conformation of glu-plasminogen. Using small lysine analogues it is well established that glu-plasminogen can undergo a lysine-dependent conformational change to a more activation-susceptible conformation (see section 1.1.1.5). Thus, the conformation of glu-plasminogen does not remain constant during a lysine-dependent interaction.

Receptors usually display high affinity for their specific ligands, with the notable exceptions of the integrin family of receptors and the cellular plasminogen receptor family. Glu-plasminogen binds to α-enolase in a lysine-dependent manner

with an apparent low affinity. Nevertheless, significant amounts of glu-plasminogen bind in a lysine-dependent manner to the cell surface plasminogen receptors since the apparent K_d for the interaction is similar to the concentration of glu-plasminogen in the blood (Chapter 2; Chapter 5; Miles *et. al.,* 1988). Thus, the low affinity binding of glu-plasminogen to these cellular receptors is physiologically relevant. This low affinity interaction dictates that glu-plasminogen would rapidly dissociate from its receptor. However, this may not be the case, since the exogenous lysine-mediated interactions of glu-plasminogen result in a conformational change, a slow on rate may not equate to a rapid off rate. If this is correct then glu-plasminogen should remain bound to the cell surface receptors for a significant period of time.

Kinetically, the binding of glu-plasminogen to α-enolase is explained using a two-step competitive interaction model (Chapter 3). The initial phase involves a competition reaction between an internal lysine residue of glu-plasminogen and an exogenous lysine residue from α-enolase. This induces a conformational change in glu-plasminogen. However once dissociated, the internal lysine residue of glu-plasminogen may competitively dissociate the bound exogenous lysine residue of the receptor from the kringle LBS unless a second interaction occurs. A second lysine-dependent binding event between another kringle domain LBS of glu-plasminogen and a second exogenous lysine residue from α-enolase stabilises the interaction such that glu-plasminogen cannot dissociate directly from α-enolase without undergoing a lysine-dependent conformational change that induces a more closed conformation.

Of the known plasminogen receptors only α-enolase and cytokeratin 8 possess a C-terminal lysine residue (table 1.4), which has been identified as an epitope involved in the plasminogen binding activity of α-enolase. The latent plasminogen receptors (class 2 receptors; table 1.4), which do not posses a C-terminal lysine

residue, may be modified at the cell surface by plasmin thereby creating a new C-terminal lysine residue on these receptors (see section 1.2.5). Since, α-enolase has a C-terminal lysine residue, the glu-plasminogen kinetic binding data can be used as a model for both group 1 and plasmin modified group 2 plasminogen receptors.

C-terminal lysine residues from class 1 plasminogen receptors or plasmin-modified class 2 receptors may act to stabilise both the binding and conformation of glu-plasminogen. The kringle domain LBS motifs of glu-plasminogen have different affinities for the different classes of lysine residues with the internal lysine analogue displaying the lowest affinity for all of the plasminogen LBS (table 1.1). Since the closed conformation of glu-plasminogen is maintained by the low affinity interactions of internal lysine residues with the LBS motifs of glu-plasminogen, the presence of a C-terminal lysine residue would efficiently bind to the LBS thereby stabilising both the binding and activation-susceptible conformation of receptor bound glu-plasminogen.

However, the interaction of glu-plasminogen with the cell surface may not be as simple as this two-step binding model suggests, since the majority of cell surface plasminogen receptor candidates are group 2 receptors (e.g. actin and annexin II) and require plasmin proteolysis for the acquisition of a C-terminal lysine residue. Indeed the known class 1 plasminogen receptors (e.g. α-enolase) were not present on the metastatic breast cancer cell surfaces. However, other unidentified class 1 plasminogen receptor molecules may be present at the surfaces of these cells. Since known class 1 plasminogen receptors were not present on the MDA-MB-231 cell surface the mechanism of glu-plasminogen binding to the cell surface may be linked to an activation event. For example, the initial competitive binding of glu-plasminogen to a class 2 plasminogen receptor may occur followed by plasminogen activation. Once formed plasmin may create a new C-terminal lysine residue capable

of the second lysine-dependent binding event that stabilises the interaction. Alternatively, glu-plasminogen may cross-link several plasminogen receptors before resulting in the open activation-susceptible conformation of glu-plasminogen. Despite this uncertainty, the lysine-dependent binding of glu-plasminogen to the surfaces of metastatic breast cancer cells involves a competition reaction between the internal lysine residues of glu-plasminogen and the lysine residues of the receptors that results in an increase in the activation rate of bound glu-plasminogen.

To understand the precise mechanism of glu-plasminogen binding to the cell surface or fibrin the spatial relationships of the kringle domains in the closed, partially open and open conformations of glu-plasminogen must be defined. Similarly, the conformation(s) of glu-plasminogen at the cell surface as well as the kringles responsible for localising glu-plasminogen to the cell surface must be defined. However, experiments designed to answer these questions must not perturb the intramolecular lysine binding network of glu-plasminogen.

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