Interaction of Basic Fibroblast Growth Factor with Mammary Cells

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by

Hai-Lan Chen

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To my parents with love

ABSTRACT

Basic fibroblast growth factor (bFGF) is the prototypic member of a family of heparin-binding growth factors which play a key role in many growth and developmental events. For example, bFGF has been implicated in the regulation of the growth of the mammary ductal tree. bFGF has two types of receptors, high-affinity tyrosine kinase receptors and low-affinity heparan sulphate (HS) proteoglycan receptors , which together constitute a dual receptor system. How the cells of the mammary gland may alter their expression of the different components of the dual receptor system for FGFs is not known and there is as yet no consensus as to the mechanism of action of the dual receptor system for FGFs. The aim of this thesis is to investigate the interaction of FGFs with cells from a model of rat mammary development. Thus it is hoped to identify some of the features of the receptors for FGFs that are involved in controlling the growth of responsive mammary cells, and hence, ultimately, certain aspects of the growth and morphogenesis of the mammary gland.

The initial work in this thesis identified the general properties of the signal(s) generated by FGFs that result in the stimulation of DNA synthesis in rat mammary (Rama) 27 fibroblasts: (i) a half-life of 1-2 h; (ii) continuous production of the signal(s) is necessary for DNA synthesis to occur; (iii) equivalence between the signal(s) produced by bFGF and epidermal growth factor; (iv) a difference in the sensitivity of the signal(s) at early and late times to growth factor concentration.

The role of HS in the dual receptor system was then investigated using two approaches. Firstly, the ability of heparin to restore the growth-stimulatory activity of FGFs in HS-deficient Rama 27 fibroblasts (the activation of FGFs) was examined. Whilst there is a clear requirement for the polysaccharide in the dual receptor system, the results show clearly that the binding of bFGF to the high-affinity receptor is not polysaccharide-dependent. Moreover, soluble polysaccharide can only partially substitute for cell-surface HS proteoglycan receptor. Secondly, HS was purified from a series of rat mammary cell lines and from different parts of the cells. For the most part, the ability of the HS to activate bFGF correlated with the known bFGF-binding and extracellular matrix-synthesis properties of the cells. However, the high activity of HS secreted by Rama 37 epithelial cells and the absence of any activity in the heparan sulphate secreted by the Rama 27 fibroblasts suggests that mammary cells may be able to regulate which particular HS proteoglycans they shed from their cell surfaces.

Finally, the post-receptor processing of bFGF by rat mammary cells was examined. After binding to its cellular receptors, bFGF is processed to polypeptides of Mr 12 kDa and 9 kDa. Moreover, the production of the 12 kDa and 9 kDa polypeptides is dependent on the presence of cellular or soluble polysaccharide receptor, and the nuclear uptake of these polypeptides is itself dependent upon the presence of intact cellular HS proteoglycan receptors.

A simple model is proposed that accounts for the above observations on the dual receptor system for FGFs. The essence of this model is that tyrosine kinase receptors for bFGF on their own can bind bFGF with high affinity but produce growth-stimulatory signals inefficiently. In contrast, in the presence of both the tyrosine kinase receptor for bFGF and the HS proteoglycan receptor for bFGF, growth-stimulatory signals are produced efficiently.

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CONTENTS

ABBREVIATIONS

aFGF	Acidic Fibroblast Growth Factor
bFGF	Basic Fibroblast Growth Factor
BM	Binding Medium
BSA	Bovine Serum Albumin
CAM	Calmodulin-dependent protein
CS	Chondroitin Sulphate
DEAE	Diethylaminoethyl
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
EDTA	Ethylenediamine Tetra-acetic Acid
EGF	Epidermal Growth Factor
FCS	Foetal Calf Serum
FGFR	Fibroblast Growth Factor Receptor
GAG	Glycosaminoglycan
GAP	Ras GTP activating protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GalN	D-galactosamine
GalNAc	2-deoxy-2-acetamido-D-galactose (N-acetyl-D-galactosamine)
GlcA	D-glucuronic acid
GlcN	D-glucosamine
GlNAc	2-deoxy-2-acetamido-D-glucose (N-acetyl-D-glucosamine)
HA	Hyaluronic acid
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HGF	Hepatocyte growth factor
HS	Heparan Sulphate

HSPG	Heparan Sulphate Proteoglycan
IdoA	L-iduronic acid
IP_3	Inositol 1,4,5 Triphosphate
kbp	kilobase pairs
K_d	Dissociation Constant
kDa	kiloDaltons
MAP	Microtubule-associated protein
MK buffer	$MgCl_2$ and KCl buffer
MKT buffer	$MgCl_2$, KCl and Triton-X-100 buffer
-NSO_3	N-sulphate group
-OSO_3	O-sulphate, ester sulphate group
PAGE	Polyacrylamide Gel Electrophoresis
PGs	proteoglycans
PI	Phosphatidylinositol
PKC	Protein kinase C
PLC	Phospholipase C
Rama	Rat mammary
RM	Routine Medium
Ser	Serine
Thr	Threonine
S-free BM	Sulphur-free Binding Medium
S-free RM	Sulphur-free Routine Medium
S-free SDM	Sulphur-free Step Down Medium
SDM	Step Down Medium
SDS	Sodium Dodecyl Sulphate
TCA	Trichloroacetic Acid
SH2	src-homology domain 2
TEMED	N,N,N',N', Tetramethylethylenediamine

Tris	Tris(hydroxymethyl)methylamine
Tween 20	Polyoxyethylenesorbitan monolaurate

Chapter One

General Introduction

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General Introduction

1.1 Overview

Many growth factors have been identified in the last 20 years and a large number of functions have been ascribed to this group of molecules. The polypeptide growth factors are analogous to hormones, since they carry information from the producing cell to target cells that have cognate receptors. However, growth factors are usually local rather than systemic agents, and the activity of the growth factors is generally thought to be related to cell growth and migration rather than metabolism, although this distinction may reflect the assays used *in vitro* rather than their true biological function. The identification of growth factors has proceeded in two ways. Firstly, growth-stimulatory activities have been identified, and then purified. Secondly, gene transfer, particularly in the 3T3 system, has identified "oncogenes" many of which are in fact growth factors.

1.2 Background

1.2.1 The FGF family of growth factors

1.2.1.1 General

The fibroblast growth factors (FGFs) are a family of heparin-binding polypeptides which currently contains nine members: acidic fibroblast growth factor (aFGF or FGF-1), basic fibroblast growth factor (bFGF or FGF-2), *int*-2 (FGF-3), *hst*/KS53 (K-FGF or FGF-4), FGF-5, FGF-6, keratinocyte growth factor (KGF or FGF-7), androgen-induced growth factor (FGF-8) and glial-activating factor (FGF-9) (Fig.1.1). The name, FGF, was derived from the assay originally used to identify the growth-promoting activity, the stimulation of DNA synthesis in fibroblasts (Gospodarowicz, 1974; Rudland *et al.,* 1974). It has since become evident that the FGFs have a broader range of target cells, including many cells of mesodermal and neuroectodermal origin (Gospodarowicz, 1983; Gospodarowicz *et al.,* 1978a, 1986) as well as some endodermal cells (Flint *et al.*, 1994). The putative functions, *in vivo*, of the FGFs include the regulation of the induction of many embryonic tissues including the mesoderm, limb bud formation and the differentiation of neural cells and in the adult, angiogenesis and wound repair (e.g. Buckley-Sturrock *et al.,* 1989; Davidson *et al.,* 1985; Finklestein *et al.,* 1988; Folkman and Klagsbrun, 1987; Kimmelman *et al.,* 1988; Montesano *et al.,* 1986; Slack *et al.*, 1988). In cultured cells the FGFs have been shown to promote not just cell proliferation and differentiation, but also cell migration and survival (Burgess and Maciag, 1989; Davidson *et al.,* 1985; Thompson *et al.,* 1988).

The observation of the correlation of the expression of FGFs and their receptors with numerous morphogenic events has suggested that these growth factors play an important role in embryonic development. Synthesis of FGF-like molecules has been studied extensively in the *Xenopus* oocyte and early stage *Xenopus* embryos. FGFs in the

Xenopus oocyte are thought to be responsible for mesoderm formation (Kimmelman and Kirschner, 1987; Kimmelman *et al.*, 1988; Slack *et al.*, 1987). Thus the addition of bovine or human bFGF to *Xenopus* embryos at stage 8 mimics the mesoderm-inducing signal of the ventrovegetal pole (Kimmelman and Kirschner, 1987; Kimmelman *et al.*, 1988; Slack *et al.*, 1987). The addition of neutralizing antibodies or the expression of dominant negative mutant FGF receptors inhibits the formation of mesoderm, causally implicating the endogenous *Xenopus* FGF (Tanaka *et al.*, 1990; Kawamoto *et al.*, 1992; Yamanishi *et al.*, 1991). Later on in development, FGFs and their receptors seem to be involved in modulating epithelial-mesenchymal interactions, for example, in limb bud formation and kidney development (Munaim *et al.*, 1988; Risau and Ekblom, 1986).

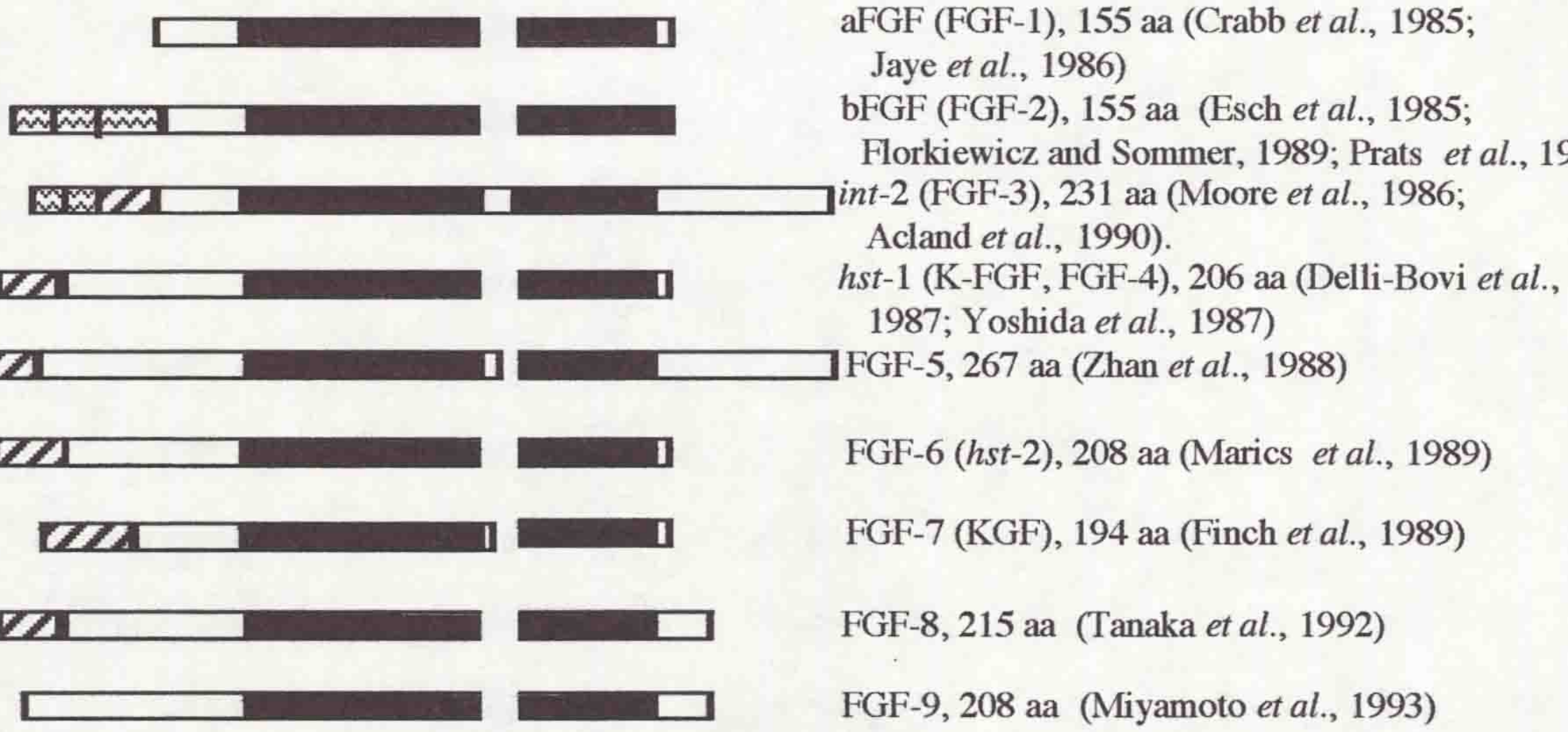


Fig. 1.1 The structure of the nine FGFs

■ Core structure of FGFs that contains the regions of the molecules required for binding to cell-surface receptors. This core structure does not, however, contain a secretory signal sequence. ▨ Secretory signal sequence. ▩ N-terminal extensions translated from upstream CUG codons which provide a nuclear localization sequence that overrides other subcellular localization signals and thus directs the extended forms of bFGF or *int*-2 to the nucleus (Acland *et al.*, 1990; Florkiewicz and Sommer, 1989; Prats *et al.*, 1989). The lengths of the primary translation products initiated from the AUG codons are also shown. This figure is adapted from Fernig and Gallagher, (1994).

1.2.1.2 The discovery of the prototypic FGFs

As early as 1939, extracts of brain were found to be a rich source of factors that promoted fibroblast proliferation *in vitro* (Trowell and Willmer, 1939; Hoffman, 1940). It was only in the early 1970s that the first growth factors were purified (Savage *et al.*, 1972; Taylor *et al.*, 1970). In 1974, Gospodarowicz and colleagues characterized a polypeptide from bovine pituitary (Gospodarowicz, 1974, 1975; Rudland *et al.*, 1974) and later from brain (Gospodarowicz *et al.*, 1978b) which stimulated the growth of 3T3 fibroblasts. Thomas and coworkers were independently able to detect the presence of a polypeptide growth factor with an acidic isoelectric point from acid extracts of bovine brain (Thomas *et al.*, 1980) and this polypeptide was named acidic FGF (aFGF).

When bovine bFGF was first sequenced it was reported to be a polypeptide of 146 amino acids with a molecular weight of about 16,500 (Esch *et al.*, 1985). However, determination of the actual molecular weight of bFGF was complicated by the susceptibility of the polypeptide to protease-mediated truncations. In particular, acid extraction of tissues, a widely used method in bFGF purification (Bohlen *et al.*, 1984; Esch *et al.*, 1985; Gospodarowicz, 1984), resulted in the activation of acid proteases that degraded bFGF at the N-terminal end and yet generated biologically active truncated forms of bFGF. Not all preparations of bFGF were truncated- : some, using acid extraction were N-terminally blocked (N-acetyl alanine) and so were presumably full-length, and others used protease inhibitors or extraction at neutral pH produced similar molecules (Klagsbrum *et al.*, 1987; Smith *et al.*, 1984a; Ueno *et al.*, 1986). Subsequent analysis of non-truncated bFGF showed bFGF to be a 155 amino acid polypeptide extended by 9 amino acids N-terminal to the 146 amino acid form (Ueno *et al.*, 1986). The cloning of bovine, human and rat cDNAs encoding bFGF predicted a 155 amino acid polypeptide (Abraham *et al.*, 1986a,b; Kurokawa *et al.*, 1987; Shimasaki *et al.*, 1988). The first 23 amino acids of bFGF are not necessary for biological activity since a highly truncated form of bFGF (24-155), isolated from adrenal

gland, kidney and corpus luteum, was still biologically active (Baird *et al.*, 1985; Gospodarowicz *et al.*, 1985, 1986).

Although bFGF was first isolated from the pituitary gland, in the mid 1980s, some 20 different growth factor activities, all with different names and isolated from different tissues and cell lines were subsequently found to be identical to bFGF (Burgess and Maciag, 1989). Thus bFGF may be among the most widely distributed growth factor in the body. The ubiquity of bFGF in normal adult tissues that are quiescent suggests that the activity of this growth factor must be highly regulated under normal physiological conditions.

1.2.1.3 Structure of the bFGF (FGF-2) polypeptides, mRNA and gene

1.2.1.3.1 Structure of the bFGF polypeptides

The bFGF originally characterized (Section 1.2.1.2) is a single chain, non-glycosylated, cationic polypeptide with a molecular weight of 18 kDa and a pI of 9.6 (Esch *et al.*, 1985). This part of the bFGF polypeptide contains the regions of the molecules that are required for binding to cell-surface receptors (Fig. 1.2). However 18 kDa bFGF does not contain a secretory signal sequence and how it is secreted by cells remains a source of controversy (Section 1.2.4).

Higher-molecular-weight forms of bFGF containing more than 155 amino acids have been isolated from guinea pig brain, rat brain, hepatoma cells and H-*ras*-transformed Rat-1 cells (Prats *et al.*, 1989; Moscatelli *et al.*, 1987, 1988; Presta *et al.*, 1988; Iberg *et al.*, 1989; Sommer *et al.*, 1987). These higher-molecular-weight forms of bFGF are N-terminal extensions of up to 55 amino acids that are generated by an unusual mechanism involving three CUG translation initiation codons (Prats *et al.*, 1989; Florkiewicz and Sommer, 1989) which are upstream of the AUG that directs the translation of the 18 kDa polypeptide. All the initiation codons are active since the transcription and translation, *in vitro*, of a full-length cDNA of 7 kb from human hepatoma cells encoding bFGF generates four polypeptides with molecular weights of

18 kDa, 21 kDa, 22.5 kDa and 24 kDa (Prats *et al.*, 1989). There is no evidence for the N-terminal extensions of bFGF acting as secretory signal sequences. However, transfection experiments indicate that the N-terminal extensions provide a nuclear localization sequence that overrides other subcellular localization signals and thus directs, posttranslationally, the extended forms of bFGF to the nucleus (Florkiewicz and Sommer, 1989; Powell *et al.*, 1991; Prats *et al.*, 1989, 1992; Renko *et al.*, 1990; Tessler and Neufeld, 1990) (Figs. 1.1, 1.2).

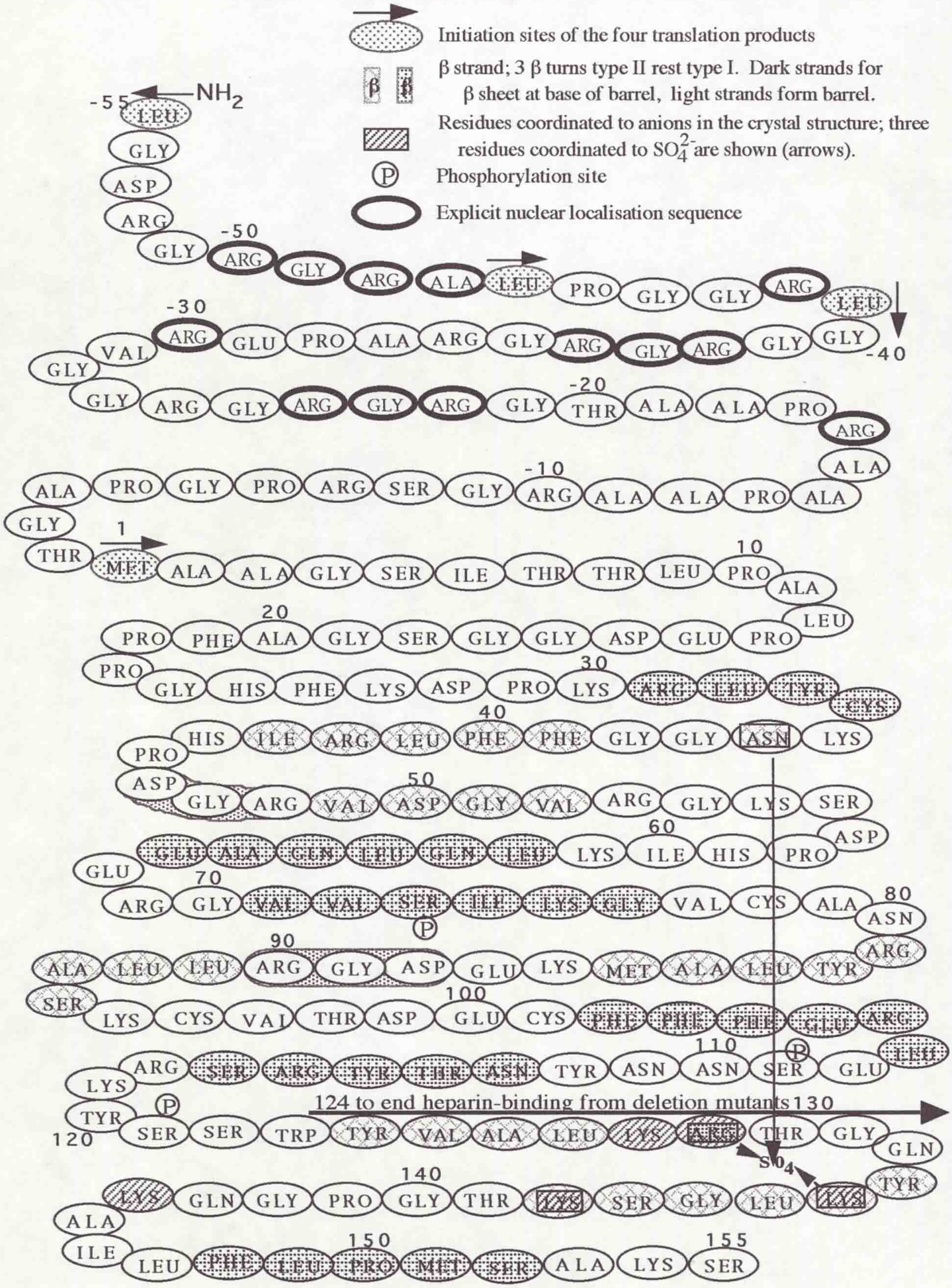
The 18 kDa bFGF polypeptide is N-terminally acetylated and has four cysteines (Fig. 1.2) (Esch *et al.*, 1985). Two of the four cysteine residues in bFGF (Cys 34 and Cys 101) are conserved in all members of the FGF family (Burgess and Maciag, 1989). The cysteine residues are not involved in disulphide bonding and treatment with reducing agents does not diminish biological activity (Hauschka *et al.*, 1986). There is also evidence for phosphorylation sites for protein kinase C at Ser^{73} and at Ser^{109} , the latter being a cryptic site (Feige *et al.*, 1989, 1991) and for protein kinase A at Ser^{121} (Feige *et al.*, 1989). The phosphorylation of these sites of bFGF has been reported to occur not just *in vitro*, but also in cultured cells where an ecto kinase is thought to be responsible for the observed phosphorylation (Feige and Baird 1989; Mascarelli *et al.*, 1989). Moreover, the specificity of phosphorylation of bFGF *in vitro* (protein kinase A versus protein kinase C) is altered by extracellular matrix components (Feige *et al.*, 1989), so some aspect of bFGF function may be regulated by phosphorylation. There are also two inverted consensus sequences for fibronectin binding (Asp-Gly-Arg) (Fig. 1.2).

1.2.1.3.2 bFGF mRNA and gene

Genomic mapping indicates that there is only one bFGF gene, and it is located on human chromosome 4 (Mergia *et al.*, 1986). The bFGF gene, which appears to be greater than 34 kb, possesses three exons separated by two large introns that are situated between the codons encoding amino acid residues 60 and 61 and residues 94 and 95 (Abraham *et al.*, 1986; Gospodarowicz *et al.*, 1987).

Fig. 1.2 Primary structure of bFGF

Amino acids -53 to 1 correspond to human and guineapig bFGF and 1 - 155 correspond to bovine bFGF



In some systems a single mRNA encoding bFGF is observed, e.g., rat mammary cells (Barraclough *et al.*, 1990), whilst in others there are two to four transcripts, e.g., human mammary cells (Ke *et al.*, 1993; Li and Shipley, 1991; Valverius *et al.*, 1990). Such heterogeneity may be due to the utilization of different polyadenylation sites (Kurokawa *et al.*, 1987). In *Xenopus* the 1.1-1.5 kb mRNA represents an antisense transcript of the bFGF gene, one exon of which overlaps the third bFGF exon (Kimelman and Kirshner, 1989; Volk *et al.*, 1989). The antisense transcript is translated into a 25 KDa protein in *Xenopus* (Kimelman and Kirshner, 1989) but has yet to be detected in other organisms (Ke *et al.*, 1993). However, the nucleotide sequence of the part of the third exon of bFGF where the sense and antisense transcripts overlap is highly conserved through evolution, and so the antisense transcript is likely to be produced in organisms other than *Xenopus*.

1.2.1.4 Structure of the aFGF (FGF-1) polypeptides, mRNA and gene

The sequence homology between bFGF and aFGF is 53% at the amino acid level (Esch *et al.*, 1985). Acidic FGF is a single chain, anionic polypeptide with a pI of 5.6 and a molecular weight of approximately 18 kDa (Crabb *et al.*,1985; Jaye *et al.*,1986). Most biological properties of aFGF are similar to those of bFGF (Burgess and Maciag 1989; Gospodarowicz *et al.*,1986) and they have long been thought to act through the same cell-surface receptor molecules (Neufeld and Gospodarowicz 1986; Ruta *et al.*,1989). aFGF also does not have a consensus signal sequence for secretion. Human aFGF contains three cysteine residues of which two are those conserved throughout the FGF family (Cys^{34} and Cys^{101}) (Fig. 1.2).

Analysis of aFGF cDNAs cloned from human libraries indicates similarity with cDNAs encoding bFGF not just at the level of sequence homology (Jaye *et al.*, 1986; Mergia *et al.*, 1986; Wang *et al.*, 1989) but also at the level of overall structure. Thus there are multiple mRNA species encoding aFGF (Myers *et al.*, 1993; Payson *et al.*, 1993). However, the aFGF cDNA open reading frame is definitively flanked by

termination codons so that the existence of higher molecular weight forms of aFGF is unlikely (Jaye *et al.*, 1986; Mergia *et al.*, 1986; Wang *et al.*, 1989). The single aFGF gene has three exons separated by two large introns in identical positions to those of bFGF which suggests that they are derived from a common ancestral gene. The aFGF gene maps to human chromosome 5 whilst the bFGF gene is found on chromosome 4.

1.2.1.5 Other members of FGF family

Since the determination of the partial amino acid sequences of the aFGF and bFGF polypeptides and the characterisation of the sequences of their corresponding cDNAs, seven other members of the FGF family have been discovered. Many of the other FGFs have been identified by the cloning of their cDNAs in transformation experiments and it is only subsequently that their functions in normal tissues have begun to be appreciated.

Int-2 (FGF-3)

The product of the *int*-2 gene was the third member of the FGFs family identified (Moore *et al.*,1986; Acland *et al.*,1990; Dickson *et al.*,1987; Smith *et al.*,1988; Wilkinson *et al.*,1988). *Int*-2 is a cellular gene that is induced to become transcriptionally active after integration (*int*) of the mouse mammary tumour virus into the mouse genome (Dickson *et al.*,1984). It codes for a protein of 240 amino acids with a 44% and 38% homology to the internal core of the primary sequence of bFGF and aFGF, respectively (Fig.1.1). The principle differences are the addition of a 16 amino acid sequence at the N-terminus and a 45 amino acid extension in the C-terminal region. The amino terminal extension contains a short non-charged sequence which acts as a signal sequence.

As with bFGF, N-terminally extended forms of *int*-2 are also found (Fig. 1.1). These extensions are translated from alternative CUG initiation codons and provide a nuclear localization sequence which directs the extended forms of *int*-2 to nucleus, overriding the secretory signal encoded from the AUG codon (Acland *et al.*, 1990).

hst/KS53 (K-FGF or FGF-4)

The fourth member of the FGFs family was identified as genomic DNA fragments derived from a human stomach tumour (*hst*) and a Kaposi's sarcoma lesion capable of inducing murine 3T3 cell transformation *in vitro* (Delli-Bovi and Basilico 1987; Delli-Bovi *et al.*,1987, 1988; Sakamoto *et al*, 1986; Taira *et al.*, 1987; Yoshida *et al*, 1987). The open reading frame encodes a polypeptide of 206 amino acids with 43, 38 and 40% amino acid sequence homology to bFGF, aFGF and *int* -2, respectively. Again the homologous sequences correspond to the central 18 kDa portion of the bFGF polypeptide (Fig. 1.1). The N-terminal domain of *hst* is extended, compared to that of bFGF, by the addition of 55 amino acids which includes a secretory signal peptide sequence. The *hst* polypeptide also possess a short COOH-terminal extension. The mature protein contains 175 amino acids (23 kDa), is glycosylated and is secreted into the medium of cells transfected with the relevant cDNA. The expression of *hst* /K-FGF is seen to increase in the mid stage of embryogenic development of the mouse but is absent from normal adult tissues. *hst*/KS53, like *int*-2 is located on chromosome 11 (Yoshida *et al.*,1989).

FGF-5

FGF-5 was isolated from the DNA of a human bladder tumour (Zhan *et al.*,1988). It contains 267 amino acids with an extensive hydrophobic sequence in the amino terminal region. The homology at the amino acid level to the core of aFGF and bFGF is 42% and 45%, respectively. The expression of FGF-5 is limited to neonatal brain and some human tumour lines (Klagsbrun, 1989).

FGF-6

This oncogene was isolated from a mouse cosmid library by screening with the *hst* gene (Marics *et al.*,1989) and accordingly shares a 70% homology at the amino acid level to the *hst* product. FGF-6 maps to chromosome 12.

KGF (FGF-7)

The cDNA for KGF was isolated from keratinocytes and contains an open reading frame encoding a 194 amino acid polypeptide with an estimated molecular weight of 22 kDa (Finch *et al.*,1989). KGF shares 37% and 39% amino acid homology to aFGF and bFGF. The N-terminus of KGF is extended compared to the core of bFGF and contains a putative hydrophobic secretion signal. KGF mRNA is present in a number of stromal fibroblast cell lines derived from embryonic neonatal and adult sources but is absent from a variety of normal glial cells and epithelial cell lines (Finch *et al.*, 1989). It was originally thought that the activity of KGF was specific for keratinocytes and epithelial cells (Rubin *et al.*, 1989). However, it has since become apparent that in adult tissues the underlying theme of KGF expression is that it is produced by stromal fibroblasts but acts on the cells of the epithelium (Bottaro *et al.*, 1990; Colemankrnacik *et al.*, 1994; Marchese *et al.*, 1990; Ron *et al.*, 1993; Taylor *et al.*, 1993)

FGF-8

Androgens were found to induce the secretion of a number of bFGF-like growth factors by an androgen-dependent mouse mammary carcinoma cell line, Shionogi carcinoma-3 (Tanaka *et al.*, 1992). These bFGF-like growth factors exhibited a significant autocrine growth-stimulating property on the Shionogi carcinoma-3 cells (Nonomura *et al.*, 1988, 1990). One of these activities was isolated and characterised as FGF-8 which shares 30-40% homology at the amino acid level with the other members of the FGF family (Tanaka *et al.*, 1992) (Fig. 1.1).

FGF-9

FGF-9 is sometimes referred to as glial-activating factor (GAF) and was originally purified from the culture supernatant of a human glioma cell line (Miyamoto *et al.*, 1993). Its amino acid homology to the other members of the FGF family is around 30%. Like aFGF and bFGF it has no secretory signal sequence, although it is released from cells (Miyamoto *et al.*, 1993).

1.2.1.6 Structure-function relationships in FGFs

Using a series of synthetic peptides, two regions of 155-amino acid bFGF have been proposed to be involved in mediating receptor-binding and hence mitogenic activity on different cell types, including fibroblasts, neural cells and endothelial cells. These regions cover amino acid residues 33-77 and 115-124 of bovine bFGF (Baird *et al.*, 1988) and the corresponding synthetic peptides act either as partial bFGF agonists or as antagonists in DNA synthesis assays on 3T3 fibroblasts (Baird *et al.*, 1988). Moreover, the binding of bFGF to cellular receptors is inhibited by a peptide corresponding to the amino acid sequence 112-155 of bFGF, but not by the region of amino acid residues 33-77 suggesting that the region of amino acid residues 115-124 represent the core sequence required for the binding of bFGF to its cellular receptors (Baird *et al.*, 1988; Walicke *et al.*, 1989). In another study two synthetic peptides encompassing amino acid residues 38-61 and 82-101 have been investigated (Presta *et al.*, 1991). Both peptides have a weak agonistic effect and are able to antagonise the stimulation of cell growth by bFGF. Antibodies raised to these synthetic peptides are able to specifically inhibit the growth-stimulatory effects of bFGF. However, the high concentrations of synthetic peptides (100-300 μg/ml) used in the above studies does raise the possibility that some of the results may be experimental artefacts.

The analysis of the activity of various recombinant mutant bFGFs suggests that the above results obtained with synthetic peptides may not be entirely correct. Thus other important sequences located at the amino terminal region of bFGF (residues 27-32) (Isacchi *et al.*, 1991) have been found to mediate the biological activity of bFGF. In addition, a recombinant bFGF mutant has been produced in which basic residues Arg^{118}, Lys^{119}, Lys^{128} and Arg^{129} have been replaced with neutral glutamine residues. This bFGF mutant (M6B-bFGF) has been used for a detailed assessment of the role exerted by these four basic amino acids in mediating the biological properties of bFGF (Presta *et al.*, 1992). When compared to wild-type bFGF, the M6B-bFGF mutant lacked significant angiogenic activity *in vivo*. However, in cultured endothelial cells, it

demonstrated a similar receptor-binding capacity and mitogenic activity, but a reduced affinity for heparan sulphate (HS) low-affinity binding sites, a reduced chemotactic activity, and a reduced capacity to induce the production of urokinase-type plasminogen activator (Presta *et al.*, 1992). Seno *et al.* (1990) have produced an exhaustive set of C-terminal deletion mutants of bFGF and they concluded that the residues between positions 116 and 141 played a major role in the mediation of the binding of bFGF to heparin. However, the stability and structure of the bFGF mutants has not been examined. Thus it is not clear whether the loss of activity of the mutant bFGFs is due to the modification of their amino acid sequence or due to the destabilization of their structure.

A fragment of aFGF (N-terminal to 122) cleaved by thrombin has a significantly reduced affinity for immobilised heparin as compared to wild-type aFGF and is at least 50-fold less potent at stimulating mitogenesis (Lobb, 1988). Moreover, reductive methylation of aFGF reduces its capacity to stimulate mitogenesis in Balb/C 3T3 cells, and this correlates with the modification of less than 3 of its 12 lysine residues, one of which is Lys^{119}, at the C-terminal of aFGF (Harper and Lobb, 1988). The methylated aFGF has both a decreased affinity for heparin and is a considerably less potent growth factor. The results indicate that the C-terminal region of aFGF is functionally important in both mitogenesis and heparin-binding (Lobb, 1988). Moreover, these results also show that in aFGF, Lys^{119} plays an important role in heparin binding and suggest that this residue and its local environment are involved both in the interaction of aFGF with heparin and in the delivery of a growth-stimulatory response (Harper and Lobb, 1988). Since the sequences of aFGF and bFGF are highly conserved in this region, it may be that the corresponding Lys in bFGF is similarly involved in binding to cellular receptors.

The crystal structures of aFGF and bFGF have been resolved by X-ray crystallography. The overall structure is a cylindrical barrel made up of 12 anti-parallel β-strands. bFGF exhibits an approximate 3-fold internal symmetry about the axis of the β-barrel (Ago *et al.*, 1991; Eriksson *et al.*, 1991; Zhang *et al.*, 1991; Zhu *et al.*,

1991). This 12 stranded β-barrel structure contains the structural motif of loop-β-β-β-β repeats that was first observed in the crystal structure of the cytokine interleukin-1β (Finzel *et al.*, 1989; Priestle *et al.*, 1989). Ago and coworkers (Ago *et al.*, 1991) calculated the electrostatic potential on the solvent accessible surface of bFGF to investigate the heparin binding sites and they found a highly positively charged region consisting of a β-turn (Lys^{119}-Trp^{123}), a β–strand (Tyr^{124}-Arg^{129}), a short β–strand (Tyr^{133}-Lys^{138}), and a loop (Thr^{139}-Leu^{147}). This region includes five basic residues (Lys^{128}, Arg^{129}, Lys^{134}, Lys^{138} and Lys^{144}), but no acidic residues and correlates with the region identified with synthetic peptides, protein modification and recombinant mutant proteins (see above). In one set of crystals of bFGF an electron-dense feature was seen in this region that appeared to correspond to a bound sulphate ion (Eriksson *et al.*, 1991). The four sulphate oxygens are hydrogen-bounded by the side chains of Asn^{36}, Arg^{129} and Lyr^{134} and the main-chain amides of Arg^{118}, Lys^{119} are also close (Eriksson *et al.*, 1991) suggesting that these amino acids may participate in forming the binding site for one of the sulphate groups of heparin. The affinity of this region for anions is further illustrated by the observation that the $IrCl_6^{2-}$ anion in a heavy atom derivative of a crystal of bFGF is located in this region (Zhu *et al.*, 1991).

1.2.2 Receptors for bFGF: FGFRs and HSPGs

1.2.2.1 General

[^{125}I]-FGF binding experiments on Swiss 3T3 cells and mouse skeletal muscle myoblasts revealed the existence of two distinct binding sites with Kd values of 1-100 pM and 1-10 nM, respectively (e.g., Neufeld and Gospodarowicz, 1985; Olwin and Hauschka, 1986). The use of serial salt and acid washes or competition with heparin suggested that the low-affinity receptors were heparan sulphate proteoglycans[1]

[1] **Footnote:** Proteoglycan nomenclature is somewhat confused, with names such as syndecan being used interchangeably for the whole proteoglycan or for its core protein. As the core protein is seldom found except as part of the proteoglycan this does not present a problem in normal use. Where there is a possibility of confusion, the terms "core-protein" or "proteoglycan" have been added to clarify the sense of the text.

(Moscatelli 1987; Olwin and Hauschka, 1986). However, the use of Scatchard analysis in these and other studies to quantify FGF binding data is invalid since the binding data are non-saturating (Klotz, 1982). Moreover, the use of serial salt and acid washes to distinguish quantitatively the high- and the low-affinity receptors has been found to be erroneous since both binding sites are, in part, salt- and acid-sensitive (e.g., Kan *et al.*, 1988; Fernig *et al.*, 1992). Nevertheless the low-affinity binding of bFGF is likely to be due to HSPGs since it is sensitive to heparinase and competition by exogenous heparin in all cells studied so far (Kan *et al.*, 1988; Fernig *et al.*, 1992). Indeed a bFGF-binding HSPG, Syndecan-1, has been isolated by expression cloning as a low-affinity receptor for bFGF (Kiefer *et al.*, 1991). The nature of the high-affinity binding site was originally identified as a tyrosine kinase. cDNAs encoding these molecules, called FGFR, have subsequently been isolated and sequenced (Section 1.2.2.2). However, it is not now certain whether, on their own, the tyrosine kinase receptors represent the high-affinity binding site.

1.2.2.2 FGFRs

The FGFR are one of nine subclasses of tyrosine kinase receptors (Fantl *et al.*,1993; Ullrich and Schlessinger, 1990). The FGFRs (for review, Jaye *et al.*,1992) are divided into four distinct types, each encoded by a separate gene, FGFR-1 (*flg* or *fms*-like gene; Pasquale and Singer 1989), FGFR-2 (*bek* or bacterial expressed kinase; Kornbluth *et al.*, 1988; Houssaint *et al.*,1990), FGFR-3 (Keegan *et al.*,1991) and FGFR-4 (Partanen *et al.*,1991).

1.2.2.2.1 The general structure of FGFRs

The FGFRs contain a single membrane-spanning domain, an extracellular region and an intracellular region. The extracellular region of FGFRs is characterized by three consensus immunoglobulin-like loops. Between immunoglobulin-like loops 1 and 2 is a short domain referred to as the acidic box domain, which is unique to the FGFRs (Fig 1.3). In the FGFR-1 protein, the acidic box domain contains a core sequence of eight

consecutive acidic residues. The intracellular region of the FGFRs possesses a relatively long juxtamembrane domain and a tyrosine kinase domain that is split by a kinase insert (Fig. 1.3). Following the tyrosine kinase domain is the C-terminal tail domain which is relatively divergent in sequence between the four FGFRs and approximately 55-66 amino acids long. Whilst not all FGF-FGFR binding interactions have been studied, it would appear that most FGFs will bind to any FGFR. For example, FGFR-1 binds aFGF, bFGF, *int*-2, hst and FGF-8, but not FGF-7, while FGFR-2 binds aFGF, bFGF, hst and FGF-7 but not FGF-5 (Fernig and Gallagher, 1994; Mansukhani *et al.*, 1992).

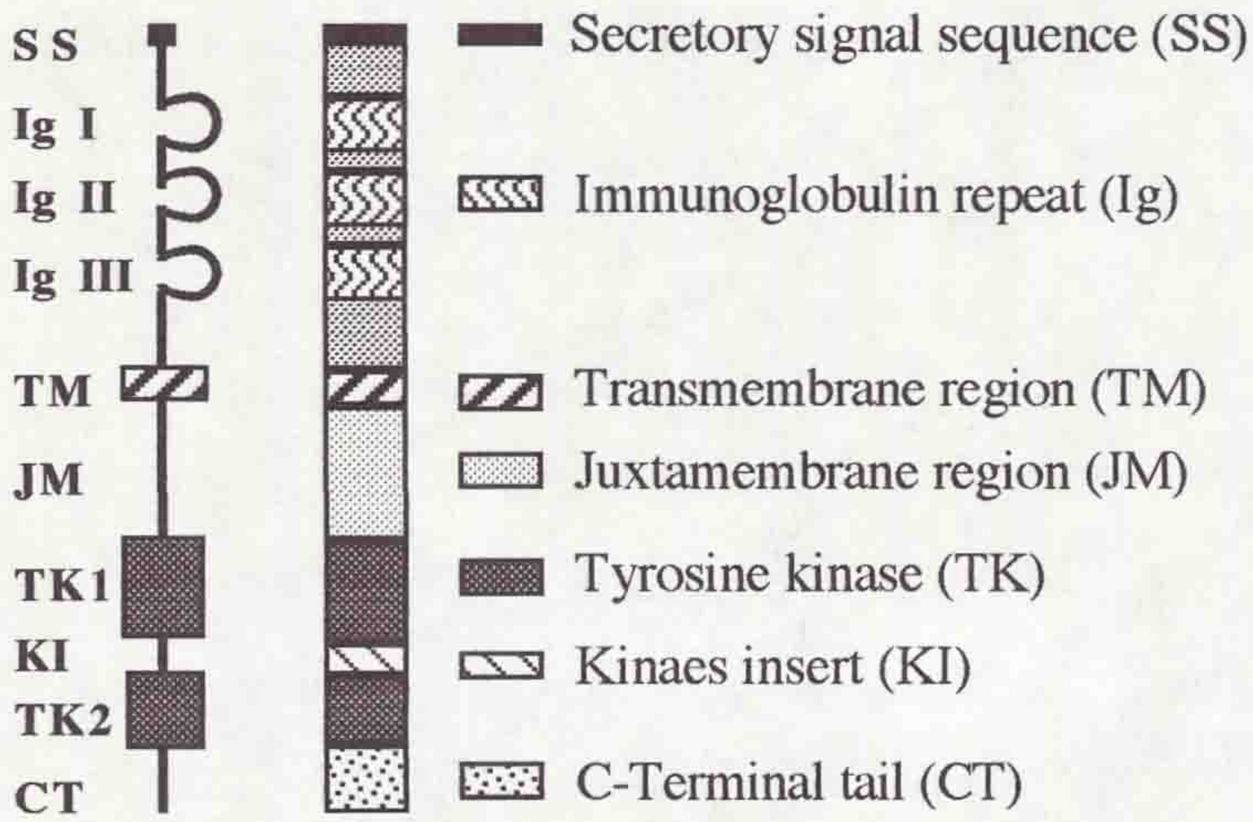


Fig. 1.3 General structure of FGFRs

Schematic general structure of the FGFRs, the fourth subclass of tyrosine kinase receptors (Section 1.2.2.2). The FGFRs are encoded by four distinct genes, FGFR-1 (flg), FGFR-2 (bek) FGFR-3 and FGFR-4 (Section 1.2.2.2.1).

The complexity of the FGFRs system is further increased by differential RNA splicing which is dictated by the exon structure of the genes encoding the FGFRs (Fig. 1.4). This exon structure which is conserved in FGFR-1, -2 and probably -3 includes the three alternative variants of exon 6 which encodes the third immunoglobulin loop (Dell and Williams, 1992; Duan *et al.*, 1992; Eisenmann *et al.*, 1991; Fujita *et al.*, 1991; Hou *et al.*, 1991). In FGFR-4, the structure of mRNA is similar except that are

no alternatively spliced variants (Vainikka *et al.,* 1992).

Six of the different FGFR-1 proteins, FGFR-1-I, -VI produced in this manner are shown in Figure 1.4. The differences in the isoforms of FGFR-1 are as follows (Fig.1.4):

(i) no secretory signal sequence (FGFR-1–VI);

(ii) deletion of the first immunoglobulin loop (FGFR-1-II, III, V and VI);

(iii) three alternative C-terminal parts of immunoglobulin-loop III (shaded box, FGFR-1-II), encoded by three different exon 6 including one with a stop codon that results in the synthesis of a secreted receptor (FGFR-1-III);

(iv) deletion of just two amino acids between the third immunoglobulin loop and the transmembrane domain (FGFR-1-II and V);

(v) a truncated tyrosine kinase domain (FGFR-1-IV).

Similar splicing events generate isoforms of FGFR-2 (Championarnaud *et al.,* 1991) and FGFR-3 (Murgue *et al.,* 1994). Thus these are a total of 48 isoforms of FGFR-1, FGFR-2 and FGFR-3, making a total of 145 different FGFR proteins.

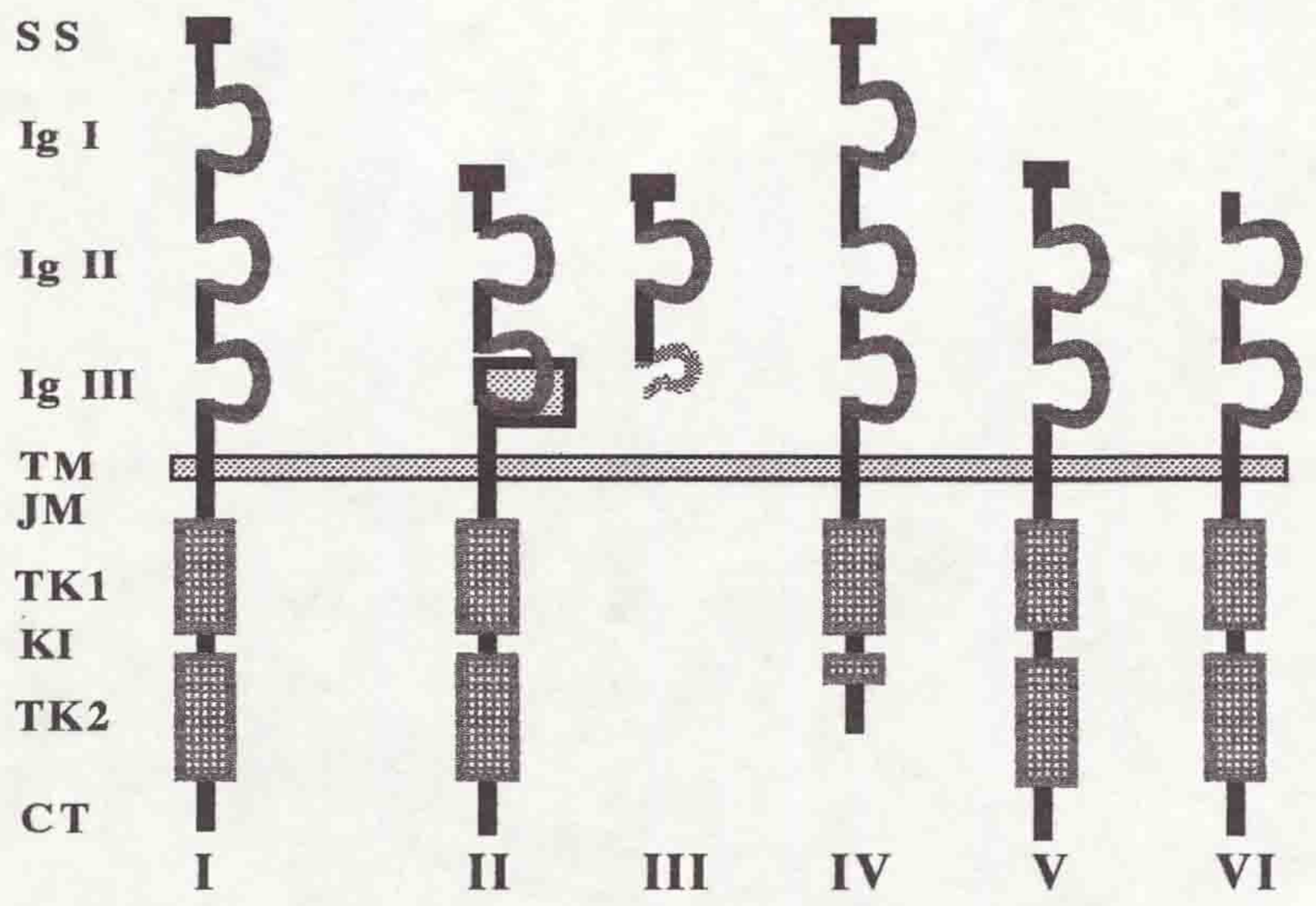


Fig. 1.4 Exon splicing generates multiple isoforms of FGFRs

Generation of FGFR-1 receptor subtypes by alternative splicing. Six of the forty eight different isoforms of FGFR-1 are shown. The region of Ig-loop III encoded by exon 6 is enclosed by a shaded box. Splice forms II and V differ by just two amino acids in their extracellular domains (Section 1.2.2.2.2)

1.2.2.2.2 Functions of the isoforms of FGFRs

The isoforms of FGFR-1 and FGFR-2 that vary in the third immunoglobulin loop region have different ligand-binding specificities. The secreted form of FGFR-1, FGFR-1-III, which uses a different exon for immunoglobulin loop 3 to the membrane bound forms (Fig. 1.4), binds bFGF but not aFGF with high-affinity (Duan *et al.*, 1992). The membrane bound forms of FGFR-1 (Fig. 1.4) may either bind aFGF and bFGF with equal affinity or only bind aFGF with high-affinity depending on which of the remaining two exons encoding the third immunoglobulin loop is used (Werner *et al.*,1992) (Fig. 1.4). The three exons that encode the third immunoglobulin loop of FGFR-2 also confer a degree of ligand specificity. One of the three splice variants binds FGF-7 and aFGF but not bFGF with high-affinity, whilst a second splice variant binds aFGF and bFGF but not FGF-7 with high-affinity (Championarnaud *et al.*, 1991). Detailed analysis of the contributions of the second and third immunoglobulin loops indicate that in this case both contribute to specifying the high-affinity ligand for FGFR-2 (Cheon *et al.*,1994; Johnson *et al.*,1991; Miki *et al.*,1992; Yayon *et al.*,1992; Zimmer *et al.*,1993).

The isoforms produced from the exon 6 variant possessing a stop codon may act as classic binding proteins or as soluble receptor antagonists (Duan *et al.*, 1992). Similarly the isoforms with a truncated tyrosine kinase (Fig. 1.4) may act as dominant negative antagonists. There is also evidence that some isoforms of the FGFRs serve to alter the binding specificity of the receptors, and that different FGFRs may have different signalling roles. For example, the two immunoglobulin loop isoforms of FGFR-1 appear to be associated with the transformed and the malignant phenotypes (Yamaguchi *et al.*, 1994; Yan *et al.*, 1992). The secreted and kinase-truncated isoforms of FGFR-1 may act as antagonists (Duan *et al.*, 1992).

Thus alternative RNA splicing imparts some degree of specificity to the FGF-FGFR binding reaction but there is still considerable overlap between the binding of the nine FGFs and the different FGFRs and their isoforms. Some of the isoforms may act potentially as antagonists. However, the reason for the existence of the estimated 145

different FGFRs is unclear at present.

1.2.2.2.3 Signalling by the FGFR tyrosine kinase

The FGFRs, like the other receptor tyrosine kinases have an extracellular ligand binding domain and an intracellular domain, which includes the protein tyrosine kinase, separated by the plasma membrane (Section 1.2.2.2.1). The activation of the receptors, caused by the binding of extracellular ligand, must be translated across the membrane barrier into the activation of the signal transduction functions of the intracellular domain. It is thought that the binding of FGFs to the extracellular domain induces the dimerization of the FGFRs, which stabilizes interactions between adjacent cytoplasmic domains and leads to activation of the tyrosine kinase function (Jaye *et al.*, 1992; Ullrich and Schlessinger, 1990). The dimerization of FGFRs is followed by receptor "autophosphorylation", which mainly occurs by one receptor molecule phosphorylating the other in the dimer (Jaye *et al.*, 1992). The phosphorylation of the tyrosine kinase receptor on tyrosine increases the activity of the enzyme and activates the binding sites for proteins that are targets of the receptor tyrosine kinase. This mechanism of ligand-induced receptor dimerization is thought to be common to the entire family of receptor tyrosine kinases (Fantl *et al.*, 1993; Ullrich and Schlessinger, 1990).

After ligand binding, receptor dimerization, and the activation of the tyrosine kinase of the receptor the receptors cluster into clathrin-coated pits and are internalized via receptor-mediated endocytosis (Fantl *et al.*, 1993; Haigler *et al.*, 1980).

Individual phosphotyrosine residues present in the cytoplasmic domains of FGFRs serve as highly selective binding sites that interact with specific cytoplasmic molecules. These signalling molecules may mediate the cellular responses to growth factors. The association between the tyrosine phosphorylated regions in growth factor receptors and signalling proteins is mediated in some cases by a conserved region of approximately 100 amino acids, termed src homology domains (SH2) (Anderson *et al.*, 1990). Tyrosine phosphorylation of the binding site serves as the receptor for SH2 association,

while the C-terminal residues provide specific recognition of the relevant SH2 domain containing target protein (Songyang *et al.*, 1994). SH2 domains are usually accompanied by another conserved domain of 50 amino acid residues termed the SH3 domain (Musacchio *et al.*, 1992) which may extend the ability of signalling proteins to complex with one another (Fig. 1.5).

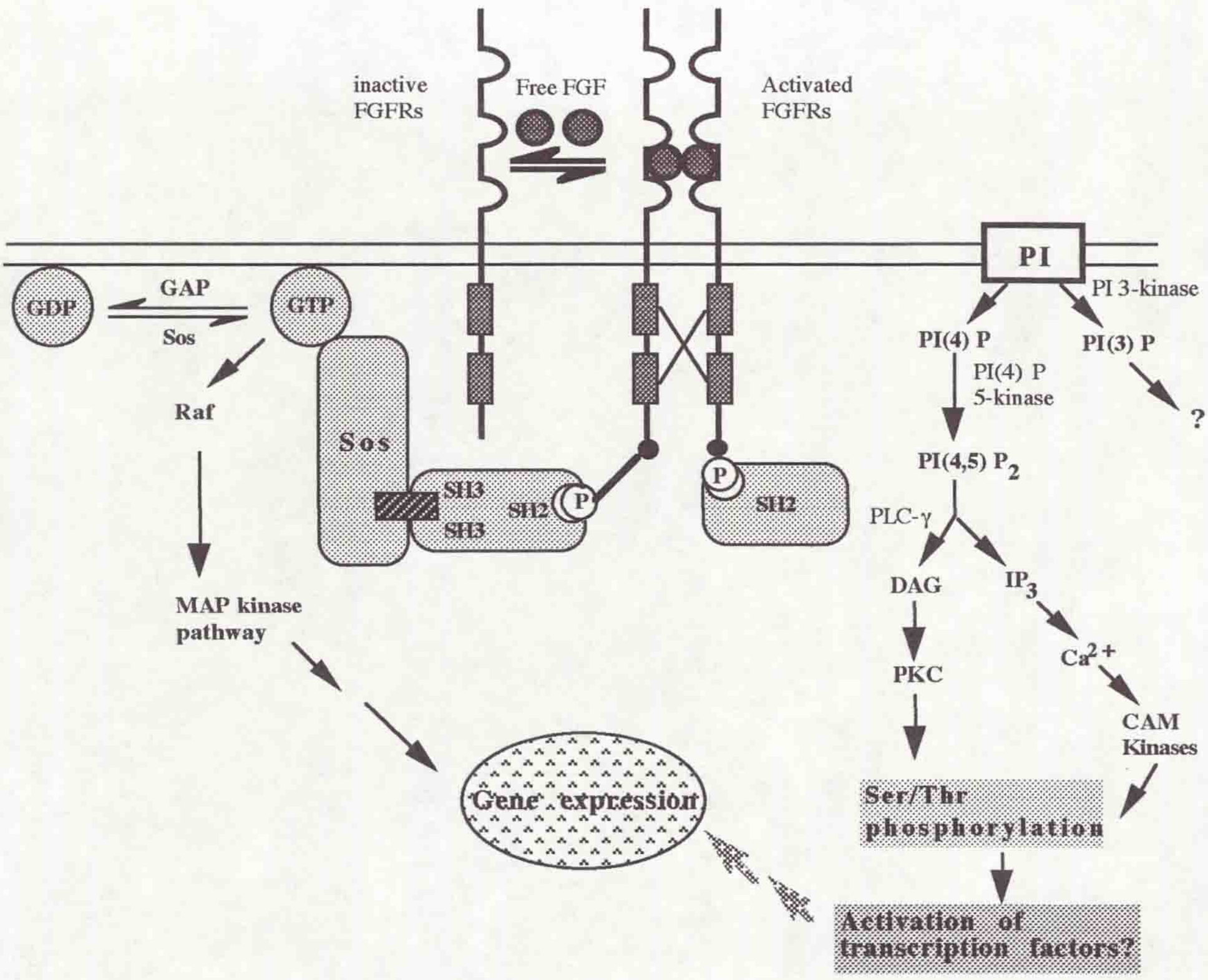


Fig. 1.5 Signalling events initiated by tyrosine kinase of FGFRs

Stimulation of binding of FGFs to FGFRs is followed by rapid receptor dimerization, tyrosine autophosphorylation and phosphorylation of signalling molecules such as phospholipase C-gamma (PLC-γ) and the ras GTPase-activating protein (GAP). PLC-γ and GTPase-activating protein bind to specific tyrosine-phosphorylated regions in growth factor receptors through their src-homology domain 2 (SH2) . Growth factor-induced tyrosine phosphorylation of PLC-gamma is essential for stimulation of phosphatidylinositol hydrolysis.

1.2.2.3 Heparan Sulphate Proteoglycans (HSPGs)

The heparan sulphate proteoglycans (HSPGs) have been shown to be the low-affinity receptors for bFGF (Kd of 10-100 nM) (Section 1.2.2.1). The HSPGs are the most complex and variable of the mammalian proteoglycans and consist of a core protein to which a variable number of glycosaminoglycan chains are linked (Gallagher et al., 1990) (Fig.1.6).

The carbohydrate components of proteoglycans are glycosaminoglycans which consist of unbranched chains of repeating disaccharide units that can be substituted with ester and amino sulphate groups. These substitutions make the glycosaminoglycans highly anionic molecules. In HSPGs the glycosaminoglycan is heparan sulphate (HS). Cell-associated proteoglycans can be found in the plasma membrane and extracellular matrix/basement membrane as well as in intracellular compartments such as the nucleus and the vesicular apparatus.

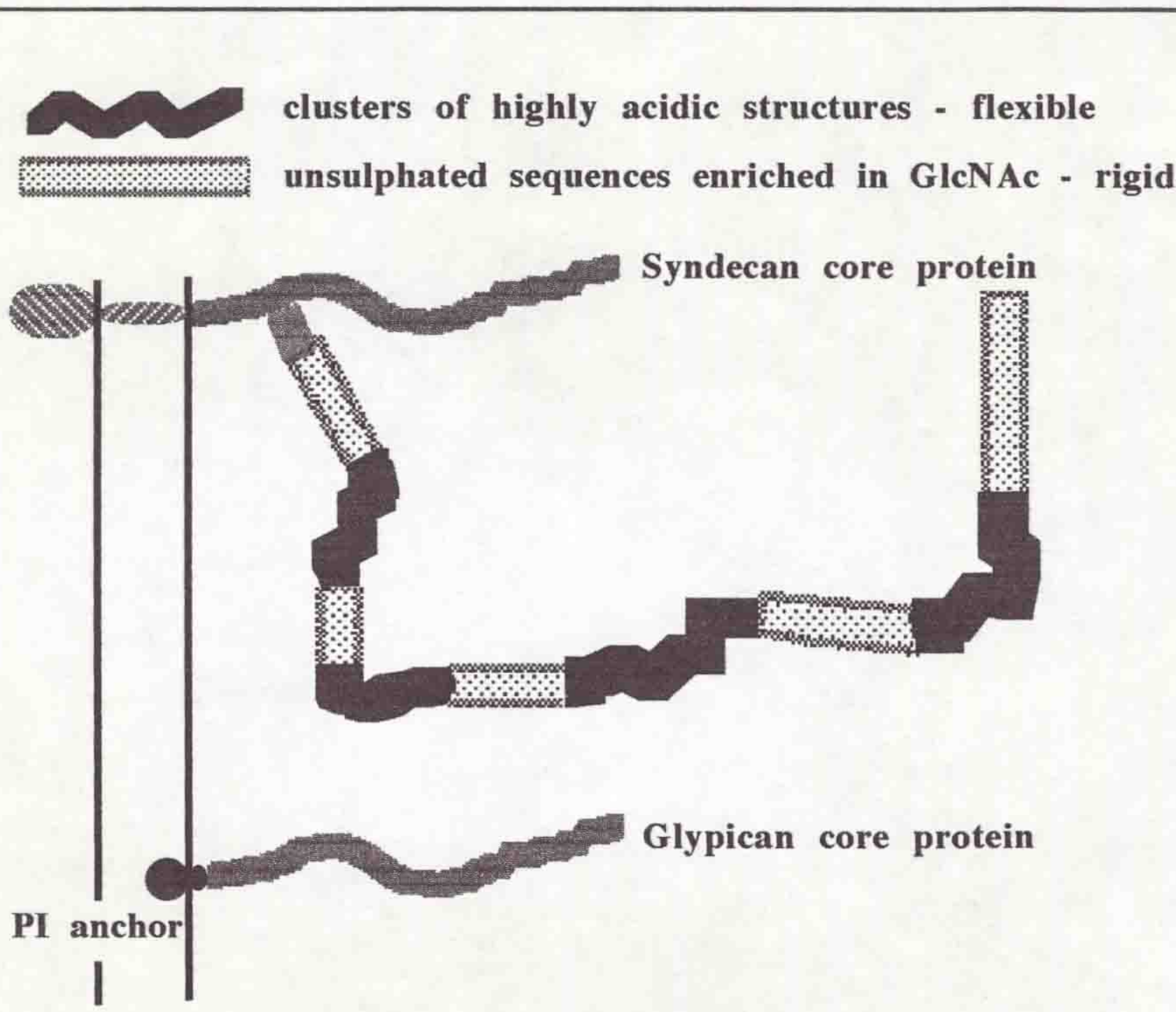


Fig. 1.6 Structure of heparan sulphate proteoglycans

HSPGs consist of heparan sulphate (HS) polysaccharide chains covalently-linked to core proteins to form HSPG (Section 1.2.2.3). HS consists of regions of low sulphation and regions of high sulphation. Most HS-binding proteins, including FGFs bind to the highly sulphated regions.

1.2.2.3.1 HSPG core proteins

Syndecans

Syndecan-1, originally isolated from mouse mammary epithelial cells was the first HSPG core protein to be characterized (Koda *et al.*,1985; Saunders and Bernfield 1988). Syndecan-1 is a composite proteoglycan containing both HS and chondroitin sulphate (CS) glycosaminoglycan chains (Rapraeger *et al.*,1985) (Fig. 1.6).

A cDNA encoding syndecan-1 was subsequently isolated and predicted a polypeptide of 311 amino-acids (32 kDa) containing a twenty-five amino acid transmembrane domain, a thirty-four residue cytoplasmic domain and an extracellular protease sensitive site near to the transmembrane domain (Saunders *et al.*, 1989). Two pairs of Ser-Gly dipeptide sequences to which HS or CS chains may be attached are located near to the amino terminus and the transmembrane domain. The subsequent isolation of a human cDNA encoding syndecan-1 showed that the the cytoplasmic and the transmembrane domains, as well as the extracellular glycanation sites were highly conserved. There was only one amino acid different between the fifty-nine residues in these regions of human and mouse syndecan-1 (Mali *et al.*, 1990).

Based on the high homology of their predicted amino acid sequences, there are currently three other members of the syndecan family of HSPGs. cDNAs encoding fibroglycan (syndecan-2) have been isolated from human fetal lung fibroblasts (Marynen *et al.*, 1989), and rat liver (Pierce *et al.*, 1992). A cDNA encoding N-syndecan (neural syndecan or syndecan-3) has been isolated from chick embryo limb buds (Gould *et al.*, 1992) and newborn rat Schwann cells (Carey *et al.*, 1992). A cDNA encoding ryudocan (syndecan-4) is expressed in chick 14 day embryos (Baciu *et al.*, 1994) and has also been reported in rat microvascular endothelial cells, smooth muscle cells and skin fibroblasts (Kojima *et al.*, 1992a,b). The extracellular domain is responsible for virtually all the structural differences between the members of the syndecan family. The conservation of the transmembrane and intracellular domains suggests that they are

important for the function of the proteoglycans although how this may occur is not yet known. However, there is evidence to suggest that the cytoplasmic domain of the syndecan core proteins interacts with cytoskeletal elements such as actin (Rapraeger *et al.*, 1986). Indeed, cell surface HSPGs are concentrated within focal adhesion structures during the spreading of fibroblasts in culture and the position of the HSPGs correlates with changes in the organization of internal microfilaments (Woods *et al.*, 1986). Such changes in intracellular organization appear to be dependent upon interactions between the HS chains and the heparin-binding domains of extracellular matrix proteins such as fibronectin (Woods *et al.*, 1986; Singer *et al.*, 1987).

Glypicans

In 1990, David and his coworkers isolated and sequenced cDNA clones for the core protein of a heparan sulphate proteoglycan from human lung fibroblasts (David *et al.*, 1990). The core protein structure and the effects of phospholipase C treatment suggest that this proteoglycan is linked to the cell surface via a glycosyl-phosphatidylinositol anchor. This proteoglycan is rapidly, quantitatively, and selectively shed from the cell surface by cultured fibroblasts and they referred to this glypiated proteoglycan by the name "glypican" (David *et al.*, 1990).

Perlecan

Perlecan, the main proteoglycan of basement membranes and pericellular space, contains one of the largest single-chain polypeptides of vertebrates. The five modules of perlecan are collated from protein building blocks evolutionarily related to molecules involved in nutrient metabolism, mitogenesis and cell adhesion (Iozzo, 1994). Perlecan has a core protein of 400 kDa and analysis of cDNA clones from mouse and human indicates the presence of conserved domains which have sequence homology to laminin β_1 and β_2 chains (Noonan *et al.*, 1988). It also contains structures analogous to a multi-loop sequence found in the neural cell adhesion molecule (N-CAM) and a domain found in the low density lipoprotein (LDL) receptor. However, the N-terminal domain

shows no homology to any other extracellular matrix component so far documented (Noonan *et al.*, 1991; Kallunki and Tryggvason, 1992; Murdoch *et al.*, 1992). In addition to basement membranes, perlecan is also found in the stroma surrounding tumours (Murdoch *et al.*, 1992) and has been isolated from fibroblast cultures (Heremans *et al.*, 1989). The Englebreth Holm Sarcoma (EHS)-derived perlecan contains three large HS chains whilst perlecan from human placenta is a hybrid molecule bearing both HS and CS or dermatan sulphate glycosaminoglycans (Isemura *et al.*, 1987) and corneal perlecan is expressed solely as a CSPG (Hassell *et al.*, 1992).

CD 44

CD44 is a polymorphic family of integral membrane glycoproteins of which the smallest form (80-90 kDa) contains no detectable GAG. It was originally identified on the surface of lymphocytes where it is involved in the process of lymphocyte homing and lymphohaemopoiesis. A 200 kDa form contains CS chains (Jalkanen *et al.*, 1988). CD44 is also found upon other leukocytes, epithelial cells, fibroblasts, glial cells, Schwann cells, smooth muscle cells, Kupffer cells and kidney mesangial cells where it is thought to play a role in cell-matrix interactions (reviewed in Brown *et al.*, 1991). The keratinocyte CD44 may also contain HS chains. The structure of the extracellular domain of CD44 is complicated by alternate splicing and variable glycosylation. One form, CD44E (Brown *et al*, 1991), contains an 132 amino acid insert. The C-terminus of this insert contains a Ser-Gly-Asp sequence, which by analogy to perlecan, may be substituted with HS.

Betaglycan

There are three types of cell surface receptors for transforming growth factor beta. The type III receptor (betaglycan) may carry HS chains but their function is unclear as they are not necessary for transforming growth factor beta binding or storage of the growth factor at the cell surface (Cheifetz and Massague, 1989). Betaglycan can also

bind basic fibroblast growth factor through the HS chains and the addition of this growth factor to cells can modify the HS content of betaglycan (Andres *et al.*, 1992). The sequence of rat betaglycan contains 853 amino acids of which 41 at the C-terminus make up the cytoplasmic domain which is rich in serine and threonine residues (Wang *et al.*, 1991). Betaglycan bears no homology to any other proteoglycan so far sequenced.

1.2.2.3.2 Heparan sulphate (HS)

HS consists of a disaccharide repeat of α,β-linked glucosamine and hexuronic acid (linkage sequence [D-glucosaminyl-(β1-4) β-D-hexuronosyl]$_n$ n=50-150). The hexuronate is present either as glucuronate (GlcA) or as its C-5 epimer, iduronate (IdoA) (Fig. 1.7). HS is highly segregated to form distinct domains, regions of low sulphation

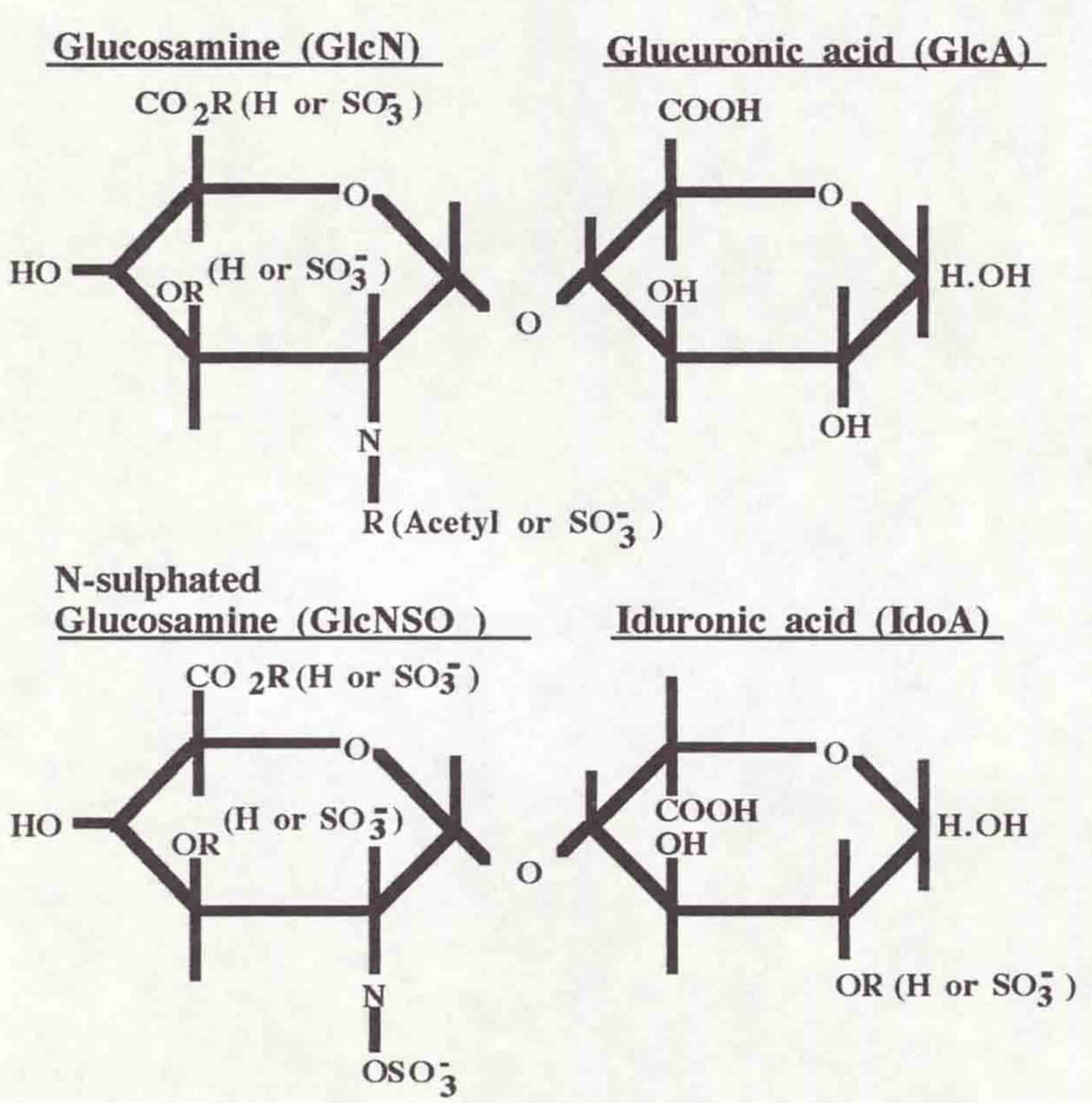


Fig. 1.7 Disaccharide units of heparan sulphate

that are separated by regions of high sulphation (Turnbull *et al.*, 1992) (Fig. 1.6). In regions of high sulphation the main repeat unit is N-sulphated glucosamine ($GlcNSO_3$) and iduronic acid (IdoA) with a variable degree of substitution with ester (O) sulphates at C-6 of $GlcNSO_3$ and C-2 of IdoA; occasionally $GlcNSO_3$ is also sulphated at C-3. In regions of low or zero sulphation the glucosamine is N-acetylated (GlcNAc) and linked to glucuronic acid (GlcA), and arrays of up to ten GlcNAc-GlcA repeats are interspersed between the sulphated domains (Turnbull *et al.*,1988).

Heparin is composed of the same carbohydrate structures as HS but it is significantly higher in sulphate content and the main disaccharide is $GlcNSO_3$,6S - IdoA,2S. It lacks the non-sulphated segments found in HS, is a specialized product of the mast cell and is not a component of the cell-surface (Lane and Lindahl, 1989; Gallagher *et al.*, 1986).

1.2.2.3.3 The biosynthesis of heparan sulphate

Core proteins are translocated to the lumen of the rough endoplasmic reticulum, since they possess secretory signal sequences. In the endoplasmic reticulum the core protein is primed by the addition of a xylose sugar to serine residues which are associated with a ser-gly motif (Section 1.2.2.3.2). Not all serines that are found associated with such motifs are modified by the addition of xylose, and some proteoglycans, such as betaglycan and CD44 can exist in either a glycanated or unglycanated form (Gallatin *et al.*, 1989; Jalkanen and Jalkanen, 1992). The linkage region in the GAG chain consists of:

Polypeptide chain - ser - xyl - gal - gal - GlcA.

where xyl is xylose, gal is galactose and GlcA is glucuronic acid

The synthesis of the HS polymer proceeds in the Golgi by sequential polymerization reactions which extend the chains on the protein-linked primers. The first amino suger residue added to the terminal uronic acid determines the type of GAG chain that will be synthesized. Thus for HS this will be a GlcNAc residue and it is likely that this key step is under the control of a specific N-acetyl-glucosaminyl transferase (Gallagher *et al.*,

1989). In the case of mixed proteoglycans such as syndecan-1 (Bernfield *et al.*, 1992) the N-acetyl-glucosaminyl transferases are able, depending upon the cell type and the physiological circumstances, to add either a GlcNAc or a GalNAc to ultimately produce either a HS or a CS chain, respectively. Polysaccharide synthesis proceeds by the alternate addition of the N-acetylated amino sugar and GlcA units which are transferred by specific glycosyltransferases from the corresponding nucleotide sugar precursors, UDP-GlcNAc and UDP-GlcA (Roden, 1980).

Modifications of the nascent heparan chain result from the action of highly specific, membrane bound, sulphotransferase and hexuronosyl epimerase enzymes (Kusche *et al.*, 1991). These enzymes are thought to act in concert with the glycosyltransferases so that the sulphation and epimerization processes occur at the same time or soon after the process of chain elongation (Lidholt and Lindahl, 1992). The specificities of the sulphotransferases and the C-5 epimerase regulate the process of sulphation as the product of each reaction acts as the substrate for the next enzyme. The modifications to the newly synthesized heparan polymer occur in a stepwise manner (Jacobsson and Lindahl, 1980; Kjéllen and Lindahl, 1991):

1. Deacetylation of GlcNAc residues
2. N-sulphation of the free amino group
3. Epimerization of the GlcA at C-5 to form IdoA
4. Ester-O-sulphation of the IdoA at C-2
5. Ester-O-sulphation of the $GlcNSO_3$ at C-6.

Examination of the frequency and distribution of these modifications in HS have demonstrated that the full potential for modification is not fulfilled since unmodified sugars are present in relatively large amounts (Gallagher and Walker, 1985). Furthermore the sulphated and non-sulphated sugars are segregated to a significant degree to give domains of high sulphation and domains of low sulphation (Turnbull and Gallagher, 1990). More recently, it has become apparent that this pattern of modification is independent of the core protein (Sanderson *et al.*, 1994). Thus the

restricted distribution of sulphate-rich residues must be finely controlled, by an as yet unidentified mechanism in the Golgi apparatus. The consequences of the control mechanism are: (i) the restriction of the epimerization of the glucuronate to iduronate so that this only occurs on GlcA residues adjacent to a $GlcNSO_3$; (ii) the O-sulphate groups are only added to N-sulphated disaccharides or adjacent N-acetylated units. Hence iduronate and O-sulphate groups will occur mainly in the regions of high $GlcNSO_3$ content, and thus in HS the ratio of $GlcNSO_3$ to GlcNAc is usually 1:1 (Gallagher *et al.*, 1985). The segregation of the regions of low and high sulphation is not absolute and minor but significant proportions of HS consist of alternating GlcNAc and $GlcNSO_3$ disaccharides (Turnbull and Gallagher, 1991; Gallagher *et al.*, 1986). The pattern of modification of heparin, a specialised HS produced by the mast cell is different to that of normal HS (Lane and Lindahl, 1989; Gallagher and Lyon, 1989). In heparin there is a significantly higher content of N-sulphated disaccharides (>80%) and a molar excess of O-sulphate to N-sulphate residues due to the presence of large numbers of the trisulphated disaccharide ($GlcNSO_3$(6S)-IdoA, 2S) which are spread in a more uniform manner along the length of the polysaccharide (reviewed in Gallagher and Lyon, 1989). Recently, heparin-like structures ($GlcNSO_3$6S-IdoA, 2S) have been found in the terminal region of the HS chains of the major HSPG of rat liver, syndecan-2 (Lyon *et al.*, 1994). Thus, whilst the gross domain structure of heparin and HS chains from various types of cell are different, it is apparent that cells are able to produce HS chains containing a wide variety of patterns of sulphation. The conversion of the N-acetyl residues to N-sulphate is the key regulatory step which permits and regulates the frequency of further modifications. Therefore it seems likely that the control of this reaction is instrumental in bringing about at least the gross structural differences between various HS chains and heparin. Now that a cDNA encoding an enzyme that catalyses the N-deacetylation/N-sulphation reaction has been cloned (Orellana *et al.*, 1994), the mechanisms that control the activity of this reaction may be elucidated in the future.

1.2.3 Functions of the receptors for FGFs

1.2.3.1 The storage/sink function of HS

An ever growing number of proteins has been found to bind to HS. The HS-binding proteins include the blood coagulation factor antithrombin III, an enzyme, lipoprotein lipase, components of the extracellular matrix/basement membrane and a large number of structurally-unrelated growth factors (Table 1.1).

Table 1.1 Heparin-binding proteins

Protein	Reference
(i) Proteins that bind HS without changing their activity	
Collagens	Bernfield et al., 1992
Fibronectin	(op. cit.)
Laminin	(op. cit.)
Interleukin-3	Roberts et al., 1988
Granulocyte macrophage colony-stimulating factor	(op. cit.)
Heregulins	Plowman et al., 1993
Interferon-γ	Lortat-Jacob and Grimaud 1991
Transforming growth factor-β	McCaffrey et al., 1992
Platelet-derived growth factor	Raines and Ross, 1992
(ii) Proteins that bind HS and undergo a change of activity	
Antithrombin III	Lindahl et al., 1984
Amphiregulin*	Johnson and Wong, 1994.
FGFs*	Fernig and Gallagher, 1994
Heparin binding-EGF*	Higashiyama et al., 1991
HGF/Scatter factor*	Lyon et al., 1994
Lipoprotein lipase	Bernfield et al., 1992
Pleiotrophin	Wellstein et al., 1992
Vascular endothelial growth factor*	Gitaygoren et al., 1992

* interaction demonstrated to be required for activity and hence the HS receptor is likely to be part of a dual receptor system.

On the basis of the functional significance of their interactions with HS, these proteins can be divided into two groups: (i) proteins that bind HS without changing their activity; (ii) proteins that, as a consequence of binding to HS undergo a change in activity/function. Since there are large amounts of HS on the plasma membrane of cells

to provide an extracellular site of attachment for HS-binding proteins. In the case of the extracellular matrix proteins, this function presumably relates to cell adhesion (Kallunki *et al.*, 1992; Murdoch *et al.*, 1992). In the case of growth factors this function may serve to provide either an extracellular storage site or a sink which acts to sequester and hence inactivate the growth factors.

There is considerable evidence for the HSPG receptors acting as storage sites for bFGF. Localization of bFGF in cultured cells and in a variety of tissues has demonstrated an association with basement membrane (Baird and Ling, 1987; Barraclough *et al.*, 1990; Clarke *et al.*, 1993; Vlodavsky *et al.*, 1987), particularly in the vicinity of quiescent cells (Rudland *et al.*, 1993). There are a large number of "spare" HS binding sites for bFGF in quiescent regions of the developing mammary gland and kidney (Rudland *et al.*, 1993; Morita *et al.*, 1994) suggesting that the basement membrane may act as a sink for bFGF. One role of this HS "sink" may be to restrict the diffusion of bFGF and to prevent bFGF and other HS-binding growth factors moving between different cellular compartments. For example, in mice carrying an *int*-2 transgene, the *int*-2 is only able to diffuse over very short distances in tissue-explants from such mice (Ornitz *et al.*, 1992a). In contrast, in growing tissues, this sink is absent (Rudland *et al.*, 1993; Morita *et al.*, 1994) indicating that under certain circumstances, bFGF is able to diffuse considerable distances both within a tissue and between tissue compartments.

Studies on cultured cells indicate a modulatory role for the storage/sink function of the HS receptor for bFGF. Cells may be "loaded" with bFGF for a short period of time, and then the exogenous bFGF removed. However, the bFGF bound to cellular HS receptors remains so and is delivered slowly, but constantly, to the FGFRs, allowing a cellular response over a prolonged period of time (Fernig *et al.*, 1992; Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989; Salmivirta *et al.*, 1992).

The structures within HS that may be responsible for the protein binding activity have only been elucidated for antithrombin III and bFGF, although partial structures are

known for some of the other HS-binding proteins.

A specific pentasaccharide sequence found in heparin and HS binds to antithrombin III, which mediates the blood anticoagulant activity of heparin/HS (Lindahl *et al.*, 1984). This sequence is composed of three GlcN units, one of which is preferentially N-acetylated, one GlcA unit, and one IdoA unit, with O-sulphate groups as follows (Kjéllen and Lindahl, 1991):

$GlcNAc/NSO_3(6S)$-GlcA-$GlcNSO_3(3S\pm6S)$-IdoA(2S)-$GlcNSO_3(6S)$

Where $GlcNAc/NSO_3(6S)$ is N-acetylated or N-sulphated, 6-O sulphated glucosamine and (3S±6S) is 3-O sulphated glucosamine with an optional 6-O sulphation.

Three oligosaccharides that bind bFGF have been isolated by affinity chromatography from the HS secreted into the culture medium by human skin fibroblasts. One of the oligosaccharides bound bFGF with the same affinity as the parental HSPG and was called Oligo-H (Turnbull *et al.*,1992). Oligo-H is composed of seven disaccharides with the following sequence:

GlcAβ1, 4 $GlcNSO_3\alpha$1, 4[IdoA(2S)α1, 4 $GlcNSO_3]_5\alpha$1, 4 IdoAα1, 4 GlcNAc

Strong binding required the N-sulphates and IdoA, 2S. However, the sequence lacks 6-O-sulphate groups which are quite common in fibroblast HS (Turnbull and Gallagher, 1990). The other two oligosaccharides that were isolated, Oligo-M and Oligo-L, had a lower affinity for bFGF than the parental HSPG. Oligo-M had an intermediate affinity for bFGF, whilst Oligo-L had a considerably lower affinity. Although the overall level of sulphation of the three oligosaccharides was similar, Oligo-M possessed only four internally repeating disaccharides of IdoA,2S - $GlcNSO_3$. Oligo-L contained more 6-O-sulphate groups, but fewer IdoA(2S), underlining the importance of the latter for bFGF binding (Turnbull *et al.*, 1992). Other studies have also stressed the importance of IdoA,2S and $GlcNSO_3$ for HS-binding to bFGF (Maccarana *et al.*, 1993).

Hepatocyte growth factor (HGF) /scatter factor is a heparan sulphate-binding growth factor with mitogenic and morphogenic activities, all of which are entirely dependent on

cells possessing both HS receptors and the C-met tyrosine kinase receptor for HGF/scatter factor (for review, Mizuno and Nakamura, 1993). Interaction of HS with HGF appears to require sequences contained within the N-sulphated domains of HS. Like bFGF, HGF is apparently selective in the specific sulphate groups required. The sequence of GlcA-$GlcNSO_3$-[IdoA-$GlcNSO_3$ (±6-S)]$_{3-5}$ - IdoA-GlcNAc, in which 2 of the $GlcNSO_3$ residues are actually sulphated at C-6, contains the essential features for specific high affinity binding of HGF to HS (Lyon *et al.*, 1994). The precise location of the $GlcNSO_3$ (6-OSO_3) residues within the recognition sequence remains to be established (Lyon *et al.*, 1994; Lyon and Gallagher, 1994).

The structures within HS responsible for binding antithrombin III, bFGF and HGF/scatter factor are different and in the case of bFGF, there are potentially three different binding sites within HS. Thus it is likely that each HS binding protein (Table 1.1) may possess a unique binding structure(s) within HS. Hence the sequence of the HS chains produced by cells will determine which, if any, of the HS-binding proteins are able to interact with the cells.

1.2.3.2 The dual receptor system

As well as the storage/sequestration function of HS (Section 1.2.3.1) HS is also required for the activity of some HS-binding proteins. The first example of HS-dependent activity was antithrombin III (Lindahl *et al.*,1984). The binding of antithrombin III to its specific sequence in heparin or HS activates the antithrombin III and enables the efficient inhibition of thrombin activity. Four years ago it became apparent that an analogous mechanism may exist for bFGF. Two different experimental systems were employed to demonstrate a requirement for HS of the growth-stimulatory activity of bFGF. Firstly, cells devoid of HS and FGFRs were transfected with a cDNA encoding FGFR-1. Cells expressing FGFR-1 could only be stimulated to grow by bFGF if exogenous heparin or HS was provided (Yayon *et al.*, 1991). The second approach was to use cells that expressed endogenous bFGF-binding FGFR and HSPGs. The synthesis of the endogenous HS was inhibited by chlorate, and under

these conditions bFGF could only stimulate cell division when exogenous heparin or HS was provided (Rapraeger *et al.*, 1991). These observations have been repeated for FGFR-1 and other FGFRs as well as four of the FGF family in diverse cellular systems using both of the above approaches (Guimond *et al.*, 1993; Li and Bernard, 1992; Mansukhani *et al.*, 1992; Olwin, and Rapraeger, 1992). The original model proposed to account for these observations was based on the activation of antithrombin-III by heparin and suggested that bFGF alone bound with only low affinity to the FGFRs. However, in the presence of heparin, bFGF bound with high-affinity to the FGFRs (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991) (Fig. 1.8).

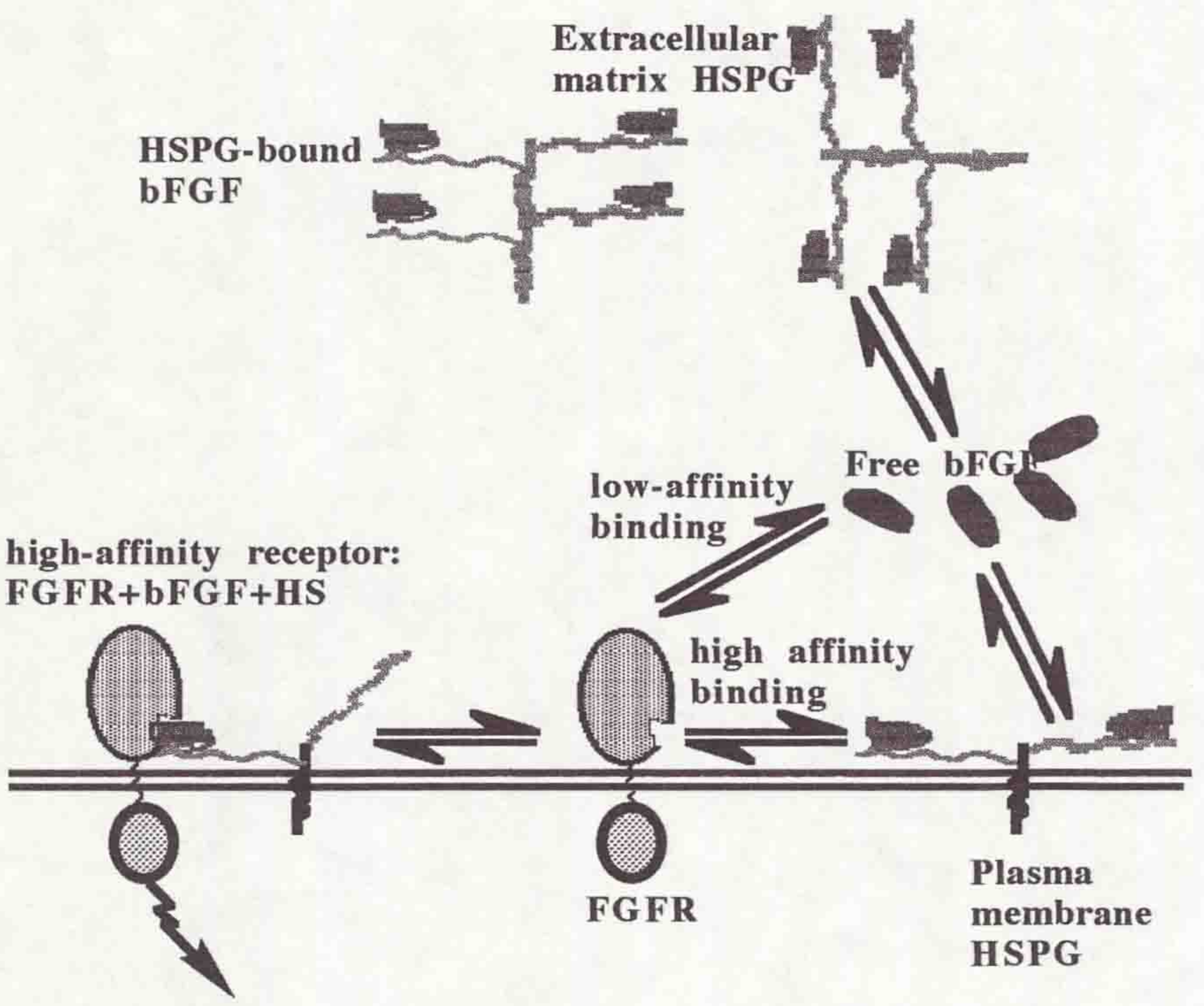


Fig. 1.8 Dual receptor system for FGFs

bFGF secreted by cells is stored on the HSPG receptors which act as a storage depot or sink for extracellular bFGF (Sections 1.2.3.1 and 1.2.3.2). Under suitable conditions the HSPG storage receptors may modulate the delivery of bFGF to the tyrosine kinase receptors (Sections 1.2.3.1 and 1.2.3.2). Free bFGF can only bind to the tyrosine kinase receptors with relatively low-affinity. However the binding of bFGF to HS induces a conformational change in bFGF that enables it to bind to the high-affinity receptors with high-affinity.

Subsequently, a number of other models for the dual receptor system have been proposed. These fall into two classes. Firstly, models where the role of the HS is not to activate bFGF, but to bind two bFGF molecules, thereby producing a dimeric ligand which can readily induce the dimerization of the FGFRs (Section 1.2.2.3.2). Secondly, models where the HS interacts with both the bFGF and the FGFR.

The structures within HS that may participate in the dual receptor system have been examined. To date no structure has been elucidated. However, composition studies indicate that a saccharide fragment of at least 10-12 sugar residues is required and that such fragments are substantially enriched in $GlcNSO_3$ and IdoA(2S) (Guimond *et al.*, 1993; Ishihara *et al.*, 1993; Walker *et al.*, 1994). Thus it would seem that these active sites in HS are similar to Oligo-H (Section 1.2.2.3.3).

1.2.3.3 The stimulation of DNA synthesis by FGFs

Addition of bFGF to cells possessing a functional dual receptor system for FGFs causes the rapid induction of a myriad of early events. These include the stimulation of the phosphorylation on tyrosine of the FGFRs and target proteins, the formation of diacylglycerol, the activation of protein kinase C activation and the mobilization of Ca^+ (Taye *et al.*, 1992). Many, if not all, of these early events are ultimately the consequence of the activation of the tyrosine kinase of the FGFR (Section 1.2.2.2.3).

However, the role of such early events in stimulating cell division is not clear. Thus whilst it has been proposed that growth factors act either as competence or progression agents (Pledger *et al.*, 1978) subsequent experimental evidence has not concurred with this model (Chana and Smith, 1991; Rudland and Jiminez de Asua, 1979). Instead it would appear that a prolonged delivery of signal(s) to cells is required to stimulate cell division. Thus not all of the early events are initiated by any particular growth factor acting on a particular target cell and the early events in themselves are not sufficient to induce DNA synthesis as synthesis does not take place if the growth factor is removed from the cells before the end of the lag period (Carpenter and Cohen, 1975; Reid and

Reid, 1987; Chana and Smith, 1991). Furthermore, conditions are known under which some of the early events take place, but DNA synthesis does not occur. For example,when platelet-derived growth factor interacts with a mutated receptor (Escobedo and Williams, 1988), when bFGF interacts with a mutated FGFR (Peters *et al.*, 1992; Mohammadi *et al.*, 1992) or when a truncated form of aFGF activates FGFRs (Imamura *et al.*, 1990, 1992).

The interaction of most polypeptide growth factors with their cellular receptors results ultimately in the rapid degradation of the growth factors in lysosomes (e.g. Haigler *et al.*, 1980). At least two of the FGFs, aFGF and bFGF, are notable exceptions to this rule. aFGF is recovered as a polypeptide of Mr 42 kDa following its interaction with its cellular receptors. The 42 kDa polypeptide results from the post-receptor covalent association of aFGF with a 20 kDa protein (Shi *et al.*, 1991). In addition, there is strong evidence to suggest that the post-receptor translocation of aFGF to the cytoplasm and thence the nucleus is a required part of the signalling mechanism that results in DNA synthesis (Imamura *et al.*, 1990, 1992; Wiedlocha *et al.*, 1994). In contrast, the interaction of bFGF with its cellular receptors results in the production of a variety of truncated polypeptides of Mr 16-14 kDa, 12-10 kDa and 9-8 kDa in endothelial cells (Baldin *et al.*, 1990; Bouche *et al.*, 1987). Moreover, there is evidence to suggest that at least in endothelial cells bFGF and the polypeptides that are produced as a consequence of the post-receptor processing of bFGF are translocated into the cell cytoplasm and into the nucleus (Amalric *et al.*, 1994; Baldin *et al.*, 1990; Hawker and Granger, 1992). Indeed, it has been suggested that the translocation of these molecules into the nucleus, and perhaps the nucleolus is an essential growth-stimulatory signal that allows the G_0 - S phase transition (Bouche *et al.*, 1987).

To elucidate how FGFs may stimulate cell division, there is clearly a need to define, more precisely the conditions under which DNA synthesis takes place. It may then be possible to determine which of the many signals initiated by the interaction of FGFs with their receptors (Sections 1.2.2.2.2 and 1.2.3.3), are necessary to promote cell division.

1.2.4 Release of FGFs from cells

A feature of all the forms of bFGF, of aFGF and of FGF-9 is that they lack a consensus signal peptide for secretion whilst the other members of the FGF family possess secretory signal sequences (Fig. 1.1). The lack of a secretory signal sequence in bFGF, aFGF and FGF-9 and the absence of bFGF immunoreactivity in the secretory pathway suggests that these proteins are not secreted by the conventional endoplasmic reticulum/Golgi pathway (Burgess and Maciag, 1989). Thus the identification of extracellular bFGF and aFGF indicated an association with the extracellular matrix in culture cells (Baird and Ling, 1987; Barraclough *et al.,* 1990; Vlodavsky *et al.*, 1987) and tissues (Consigli *et al.*, 1991; Cordon-Cardo *et al.*, 1991; Fu *et al.*, 1991; Gonzalez *et al,* 1990; Gomm *et al.*, 1991; Kardami *et al.*, 1991; Rudland *et al.*, 1993). However, whether bFGF and aFGF were actually released from cells was controversial because of the difficulty in identifying the growth factors in the culture medium - a consequence of their binding to HS on the cell surface and in the extracellular matrix. Hence it has been suggested that bFGF and aFGF are only released from dying/dead cells and that under normal circumstances these growth factors are intracellular. However, some studies indicated that aFGF and bFGF could also be recovered from the medium of cultured cells (Bunnag *et al.*, 1991; Jouanneau *et al.*, 1991, 1994; Mignatti and Rifkin, 1991; Sato *et al.,* 1989). Consequently, cell injury has been suggested to be the mechanism of aFGF and bFGF release *in vivo.* For example, the migration of endothelial cells following irradiation injury (Witte *et al.,* 1989) *in vitro* could be inhibited by the addition of neutralizing anti-bFGF-antibodies as well as by protamine sulphate or suramin which blocks the interaction of bFGF with its receptors (Sato *et al.,* 1988). In addition the release of bFGF was demonstrated using endotoxin in aortic endothelial cells and irradiation in bovine, porcine and human endothelial cells (Gajdusek and Carbon, 1989; Witte *et al.,* 1989). Therefore bFGF would appear to be released by cells, though not by the conventional endoplasmic reticulum/Golgi pathway. Transient and reversible mechanical induced disruption of the plasma membrane caused the release of cytoplasmic bFGF from cultured endothelial

cells (D'Amore, 1990; Haimovitzfriedman *et al.*, 1991; McNeil *et al.*, 1989; Muthukrishnan *et al.*, 1991; McNeil and Ito 1989; McNeil, 1993) and muscle fibres *in vivo* (Clarke *et al.*, 1993). Although cell death could not be excluded in the above studies, in single, isolated, NIH 3T3 cells transfected with a cDNA encoding bFGF, bFGF is exocytosed into the extracellular medium and stimulates migration of the cells via an autocrine mechanism (Mignatti *et al.*, 1991, 1992; Mignatti and Rifkin, 1991). The release of aFGF from cultured cells by exocytosis has been observed to be promoted by heat shock (Jackson *et al.*, 1992), suggesting a secretory pathway analogous to that proposed for interleukin-1β (Rubartelli *et al.*, 1990).

As a result, two alternative pathways have been suggested for the release of bFGF by cells: (i) transient cell wounding ; (ii) exocytosis. Evidence for the the transient cell wounding pathway (McNeil, 1993) has been obtained from studies *in vivo* (Clarke *et al.*, 1993), and in normal cultured cells (e.g., D'Amore, 1990; Haimovitzfriedman *et al.*, 1991; McNeil *et al.*, 1989; Muthukrishnan *et al.*, 1991). However, the contribution of cell death to the observed release of bFGF cannot be ruled out. The evidence for the exocytotic pathway has been obtained from the investigation of the release of bFGF and aFGF from single cells under conditions where cell death is unlikely to be a contributing factor (Jackson *et al.*, 1992; Mignatti and Rifkin, 1991; Mignatti *et al.*, 1991, 1992). However, the use of cells transfected with a cDNA encoding aFGF or bFGF and consequently overexpressing the protein may have caused the artefactual release of these FGFs. Since there are, however, examples of cells in culture producing an endogenous FGF which is released into the culture medium in the absence of mechanical or other stresses the exocytotic pathway is probably valid (Bunnag *et al.*, 1991; Jouanneau *et al.*, 1991, 1994; Mignatti and Rifkin, 1991; Sato *et al.*, 1989). One attraction of the exocytotic pathway is that it does not require mechanical stress to break the cell and hence it may operate in situations such as tissue development where the spatial and temporal regulation of the release of bFGF are important and yet there is no evidence for transient cell wounding.

1.2.5 Mammary gland

1.2.5.1 The structure and the cells of the mature mammary gland

The mature mammary gland consists of a highly branched parenchymal system of ducts that undergo characteristic changes at different periods of life and its function is to secrete milk to nourish newborns.

The lumen of the ducts is continuous throughout the ductal tree and is lined by a layer of one or more epithelial cells. Surrounding the epithelial cells is a more or less continuous layer of elongated myoepithelial cells which possess smooth muscle-like myofilaments and pinocytotic vesicles. The ends of the ducts terminate in various budded structures, depending on the state of differentiation of the gland. During lactation, when the mammary gland reaches full functional differentiation, the ducts terminate in alveoli, the lumen of which is bordered by a single layer of alveolar cells. The alveolar cells are secretory and produce the various components of milk. At earlier stages in development, the ducts terminate in alveolar buds, the precursors of the alveoli and in terminal end buds (TEBs). The TEBs contain cells that are intermediate in character between epithelial and myoepithelial, and are the most active site of cell proliferation in the developing gland (Joshi *et al.*, 1986). The mammary gland epithelium is separated from the stroma by basement membrane. Surrounding the mammary gland is stromal connective and adipose tissue. The stroma contains fibroblasts, adipocytes, endothelial cells of the blood vessels and nerves, all embedded in extracellular matrix. This loose, vascular connective tissue allows for easy distension when the alveoli multiply and enlarge for lactation.

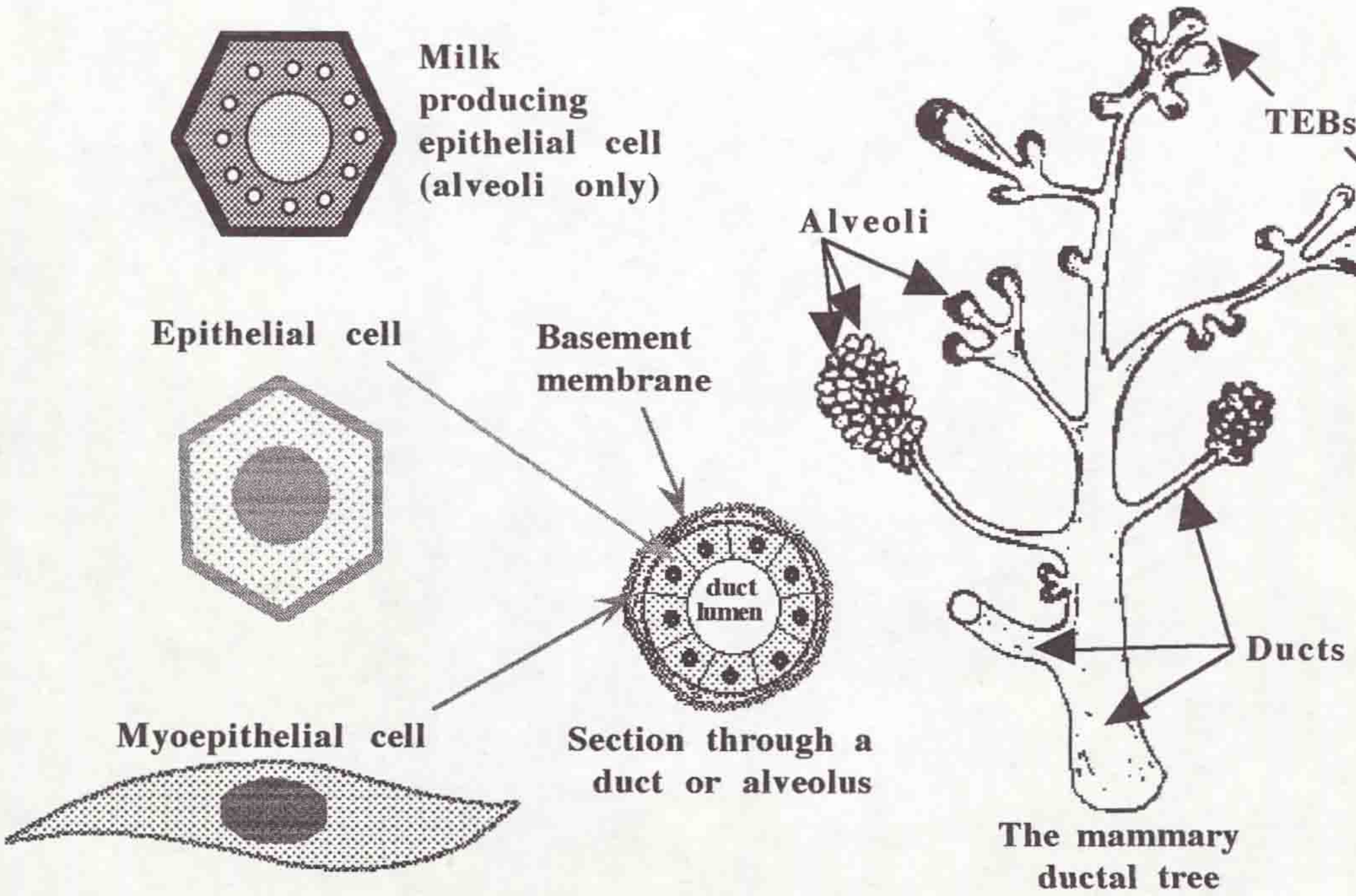


Fig. 1.9 The mammary gland and its cells

1.2.5.2 Development of Mammary gland

Most organs have completed morphogenesis by the time of birth and their subsequent development consists of enlargement or replication of preexisting structures. However, the mammary gland undergoes both morphogenesis and enlargement after birth and the growth of the tissue is at times allotropic. Therefore, we can investigate the development of the mammary gland, and the roles of FGFs in that developing system without having recourse to the embryonic animals.

In newborn rats, primitive mammary ducts with a lumen, epithelial and myoepithelial cells are apparent within 7 days. Between birth and puberty, the ductal tree extends to the limit of the fat pad by elongation and branching of existing ducts (Fernig *et al.*, 1990a). The ends of some of the ducts are formed by budded structures called TEBs. At the onset of puberty, the number of TEBs increases, and then as these differentiate into alveolar buds with each oestrus cycle, their number decreases (Ormerod and Rudland, 1984).

1.2.5.3 Growth factors in the mammary gland

The growth and differentiation of the mammary gland was shown to be controlled by systemic hormones (e.g., oestrogen and prolactin) in a series of endocrine gland ablation and hormone replacement experiments (Lyons *et al.*, 1958; Nandi, 1958). However, in cultured mammary cells a number of growth factors were found to be potent stimulators of cell growth whilst the systemic hormones did not have a significant growth-stimulatory effect (Topper & Freeman, 1980; Kano-Sueoka, 1983; Rudland *et al.*, 1977). These results were interpreted to suggest that the hormones had a 'priming' effect upon the cells of the mammary gland, whilst the growth factors were ultimately responsible for stimulation of cellular growth (Sirbasku, 1978). In addition, the growth-stimulatory effects of some of the growth factors were cell-type specific. For example, bFGF was found to stimulate the growth of myoepithelial cells and stromal fibroblasts, but not epithelial cells (Smith *et al.*, 1984b).

Since the discovery of the growth-stimulatory effect of growth factors on mammary cells, growth factors have also been shown to affect cell growth and tissue morphogenesis *in vivo*. For example, slow-release pellets impregnated with alpha transforming growth factor or EGF promote ductal growth and branching (Daniel and Silberstein, 1991; Vonderhaar, 1988). Mice carrying an *int*-2 transgene under control of a mammary specific promoter develop mammary hyperplasias (Ornitz *et al.*, 1992a)

bFGF is produced by stromal fibroblasts and myoepithelial cells derived from the mammary gland but not by the epithelial cells (Barraclough *et al.*, 1990; Ke *et al.*, 1993). In cells intermediate in character between myoepithelial and epithelial that are analogous to the cells found in TEBs, the expression of bFGF increases as the myoepithelial characteristics of the cells increase (Barraclough *et al.*, 1990). The expression of both receptors for bFGF, HS and FGFR, parallels that of bFGF: these receptors are absent from epithelial cells and are expressed in increasing amounts as the myoepithelial characteristics of the cells increase (Fernig *et al.*, 1990b, 1992, 1993; Ke *et al.*, 1993).

Immunocytochemical detection of bFGF in the developing rat mammary gland

indicates that bFGF is associated with the basement membrane and to a lesser extent with the myoepithelial cells along resting ducts but it is not associated with the luminal epithelial cells. bFGF is associated with the intermediate cells of the TEBs, while the basement membrane surrounding these structures is only weakly stained (Rudland *et al.*, 1993). When bFGF is added with the antibody, a profound increase in staining is observed for the basement membrane and to a lesser extent the myoepithelial and epithelial cells of resting ducts. Since heparin can inhibit the increase in staining, it seems likely that a large number of spare HS receptors for bFGF exist in the basement membrane as well as on the cells of quiescent areas of the mammary gland. In contrast, no increase in staining is observed in TEBs when bFGF is added to sections with the antibody. Hence in TEBs there is not a large number of spare HS receptors for bFGF (Rudland *et al.*, 1993). Therefore, it seems likely that bFGF is unable to diffuse when it is released by myoepithelial cells in resting ducts, whilst within growing structures such as TEBs, bFGF is able to diffuse freely not just within the epithelium, but also into the surrounding stroma. Thus bFGF may not just have an autocrine/paracrine growth-stimulatory function within the mammary epithelium, but it may also serve to stimulate events in the surrounding stroma, such as angiogenesis. In this way, bFGF may be involved in the stimulation of the growth of the mammary ductal tree and the coordination of the vascularization of the ductal tree.

In a rat model of breast cancer bFGF and its high-affinity receptors are expressed by malignant epithelial cells (Fernig *et al.*, 1993). Human malignant mammary epithelial cells consistently express high-affinity receptors for bFGF, and about a quarter of these cells produce bFGF (Peyrat *et al.*, 1992; Anandappa *et al.*, 1994). However, neither the rat nor the human mammary cells produce low-affinity receptors for bFGF (Fernig *et al.*, 1993; Peyrat *et al.*, 1992). The loss of the myoepithelial cell is a hallmark of malignant mammary tumours in rats and humans (Warburton *et al.*, 1982). Therefore the expression of high-affinity receptors for bFGF and of bFGF itself by the malignant cells is ectopic. Despite the loss of the low-affinity receptor the malignant epithelial cells

are able to respond to bFGF, indicating that they have a functional dual receptor system (Peyrat *et al.,* 1992; Smith *et al.,* 1994). Thus it seems likely that alterations to the HS receptors for bFGF on the malignant cells results in the maintenance of the activation function of this HS, but a loss of the sink function.

aFGF is likely to be a product of the mammary stroma (Barraclough *et al.,* 1990). FGF-7 mRNA has been detected in the developing mouse mammary gland (Colemankrnacik and Rosen, 1994). FGF-8 is produced by cells from the Shionogi carcinoma. When human mammary cells are engineered to express *hst* they become metastatic in the nude mouse (McLesky *et al.,* 1993). Therefore it would appear that several FGFs may be involved in the development of the normal mammary gland, and that the ectopic expression of the receptors for FGFs and of FGFs themselves may be an important step in the generation of malignant cells.

1.3 Aims

The FGFs play a key role in the development of numerous tissues (Section 1.2.1.1) including that of the mammary gland (Section 1.2.5.3). The complexity of the receptor system used by FGFs (Sections 1.2.3) to stimulate cellular processes such as cell division allows for a great deal of control to be exerted by cells on their responsiveness to FGFs. However, how cells may alter their expression of the different components of the dual receptor system for FGFs is not known. Moreover, there is as yet no consensus as to the mechanism of the dual receptor system for FGFs (Section 1.2.3.2). The aim of this thesis is to examine the interaction of FGFs with cells from a model of rat mammary development. Thus it is hoped to identify some of the features of the receptors for FGFs that are involved in controlling the growth of responsive mammary cells, and hence, ultimately, certain aspects of the growth and morphogenesis of the mammary gland.

Chapter Two
Materials and Methods

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 General Materials

2.1.1.1 Tissue culture

Dulbecco's modified Eagle's medium (DMEM)	Gibco Bio-Cult, Paisley, Scotland.
Dimethyl sulphoxide (DMSO)	Sigma Chemical Co, Poole, UK.
Foetal calf serum (FCS)	Seralab Biological Industries, Israel.
L-glutamine (200 mM)	Sigma.
Hydrocortisone	Sigma.
Insulin	Sigma.
Isoton II counting fluid.	Coulter Electronics, Luton, UK.
Trypsin, 2.5% (w/v) in PBS	Gibco.
Versene (0.5 mM EDTA in PBS, pH 7.2)	Gibco.
Cryotubes	Nunc.
Filters	Millipore, Bedford, MA, USA.
Sterilin tubes	Bibby Sterilin Ltd, Stone, UK.
24-well tissue culture plates	Gibco.
Tissue culture grade petri dishes	Nunc Roskilde, Denmark.

2.1.1.2 Preparation of HS-deficient cells

DMEM without $MgSO_4$, Cysteine & Methionine	Gibco.
L-Cysteine	Sigma.
L-Methionine	Sigma.
$MgCl_2$	BDH, Poole, UK.

$NaClO_3$	Fluka Chemika, Gillingham, UK.

2.1.1.3 DNA synthesis assay

Bovine serum albumin (BSA)	Sigma.
Heparin porcine intestinal mucosa (187 USP units/mg)	Sigma.
[^{3}H]-thymidine	ICN, Thame, Oxfordshire, U.K.
NaOH	BDH.
Trichloroacetic acid (TCA)	BDH.
ULTIMA Scintillant	Packard Canberra, Netherlands.

2.1.1.4 Binding assay

Chloroform	BDH.
Gelatin	BDH.
Iodogen	Pierce Warriner, Chester, UK.
KI	BDH.
$Na_2S_2O_5$	BDH.
Sephadex G25	Pharmacia, Uppsala, Sweden.
[^{125}I]-sodium iodide (NaI)	Amersham Int., Amersham, U.K.

2.1.1.5 Purification of HSPGs

Chondroitinase ABC (E.C.4.2.2.4)	Seikagaku Kogyo Co. Ltd, Japan.
D-[^{3}H] glucosamine hydrochloride	ICN.
DEAE Sepharose fast flow	Pharmacia.
Heparinase (E.C.4.2.2.7)	Sigma.
$Na_2{}^{35}SO_4$	ICN.
Pronase	Boehringer Mannheim, Sussex, UK.

2.1.1.6 SDS-PAGE

Ammonium persulphate	BDH.
Bromophenol blue	Fisons, Loughborough, UK.
Coomassie brilliant blue R-250	Sigma.
Ethylenediamine tetra-acetic acid (EDTA)	BDH.
Glycine	BDH.
Isopropanol	BDH.
Kodak X-ray film	Kodak Ltd.
N,N,N' ,N' ,Tetramethylethylene diamine	BDH.
Sodium azide	BDH.
Sodium dodecyl sulphate (SDS)	BDH.
Tris(hydroxymethyl)methylamine (Tris)	BDH.
Triton-X-100	Sigma.
Tween-20	Sigma.

2.1.1.7 Cellular subfractionation

Sucrose	BDH.

All other chemicals were of Analar grade from BDH or of equivalent grade from other suppliers.

2.1.1.8 SDS-PAGE molecular weight markers

Molecular weight markers were used as follows (apparent molecular weight (kDa) of the markers in parenthesis:

(i) for immunoblots, high molecular weight range prestained SDS-PAGE molecular weight markers (BioRad, Hemel Hempstead, UK): myosin (205); β-galactosidase (116.5); bovine serum albumin (80); ovalbumin (49.5).

(ii) for immunoblots that were subsequently stained, and for SDS-PAGE aimed at separating large polypeptides: high range SDS-PAGE molecular weight markers (BioRad):

myosin (200); β-galactosidase (116.25); phosphorylase B (97.4); bovine serum albumin (66.2); ovalbumin (45).

(iii) for SDS-PAGE aimed at separating small polypeptides: low range SDS-PAGE molecular weight markers (BioRad): phosphorylase B (97.4); bovine serum albumin (66.2); ovalbumin (45); carbonic anhydrase (31); trypsin inhibitor (21.5); lysozyme (14.4).

2.1.1.9 Sources of growth factors

Human recombinant bFGF and aFGF were the kind gift of Dr. Y. Ke, Department of Biochemistry, University of Liverpool. bFGF and aFGF were produced in E. coli and then purified by heparin-affinity chromatography (Ke *et al.*, 1990, 1992). EGF, purified from mouse submaxillary glands (Smith *et al.*,1984b), was a kind gift of Dr. J.A. Smith Department of Biochemistry, University of Liverpool.

2.1.2 Reagents and Buffers

2.1.2.1 Reagents and Buffers for tissue culture

Routine Medium (RM)	DMEM (1x), 0.75% (w/v) sodium bicarbonate, 20 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5% (v/v) FCS, 50 ng/ml Insulin, 50 ng/ml Hydrocortisone.
Step Down Medium (SDM)	DMEM (1x), 250 μg/ml BSA.
Freezing Medium	DMEM supplemented with 20% (v/v) FCS,

	7.5% (v/v) Dimethyl sulphoxide.
Trypsin/EDTA solution	25 ml versene containing
	2.5% (w/v) trypsin.
Phosphate buffered saline (PBS)	137 mM NaCl,
	2.7 mM KCl,
	8 mM Na_2HPO_4,
	1.5 mM KH_2PO_4,
	pH 7.2.
Hydrocortisone	1mg/ml in 100% (v/v) ethanol.
Insulin	1mg/ml in 5 mM HCl and 150 mM NaCl.

2.1.2.2 Preparation of HS-deficient cells

Sulphate-free-Routine Medium	S-free DMEM,
(S-free RM)	supplemented with 0.8 mM $MgCl_2$,
	15 mM $NaClO_3$,
	0.04 mM L-cysteine,
	0.02 mM L-methionine,
	5% (v/v) dialysed FCS,
	50 ng/ml insulin,
	50 ng/ml hydrocortisone.
	Filter sterilize through a
	0.2 μm Millipore filter, store at 4°C.
Sulphate-free-Step Down Medium	S-free DMEM,
(S-free SDM)	0.8 mM $MgCl_2$,
	15 mM $NaClO_3$,
	0.04 mM L-cysteine,
	0.02 mM L-methionine,
	250 μg/ml BSA.

	Filter sterilize through a 0.2 μm Millipore filter, Store at 4°C.

2.1.2.3 Reagents and buffers for DNA synthesis assay

Heparin	Dissolved in PBS to a concentration of 10 mg/ml, Filter sterilize through a 0.2 μm Millipore filter, Store at 4°C.

2.1.2.4 Reagents and buffers for binding assay

Binding Medium (BM)	DMEM supplemented with 20 mM HEPES, pH 7.2 and 1% (w/v) BSA, Filter sterilize through a 0.2 μm, Millipore filter, Store at 4°C.
Sulphate-free-Binding Medium (S-free BM)	S-free DMEM, 0.8 mM $MgCl_2$, 15 mM $NaClO_3$, 0.04 mM L-cysteine, 0.02 mM L-methionine, 1% (w/v) BSA, 20 mM HEPES, pH 7.2, Filter sterilize through a 0.2 μm Millipore filter, Store at 4°C.

2.1.2.5 Reagents and buffers for purification of HSPGs

Chondroitinase ABC/ pronase buffer	50 mM NaCl, 50 mM Tris, pH7.8.

Ion exchange buffers	PBS and 0.6 M or 2 M NaCl in 20 mM phosphate, pH 6.8.

2.1.2.6 Reagents and buffers for SDS-PAGE

Premixed acrylamide stock solution	Severn Biotech Ltd, Kidderminster UK. 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide.
SDS-PAGE resolving gel	7.5% or 15% (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.033% (v/v) NNN'N'- Tetramethylethylenediamine (TEMED), 0.033% (w/v) ammonium persulphate.
SDS-PAGE stacking gel	4% (w/v) acrylamide, 0.125M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (v/v) TEMED, 0.05% (w/v) ammonium persulphate.
SDS-PAGE sample buffer (1x)	10% (v/v) glycerol, 2% (w/v) SDS, 125 mM Tris-HCl, pH 6.8, 5% (v/v) β -mercaptoethanol, 0.002% (w/v) bromophenol blue.
Running buffer	192 mM Glycine, 50 mM Tris-HCl, 0.1% (w/v) SDS.
Coomassie blue dye staining	0.25% (w/v) Coomassie brilliant blue R, 50% H_2O,

Destaining solution	40% (v/v) methanol, 10% (v/v) glacial acetic acid. 50% H_2O, 40% (v/v) methanol, 10% (v/v) glacial acetic acid.

2.1.2.7 Regents and buffers for subcellular fractionation

MK buffer	5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 7.2.
MKT buffer	MK buffer with 0.1% (v/v) Triton-X-100.
Sucrose	2.2 M in MK buffer.

2.2 Methods

2.2.1 Culture of rat mammary cell lines

2.2.1.1 The cell Lines

The cell lines used were all single-cell-cloned and their derivation and characterization is summarized in Table 2.1.

Table 2.1. Origins of the rat mammary cell lines

	Cell line	Identity	Reference
Mammary tissue:			
Normal rat, fast sticking fraction	Rama 27	fibroblastic	Rudland *et al.*, 1984
Furth-Wistar inbred rat, benign, DMBA-induced tumour	Rama 37 CL-A3	epithelial	Dunnington *et al.*, 1983
Normal rat mammary gland	Rama 401	myoepithelial	Warburton *et al.*, 1981
Transplantable metastatic tumour, TMT-081	Rama 800	malignant, epithelial	Dunnington *et al.*, 1984
Transplantable weakly-metastasizing tumour, TR2CL	Rama 600	malignant, epithelial	Williams *et al.*, 1985

2.2.1.2 Routine culture of cells

Rat mammary (Rama) cells were seeded into 9 cm diameter tissue culture dishes and grown in monolayer culture in RM at 37°C in a humidified atmosphere of 10% carbon dioxide: 90% air.

The strongly adherent Rama 27, Rama 401, Rama 37 and Rama 600 cells were subcultured at a ratio of 1:8 when the cell monolayer was 80% confluent. The cells were washed twice with PBS. In the case of the Rama 37 cells, the cells were then incubated for 3 min in 5 ml versene at 37°C. The cell monolayers were then incubated with 1 ml 0.5%

trypsin (w/v) in versene at 37°C until the cell monolayer was detached from the culture dish. The weakly adherent Rama 800 cells were collected by repeated pipetting followed by centrifugation at 1000 rpm for 5 min.

The detached cells were resuspended in 7 ml RM and 1 ml of the resuspended cells was placed into 9 cm diameter tissue culture dishes. In order to ensure that the cells retained their growth characteristics, cells were used within a narrow passage range as follows: Rama 27 fibroblasts between passages 30 and 40; Rama 401 myoepithelial-like cells between 30 and 40; Rama 37 epithelial cells between 30 and 40; Rama 600 malignant epithelial cells between 20 and 30; Rama 800 malignant epithelial cells between 20 and 30.

2.2.1.3 Determination of cell number

The number of cells in a suspension was determined using a Coulter Electronics particle counter. A cell suspension (0.5 ml), obtained by Method 2.2.1.2, was mixed with 9.5 ml of Isoton II. Two counts were performed upon each suspension of cells and the mean was used to calculate the number of cells in the original suspension.

2.2.1.4 Freezing cells

When cell monolayers were ready for passaging, they were detached (Method 2.2.1.2). The resuspended cells were transferred to a 30 ml universal tube and the total number of cells determined (Method 2.2.1.3). The cell suspension was centrifuged at 1000 rpm for 5 min in a benchtop MSE centrifuge, and the supernatant removed by aspiration. The cell pellet was resuspended in an appropriate volume of freezing medium to produce a final cell density of 1-1.5 x 10^6 cells/ml and 1 ml aliquots were transferred to cryotubes. The cryotubes were placed on dry ice for 2 hours and then stored at -70°C for 2-3 days before being transferred to liquid nitrogen.

2.2.1.5 Thawing cells

Frozen cells were thawed quickly in a water bath at 37°C. The cells were transferred to a 30 ml plastic universal to which 10 ml of DMEM, supplemented with 20% FCS(v/v), was added slowly. The cell suspension was then centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 9 ml RM. The cell suspension was transferred to three 9 cm-diameter dishes and RM was added to give a final volume of 15 ml.

2.2.2 Preparation of HS-deficient cells

Near-confluent Rama 27 fibroblasts grown in 9 cm-diameter tissue culture dishes were washed twice with 10 ml PBS and then 10 ml S-free RM (Section 2.1.2.2) was added to each dish. Following a 6 hour incubation in this medium the cells were detached with 1 ml 0.5% trypsin (w/v) in versene (Section 2.2.1.2), resuspended in 9 ml S-free-RM and the number of cells counted (Section 2.2.1.3). The cells were collected by centrifugation at 1000 rpm for 5 min and the cell pellet was resuspended in S-free-RM to give 40,000 cells/ml and then 20,000 cells/well were seeded into 24-well plates. In some experiments the resuspended cells were plated out into 9-cm-diameter dishes.

2.2.3 Measurement of DNA synthesis in quiescent Rama 27 fibroblasts

2.2.3.1 General method

Rama 27 cells were plated into 24-well plates at a density 1-1.5 x 10^4 cells per 1 cm-diameter well in 0.5 ml RM. After 24 hours, the medium was removed and the cells were washed twice in PBS, and 0.5 ml SDM was added. After 24 hours, serum or the growth factors were added to the cells as required. In most experiments the 0.5 ml SDM in the wells was replaced with fresh SDM 1 h prior to the addition of growth factors or serum. In addition, heparin at a final concentration of 2 μg/ml was sometimes added to the SDM at this stage. After a further 18 h, 20 μl of 40 μCi/ml [^{3}H]-thymidine (0.8 μCi per well) containing

40 μM unlabelled thymidine was added to the cells. After a 1 hour incubation at 37°C, the cells were washed twice with PBS and then 0.5 ml of ice-cold 5% TCA (w/v) was added to precipitate macromolecules. The cells were left in TCA for at least 1 hour at 4°C. The TCA was removed and the cells were washed once with 0.5 ml of ice-cold 5% TCA, and twice with 0.5 ml 95% ethanol at 4°C. After drying the TCA precipitates on the bench for 2 h, 0.5 ml of 0.2 M NaOH was added to each well. The TCA precipitates were left at room temperature overnight to allow their solubilization and then 0.3 ml of solubilised macromolecules was added to scintillation vials, followed by 1 ml of ULTIMA scintillant. Radioactivity was determined by counting for 10 min in a Packard TRI Carb 1900TR scintillation counter.

2.2.3.2 Kinetics of DNA synthesis

In these experiments, Method 2.2.3.1 was modified such that the [^{3}H] thymidine was added for 1 hour at different times after the addition of growth factors or serum. The cells were then processed for scintillation counting as in Method 2.2.3.1.

2.2.3.3 Changing effectors

In these experiments, Method 2.2.3.1 was followed up to the addition of growth factors or serum. After the addition of the growth factors and serum, at times described in the Figure legends (generally 9 h after the initial addition of growth factors), the cells were washed twice with PBS, and fresh SDM that contained a different growth factor was added.

In order to interpret results clearly and to ensure that the experimental manipulations were not causing artefactual responses in the cells, several controls were required in these experiments. Each 24-well plate had the following controls in triplicate:

I. For homologous exchange experiments:

(i) no addition;

(ii) no addition, cells washed and fresh SDM added;

(iii) 1% FCS;

(iv) 1% FCS, cells washed and fresh SDM containing 1% FCS added;

(v) growth factor being assayed;

(vi) growth factor being assayed, cells washed and fresh SDM with the same growth factor added;

(vii) growth factor being assayed, cells washed and the original medium replaced.

II. For homologous exchange of growth factor at different concentrations:

A: For concentration exchange from a **low** concentration to a **high** concentration of growth factor:

(i) no addition;

(ii) no addition for 9 h, then the cells were washed and fresh SDM added with growth factor at the **high** concentration;

(iii) growth factor being assayed at the **low** concentration;

(iv) growth factor being assayed at the **low** concentration, cells washed and fresh SDM with the same **low** concentration of growth factor added;

(v) growth factor being assayed at the **high** concentration;

(vi) growth factor being assayed at the **high** concentration, cells washed and fresh SDM with the same **high** concentration of growth factor added;

(vii) growth factor being assayed at the low concentration, cells washed and the original medium replaced.

B: For concentration exchange from a **high** concentration to a **low** concentration of growth factor:

(i) no addition;

(ii) no addition for 9 h, then the cells were washed and fresh SDM added with growth factor at the **low** concentration;

(iii) growth factor being assayed at the **low** concentration;

(iv) growth factor being assayed at the **low** concentration, cells washed and fresh SDM with the growth factor at the same **low** concentration added;

(v) growth factor being assayed at the **high** concentration;

(vi) growth factor being assayed at the **high** concentration, cells washed and fresh SDM with the growth factor at the same **high** concentration added;

(vii) the **high** concentration of growth factor for 9 h, cells washed and fresh SDM added.

III. For heterologous exchange of growth factors at different concentrations:

A: For concentration exchange from a **low** concentration of a **first** growth factor to a **high** concentration of a **second** growth factor:

(i) no addition;

(ii) no addition for 9 h, then cells washed and fresh SDM added with a **high** concentration of the **second** growth factor;

(iii) **low** concentration of the **first** growth factor;

(iv) **low** concentration of the **first** growth factor for 9 h, cells washed and fresh SDM with same growth factor at the same concentration added;

(v) **high** concentration of the **second** growth factor;

(vi) **high** concentration of the **second** growth factor for 9 h, cells washed and fresh SDM with the same growth factor at same concentration added;

(vii) **low** concentration of the **first** growth factor, cells washed and the original medium replaced.

B: For concentration exchange from a **high** concentration of the **first** growth factor to a **low** concentration of the **second** growth factor:

(i) no addition;

(ii) no addition for 9 h, then cells washed and fresh SDM added with a **low** concentration of the **second** growth factor;

(iii) **high** concentration of the **first** growth factor;

(iv) **high** concentration of the **first** growth factor for 9 h, cells washed and fresh SDM with the same growth factor at the same concentration added;

(v) **low** concentration of the **second** growth factor;

(vi) **low** concentration of the **second** growth factor for 9 h, cells washed and fresh SDM with the same growth factor at the same concentration added;

(vii) **high** concentration of the **first** growth factor for 9 h, cells washed and fresh SDM added.

The subsequent addition of [^{3}H]-thymidine and the processing of the cells for scintillation counting were as described in Method 2.2.3.1.

2.2.3.4 Decay of mitogenic signal

These experiments followed Method 2.2.3.1 as far as in Method 2.2.3.3 above. After the addition of growth factors, the cells were incubated for 9 h. The cells were then washed twice with PBS, sometimes containing 2 μg/ml heparin, and fresh SDM was added. After varying periods of time, growth factor was added back to the cells at the original concentration, and 18 h after the initial addition of growth factor, [^{3}H]-thymidine was added and the cells were processed according to Method 2.2.3.1.

2.2.4 Iodination of bFGF

bFGF was iodinated using IODOGEN as the oxidant (Fernig *et al.*, 1990). IODOGEN was dissolved in chloroform to give a final concentration of either 1 mg/ml or 100 μg/ml, and 50 μl was added to 1.5 ml microfuge tubes. The chloroform was evaporated by hand-warming, and the IODOGEN-coated tubes were stored at 4°C for up to three months. One day before the iodination a centrifugal desalting column (Christopherson, 1983) was prepared. The tip of a 1 ml syringe was packed tightly with glass wool. The plunger was then removed from the syringe which was filled to the top with preswollen Sephadex G25. The column was washed with 10 column volumes of PBS containing 0.2% gelatin and 0.02% azide (both w/v), and then the column was stored overnight at 4°C.

To the IODOGEN-coated tube, 40 μl of 0.5 M NaH_2PO_4 pH 7.4 was added, followed by 2 μg of growth factor (the actual amount depended on the scale of the preparation and the concentration of the growth factor stock; growth factor and [^{125}I] were always added in the ratio of 1 μg : 100 μCi), and 2 μl of [^{125}I] (200 μCi, the actual amount depending on the

scale of the preparation). The tube contents were mixed by gentle tapping with a finger, and the reaction was allowed to proceed for 10 min. While the reaction took place, the 1 ml Sephadex G25 column (prepared the preceding day) was centrifuged for 2 min at 1600g in an MSE Centaur benchtop centrifuge to remove excess buffer. The iodination reaction was stopped by transfer of the soluble reactants to a microfuge tube containing 5 μl 100 mM KI, 5 μl 100 mM $Na_2S_2O_5$ (made freshly), and 50 μl PBS containing 0.2% gelatin and 0.02% sodium azide (both w/v). The quenched iodination reaction was loaded onto the Sephadex G25 column, which was then centrifuged as above. Free [^{125}I] remained in the G25 column, while the [^{125}I]-labelled bFGF was in the eluate.

Measurement of the growth-stimulatory activity of the iodinated bFGF indicated that 100% of the original growth-stimulatory activity was recovered after iodination. The [^{125}I]-bFGF had specific activity of 7.7 - 8.2 x 10^{17} dpm/mole, and was used immediately.

2.2.5 Processing of [^{125}I]-bFGF

2.2.5.1 Processing of [^{125}I]-bFGF in whole cultured cells

Rama 27 cells were plated at a density $2x10^5$ cells in 5 cm-diameter culture dishes in 5 ml RM. After 24 hours the cells were washed twice with PBS, and 5 ml SDM added. After a further 24 h, the cells were washed twice with PBS, and 2 ml BM (Section 2.1.2.4) at 4°C for experiments at 4°C or 2 ml SDM for experiments at 37°C was added together with 20 ng/ml of [^{125}I]-bFGF. The cells were then incubated at 4°C or at 37°C on an orbital shaker for the time specified in the Figure legends. Following the incubation the culture medium was removed and the cells were washed twice with PBS (4°C). The cells were then scraped in the residual PBS with a rubber policeman and put into microfuge tubes. Twenty microlitres of 2x SDS-PAGE sample buffer (Section 2.1.1.6) was added to the tubes to give ratio of sample buffer to the cell suspension of 1:1 and the samples were sonicated with Dawe Ultrasonic Generator (Branson Sonic Power Company, London UK) set on 20% power with a 30% pulsed output for 1 minute to shear DNA and RNA. The samples were

then heated to 100°C for 3 minutes and polypeptides separated by electrophoresis (Section 2.2.9.1).

2.2.5.2 Subcellular fractionation

Rama 27 cells were grown in RM or S-free RM in 9 cm dishes. When the cells were 90% confluent, the cells were washed twice with PBS and 5 ml of the appropriate SDM added. After a further 24 h, 3 ng/ml of [^{125}I]-bFGF was added to the cells. After a 9 h incubation at 37°C, the culture medium was removed and kept on ice, to produce the " culture medium" fraction.

The cells were then washed twice with 5 ml PBS (4°C) and 0.5 ml MKT buffer (Section 2.1.2.7) at 4°C was added. The cells were scraped with a rubber policeman in the MKT buffer and placed in a Dounce homogenizer kept on ice. The culture dish was washed by scraping with a further 0.5 ml MKT buffer which was added to the original 0.5 ml. The cells were homogenized in the Dounce homogenizer with 20 strokes and centrifuged at 13,000g for 2 min. The supernatant was stored temporarily on ice while the cell pellet was resuspended in 1 ml MKT buffer, re-homogenized and nuclei were collected by centrifugation at 13,000g for 2 min. The second supernatant was pooled with the first - this fraction represents the cytoplasmic compartments of the cell, the "cytoplasmic" fraction. The pellet was carefully resuspended in 0.25 ml MKT buffer (4°C) and then 2.5 ml 2.2 M sucrose in MK buffer (4°C; Section 2.1.2.7) was slowly added to the pellet. A cushion of 2 ml 2.2 M sucrose in MK buffer was added to a 6 x 14 ml Kontron swingout tube. The resuspended nuclei in 2 M sucrose were carefully overlaid on the cushion, and then the tube was filled with 8 ml 0.22 M sucrose in MK buffer. Following centrifugation at 40,000 g for 1 h at 4°C, the supernatant was removed. The pellet, which contains purified nuclei, as determined by electron microscopy (Yu, L. and Fernig, D.G., unpublished result), was stored at -20°C.

2.2.6 Binding of [^{125}I]-bFGF to Rama 27 fibroblasts - surface receptors

The binding of [^{125}I]-bFGF to cells in monolayer culture was performed using a method described previously (Fernig *et al.*,1990, 1992). The cells were plated out into 24 well plates at $2x10^4$ cells per well in RM or in S-free RM (Sections 2.2.1.2 and 2.2.2). Two days after plating, the cells were washed twice with 500 μl of PBS at 4°C and once with 200 μl of Binding Medium or S-free BM (Section 2.1.2.4), and then 200 μl of BM or S-free BM was added. [^{125}I]-bFGF, in a range of concentrations that increased linearly from 0.5 ng/ml to 250 ng/ml was added to quadruplicate sets of wells. At concentrations of 1 ng/ml and 4 ng/ml of [^{125}I]-bFGF, a 200-fold excess of unlabelled bFGF was added to determine the non-specific binding. In some experiments 30 ng/ml heparin was included in the BM or S-free BM. The cells were incubated at 4°C on an orbital shaker for 2 h, by which time the binding of [^{125}I]-bFGF had reached a maximum (Fernig *et al.*,1990; Ke *et al.*, 1990). After incubation at 4°C, unbound radioactivity was removed by washing twice with 200 μl BM (in some experiments, S-free BM) and three times with 500 μl PBS at 4°C. Cell-associated radioactivity was solubilized with 0.5 M NaOH and then measured in a Wilj gamma counter. The number of cells in each 24-well plate was determined in four wells that were not used for binding using a Coulter counter as described in Method 2.2.1.3. The results were analysed by non-linear curve fitting with the aid of the LIGAND programme (Munson and Robard, 1980).

2.2.7 Purification of HSPGs

This method is a modification of that described by Gallagher and Turnbull (1988, 1991a,b). HS chains were isolated and purified from four different rat mammary cell lines: Rama 27 fibroblasts; Rama 401 myoepithelial-like cells; Rama 37 epithelial cells and Rama 800 malignant epithelial cells (Section 2.2.1.1).

2.2.7.1 Labelling

The cells were cultured in RM or S-free RM according to Methods 2.2.1.2 and 2.2.2. When the cells were 70% confluent, $Na_2{}^{35}SO_4$ (20 μCi/ml) and [^{3}H]-glucosamine (10

μCi/ml) were added to the culture medium. Cells were maintained in this medium for 48 hours prior to the preparation of HSPGs.

2.2.7.2 Preparation of HS from different cellular fractions:

Medium from unlabelled cells or metabolically labelled cells (Section 2.2.7.1) was removed and the cell monolayer washed with 2 x 3 ml PBS per 15 cm diameter culture dish. The PBS washes were pooled with the medium and then filtered through a 0.2 μm 47 mm diameter filter to remove any floating cells and cell debris. This medium sample is called "Conditioned Medium".

The cell layer was incubated with 2 ml 0.5% trypsin (w/v) in versene per 15 cm dish for 10 min at 37°C to detach the cells which were then centrifuged at 3000 rpm for 10 min in 30 ml universal tubes. The supernatant was collected and the cell pellet was washed with 5 ml PBS by centrifugation as above. The second supernatant was pooled with the first one to produce a sample called the "Trypsinate".

The cell pellet was solubilized by shaking vigorously in 5 ml 6 M urea, 0.1% Triton X-100 in PBS, pH 6.8 at 4°C overnight. The solubilized cells produced a sample called "urea/Triton" or "U/T" sample. All samples were either immediately subjected to anion-exchange chromatography or were stored at -20°C.

2.2.7.3 First DEAE-Sepharose anion-exchange chromatography

The Conditioned Medium, Trypsinate and the U/T samples were loaded onto a DEAE-Sepharose fast flow column (about 30 ml gel: 1.5 x 16 cm) pre-equilibrated in PBS, pH 6.8, at a flow rate of 2 ml/min. Following the application of the samples, the column was washed with 5 column volumes of PBS to remove unincorporated label. Bound material was eluted with a linear gradient of 0.15-2 M NaCl in 20 mM phosphate buffer, pH 6.8, and 100 x 2 ml fractions were collected. In the case of the U/T samples, the column was pre-equilibrated in 0.1% (v/v) Triton-X-100 in PBS pH 6.8. Following the application of the U/T sample, the column was treated in an identical manner to the Conditioned Medium and Trypsinate

samples except that all buffers contained 0.1% (v/v) Triton X-100. A small volume (5 or 10 μl) of each fraction was added to 1 ml Ultima Gold scintillant and the [^{3}H] and [^{35}S] radioactivity was determined by dual label scintillation counting in a Packard Tri Carb 1900 TR scintillation counter. The early eluting material (0.15-0.2 M NaCl) which contained only [^{3}H]-radiolabel has been shown to consist of hyaluronic acid (HA) by its susceptibility to hyaluronidase (Gallagher and Turnbull 1988). The material eluting at approximately 0.5 - 0.7 M NaCl, fractions 30-55, showed a bimodal distribution and contained both [^{3}H] and [^{35}S] labels and consists of sulphated proteoglycans (Gallagher and Turnbull 1988). These latter fractions were pooled, dialysed against 4 x 500 ml of distilled water and then freeze dried. Alternatively, the pooled fractions were concentrated to about 5 ml by reverse osmosis against polyethylene glycol 20,000 and then dialysed against 4 x 500 ml of chondroitinase buffer (Section 2.1.2.5) with the addition of 0.1% Triton X-100 in the case of the U/T cell extracts.

2.2.7.4 Enzyme treatment

The freeze dried samples were dissolved in chondroitinase buffer (Section 2.1.2.5). To hydrolyse chondroitin sulphate and dermatan sulphate, the samples were then digested for 16 hours at 37°C with 1.0 mU/ml of chondroitinase ABC. Core proteins were subsequently digested by an incubation with 10 μg/ml of pronase at 37°C for a further 16 hours.

2.2.7.5 Second DEAE sepharose anion-exchange chromatography

The above enzyme digests were loaded onto a DEAE-Sepharose column (about 5 ml gel: 0.7 x 5 cm) pre-equilibrated in PBS, pH 6.8, at a flow rate of 15 ml/h. Following sample application the column was washed with 5 column volumes of PBS to remove unincorporated label and unbound material. Bound material was eluted with a linear gradient of NaCl (0.15 - 0.8 M) in 20 mM PBS and 50 x 1 ml fractions were collected. Aliquots of each fraction (usually 5-10μl) were monitored for radioactivity by dual label scintillation counting (Section 2.2.7.3). The material eluting between 0.5 M NaCl and 0.7 M NaCl

showed a bimodal distribution and contained both [^{3}H]- and [^{35}S]-radiolabels and has been shown to consist of HS chains by its susceptibility to heparinases (Gallagher and Turnbull, 1988). Consequently the fractions containing both [^{3}H] and [^{35}S] that eluted between 0.5 M to 0.7 M NaCl were pooled and freeze dried. The dried samples were dissolved in a small volume (1-2 ml) of distilled water.

2.2.7.6 Desalting

Gel filtration on Sephadex G-25 was employed to desalt some samples of HS using a column of 25 x 1.2 cm equilibrated in distilled water at a flow rate of 50 ml/h. Alternatively the samples of HS were dialysed exhaustively against water. Following desalting the samples of HS were freeze dried, dissolved in 0.5 ml of distilled water and stored at -20°C.

2.2.8 Assay for the activation of FGFs by HS and heparin

After incubating HS-deficient Rama 27 cells in 24-well plates in S-free RM (Method 2.2.2) for 24 hours, the medium was removed and the cells were washed twice with 0.5 ml PBS, and 0.5 ml S-free-SDM was added (Section 2.1.2.2). The cells were incubated in this medium for 24 hours to render them quiescent and fresh S-free SDM (0.5 ml/well) was added. One hour later, growth factors, heparin or HS purified from different mammary cell lines were added to the cells as indicated in the Figure legends. The cells were then processed exactly as described for DNA synthesis assays (Section 2.2.3.1)

2.2.9 Separation of Polypeptides by SDS-PAGE

2.2.9.1 Preparation of SDS-PAGE

SDS-PAGE was carried out according to published methods (Sambrook *et al.*, 1989). SDS-PAGE was carried out using 7.5% (w/v) or 12% (w/v) acrylamide resolving gels with 4% (w/v) acrylamide stacking gels (Section 2.1.2.6) using the Bio-Rad mini protein gel system with 1 mm spacers. Electrophoresis was carried out at 30 mA, 100 V. When the dye

front reached the bottom of the gel, the gel mould was removed from the electrophoresis apparatus, and then taken apart.

2.2.9.2 Identification of polypeptides separated by electrophoresis

2.2.9.2.1 Fixing and staining

To fix and stain the gel after electrophoresis, the gel was incubated with Coomassie blue dye staining solution (Section 2.1.2.6) for 1 hour, and then incubated in several changes of destain solution until the background of the gel became very light blue or clear. The gel was then dried under vacuum with a Bio-Rad Model 583 Gel Dryer.

2.2.9.2.2 Autoradiography

Fixed, stained and dried gels were autoradiographed with a Kodak X-ray film at -70°C for between 3 and 20 days.

Chapter Three

General Properties of the Stimulation of DNA by FGFs

Chapter Three
General Properties of the Stimulation of DNA by FGFs

3.1 Introduction

bFGF, like other growth factors, has been found to induce the stimulation of many intracellular signalling pathways, and some of these are outlined in Sections 1.2.2.2.3 and 1.2.3.3. In this chapter the concentration- and time-dependence of the stimulation of DNA synthesis in Rama 27 fibroblasts by EGF, bFGF and aFGF is explored. The aim is to select suitable assay conditions which may be used for the investigation of the interactions of FGFs with mammary cells. In addition, the conditions under which three growth factors, aFGF, bFGF and EGF, generate the late signal(s) necessary for cell division are investigated, as is the relationship between these growth factors and this final signal(s).

3.2 Results

3.2.1 Response of quiescent Rama 27 cells to growth factors

3.2.1.1 Dose response

Increasing concentrations of EGF, aFGF and bFGF were added to quiescent Rama 27 cells to determine the dependence of DNA synthesis on growth factor concentration (Method 2.2.3.1). In the case of EGF, an increase in the rate of DNA synthesis occurred between concentrations of 1 pg/ml and 1 ng/ml of EGF (Fig. 3.1a). bFGF began to stimulate DNA synthesis at a concentration of 10 pg/ml. Half maximal stimulation occurred at 100 pg/ml, while maximal stimulation was observed at 3 ng/ml (Fig. 3.1b). aFGF was considerably less potent than bFGF with stimulation of DNA synthesis beginning at a concentration of 100 pg/ml, half maximal stimulation occurring

at 30 ng/ml, and maximal stimulation at 300 ng/ml (Fig. 3.1c).

Heparin at 2 μg/ml did not affect the stimulation of DNA synthesis by EGF or bFGF (Figs 3.1a,b). In contrast, the amount of aFGF required to achieve maximal stimulation of DNA synthesis is reduced 300-fold in the presence of 2 μg/ml heparin (Fig. 3.1c).

3.2.1.2 Kinetics of stimulation of DNA synthesis

The incorporation of [^{3}H]-thymidine was measured at specific times after the addition of FCS, EGF, bFGF or aFGF in the absence or the presence of 2 μg/ml heparin, as described in Methods 2.2.3.1 and 2.2.3.2.

In the absence of any additions, Rama 27 cells incorporated [^{3}H]-thymidine at a low rate (Fig. 3.2a). No stimulation above baseline was observed until 9 h after the addition of the growth factor. In the presence of 2% FCS, incorporation of [^{3}H]-thymidine into the DNA of Rama 27 cells started at 9 h and reached a maximum at 18 h (Fig. 3.2a). When the cells were exposed to 100 pg/ml and 1 ng/ml of EGF, DNA synthesis peaked at 18 h (Fig. 3.2b). Although the maximal response elicited by 100 pg/ml EGF was lower than that observed with 1 ng/ml EGF, the time-dependence of the stimulation of DNA synthesis in Rama 27 cells was indistinguishable at either concentration. The increase in DNA synthesis induced by bFGF occurred between 12 h-18 h, the maximum being at 18 h (Fig. 3.2c). The time of maximum stimulation is also independent of concentration. The stimulation of DNA synthesis by aFGF is maximal at 18 h and the kinetics of the stimulation of DNA synthesis are, once again, independent of concentration (Fig. 3.2d).

In the presence of heparin the kinetics of stimulation of incorporation of [^{3}H]-thymidine into DNA synthesis observed with EGF, aFGF or bFGF are similar to those observed in the absence of heparin (Figs 3.2b-d, insets).

3.2.2 Requirement for late signals

Method 2.2.3.3 was used to determine the effect of removing the growth factor stimulating Rama 27 fibroblasts following an initial 9 h period of stimulation. When the

SDM on the cells was removed after 9 h and replaced with fresh SDM , the level of DNA synthesis was identical, within the experimental error, (Figs 3.3a-d) to that observed in undisturbed cells. Similarly when the SDM containing 1% (v/v) FCS was removed and replaced with fresh SDM containing 1% (v/v) FCS (1508±101 cpm) the observed level of DNA synthesis was indistinguishable from that seen in the undisturbed cells (1493±93 cpm). Moreover, when the SDM containing either 1 ng/ml EGF (Figs. 3.3 a,c: EGF control) or 10 ng/ml bFGF (Figs 3.3b,d: bFGF control) was removed and replaced with either the original medium or fresh SDM containing the same growth factor at the same concentration, the level of DNA synthesis was identical. Therefore the manipulations carried out on the cells 9 h after the initial addition of growth factors did not affect the response of the cells to EGF or bFGF since the level of DNA synthesis observed in manipulated cells and undisturbed cells was equivalent (Figs 3.3a-d and Figs 3.4a-d; controls).

However, when EGF (1 ng/ml) was removed 9 h after its initial addition to the Rama 27 cells and replaced with just SDM, the level of incorporation of [^{3}H]-thymidine into DNA was reduced to 25%-30% of that observed in Rama 27 cells which were exposed to 1 ng/ml EGF for the full length of the experiment (Fig. 3.3a). In addition, in the presence of 2 μg/ml heparin a similar result was obtained (Fig. 3.3c).

In contrast to EGF, if bFGF was removed 9 h after its initial addition and replaced by SDM, the level of DNA synthesis was identical to that observed in cells exposed to bFGF for entire experimental period (Fig. 3.3b). However, in the presence of heparin (2 μg/ml) the removal of bFGF at 9 h caused a reduction of 50% in the level of incorporation of [^{3}H]-thymidine into DNA compared to the level observed in cells exposed to bFGF for the duration of the experiment (Fig. 3.3d).

Thus it would appear that in Rama 27 fibroblasts, there is a requirement for the signal(s) delivered by growth factors to be produced over a long period of time, if quiescent cells are to enter S-phase and replicate their genome.

3.2.3 Equivalence of late signals

To determine whether the requirement for late signals for the stimulation of DNA synthesis in Rama 27 cells by EGF and bFGF was dependent on the growth factor used to stimulate the cells initially, the growth factors were exchanged 9 h after their initial addition.

The replacement of 1 ng/ml EGF by 10 ng/ml bFGF produced the same level of incorporation of [^{3}H]-thymidine into the DNA of Rama 27 cells as those cells that remained in SDM containing EGF for the duration of the experiment (Fig. 3.4a). The same result was obtained in the presence of 2 μg/ml heparin (Fig. 3.4c).

As would be expected from Fig. 3.3b, replacing 10 ng/ml bFGF with 1 ng/ml EGF in the absence of heparin did not alter the level of DNA synthesis (Fig. 3.4b). In the presence of 2 μg/ml heparin replacing bFGF by EGF did not alter the level of DNA synthesis compared to the level in Rama 27 cells continuously exposed to bFGF (Fig. 3.4d).

3.2.4 Differential homologous stimulation of early and late signals

Rama 27 cells required a more or less continuous exposure to EGF or bFGF to produce a maximal response to these growth factors (Figs 3.3, 3.4). Therefore, to ascertain whether this requirement for long-term exposure of Rama 27 cells to growth factors was dependent on their concentration, the concentrations of EGF and bFGF were altered 9 h after their initial addition to the cells (Methods 2.2.3.1 and 2.2.3.3).

In one experiment a low concentration of EGF (10 pg/ml) was replaced by a high concentration of EGF (1 ng/ml) 9 h after the initial addition of EGF. The expected level of DNA synthesis would be the sum of the levels observed when the concentration of EGF was maintained at 10 pg/ml for the duration of the experiment (Fig. 3.5a) and when EGF was added at 1 ng/ml to cells that were incubated in SDM alone for the initial 9 h (Fig. 3.5a). However, the observed level of incorporation of [^{3}H]-thymidine into the DNA of Rama 27 cells was 3-fold more than the expected level when the

concentration of EGF was increased 9 h after its initial addition (Fig. 3.5a; Table 3.1). The replacement of 1 ng/ml EGF 9 h after its initial addition with 10 pg/ml EGF also increased the level of incorporation of [^{3}H]-thymidine into the DNA of Rama 27 cells above the expected level, though to a lesser extent (Fig. 3.5b; Table 3.1).

In the presence of heparin (2 μg/ml), the replacement of bFGF at a concentration of 10 pg/ml by bFGF at a concentration of 3 ng/ml also resulted in an increased level of DNA synthesis, although the effect was less pronounced (33% increase, Fig. 3.5c; Table 3.1). Decreasing the concentration of bFGF from 3 ng/ml to 10 pg/ml 9 h after the initial addition of bFGF resulted in a slight increase in the level of DNA synthesis compared to the expected level (Fig. 3.5d; Table 3.1).

3.2.5 Differential heterologous stimulation of early and late signals

The requirement of Rama 27 fibroblasts for a prolonged exposure to EGF or bFGF to induce maximal levels of DNA synthesis was dependent on the concentration of the growth factors during the course of the experiment (Fig. 3.5; Table 3.1). To determine whether this dependence was itself dependent on the nature of the growth factor, 9 h after the initial addition of growth factor to the cells, both the growth factors and their concentrations were changed.

The sum of the levels of DNA synthesis observed when the concentration of EGF was maintained at 10 pg/ml and when bFGF was added at 3 ng/ml to cells that were incubated in SDM alone for the initial 9 h was 593 cpm (Fig. 3.6a; Table 3.2). However, when the low concentration of EGF (10 pg/ml) was replaced by a high concentration of bFGF (3 ng/ml) 9 h after the initial addition of EGF the resulting level of DNA synthesis was increased by 50% (Fig. 3.6a; Table 3.2).

Similarly, the replacement of a low concentration of bFGF (10 pg/ml) by a high concentration of EGF (1 ng/ml) resulted in a 90% increase in the observed level of DNA synthesis when compared to the expected value (10 pg/ml bFGF present throughout plus 1 ng/ml EGF present for the final 9 h; Fig. 3.6c; Table 3.2).

In a parallel series of experiments in which the initial concentration of growth factor

was high a similar pattern was observed. Thus when a high concentration of EGF was replaced 9 h after its addition by a low concentration of bFGF, the observed level of DNA synthesis was 40% higher than expected (Fig. 3.6b; Table 3.2). The observed level of DNA synthesis was also increased by 40% over the expected value when a high concentration of bFGF (3 ng/ml) was replaced by a low concentration of EGF (10 pg/ml; Fig. 3.6d; Table 3.2).

3.2.6 Decay of the mitogenic signal

Methods 2.2.3.1 and 2.2.3.4 were used to monitor the decay of the mitogenic signal induced by EGF and bFGF in Rama 27 cells. When EGF was replaced at different times following its removal from the cells 9 h after its initial addition, a 14% decrease was observed in the rate of DNA synthesis after a 30 min interval (Fig. 3.7a). By 3 to 5 h the decrease was maximal, and the approximate half-life of the mitogenic signal would appear to be about 2 h (Fig. 3.7a).

bFGF at a concentration of 1 ng/ml was added to the cells and removed at 9 h for different periods of time. When bFGF was added back to the cells after an absence of 10 min the rate of DNA synthesis decreased by 14% (Fig. 3.7b). After an absence of 2 h the rate of DNA synthesis (measured at 18 h) dropped to 49% of the stimulation observed in control cells and the half-life of the mitogenic signal would appear to be 1-2 h (Fig. 3.7b).

3.3 Discussion

The dose-response experiments were important to establish the most suitable concentration of growth factors required to obtain a clear stimulation when the cells were exposed to a growth factor. The concentrations of growth factors required to elicit a maximum stimulation of DNA synthesis in Rama 27 cells, 1 ng/ml EGF, 3 ng/ml bFGF and 100-300 ng/ml aFGF (Fig. 3.1) are in agreement with those reported previously (Smith *et al.*, 1984b; Ke *et al.*, 1990). The inclusion of 2 μg/ml heparin in the SDM

had little or no effect on the stimulation of DNA synthesis in Rama 27 cells by EGF or bFGF. However, heparin at this concentration potentiated the stimulatory effect of aFGF by over 300-fold, rendering aFGF as potent as bFGF. The potentiation effect of heparin on aFGF is well-characterized, and it has been suggested that it is caused by heparin stabilizing aFGF (Damon, *et al.*, 1989; Sections 4.2.6 and 4.2.7).

The presence of the growth factor for 9 h was only just sufficient to cause an increase in the rate of DNA synthesis. A maximal signal was seen after the exposure of the cells to serum, EGF, bFGF or aFGF for 18 h. The second increase in incorporation of [^{3}H]-thymidine into the DNA of Rama 27 cells stimulated by FCS seen at 30 h is probably due to cells that have completed mitosis and are entering S-phase for a second time (Fig. 3.2a). Since a similar time course of DNA synthesis is obtained with each growth factor, this suggests that the same general processes are involved in stimulating DNA synthesis in Rama 27 cells. Because the maximum incorporation of [^{3}H]-thymidine is always at 18 h, we can measure DNA synthesis at this time, and we do not need to do a time course for each experiment. A comparison of the time courses obtained with different concentrations of growth factors shows that the time course of the response is not dependent on the concentration of growth factor added. Thus maximum DNA synthesis is observed at 18 h (Fig. 3.2), and so the concentration of EGF, bFGF or aFGF only affects the amount of synthesis of DNA but not the timing of the cell cycle. In addition, heparin does not appear to affect the kinetics of stimulation of DNA synthesis (Fig. 3.2).

It has been shown by others that once a cell is committed to DNA synthesis the growth factor could be removed from the cell culture medium, and that prolonged exposure to the growth factor (12 to 16 h) is required to reach this point (Chana and Smith, 1991; Rudland and Jiminez de Asua, 1979). This has led to the hypothesis that either one signal must be given over a prolonged period of time or that two signals, one early and the other late, must be given if a cell is to commit itself to DNA synthesis (Chana and Smith, 1991; Rudland and Jiminez de Asua, 1979). To investigate this

hypothesis, experiments were carried out in which the cells were washed 9 h after the initial addition of growth factor, and either a different growth factor, or the same growth factor at a different concentration was added to the cells. To be able to interpret the results, many controls were performed. Controls for the manipulation of the cells at 9 h are described in Method 2.2.3.3. It is clear that manipulating the cells at 9 h had no detectable effect on the magnitude of DNA synthesis (Figs 3.3a-3.3d).

When EGF is removed after 9 h, the incorporation of [^{3}H]-thymidine into DNA is reduced to 30% of that observed in the controls (Fig. 3.3a). This shows that EGF must be present for more than 9 h to stimulate DNA synthesis in Rama 27 cells as has been shown in 3T3 cells (Chana and Smith, 1991).

In contrast to EGF, the rate of DNA synthesis was not decreased if the bFGF was removed at 9 h (Fig. 3.3b). This is presumably because much of the bFGF is stored on its low-affinity HSPG receptors and then delivered after the wash to the high-affinity receptor as has been found in endothelial cells (Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989) and rat mammary myoepithelial cells (Fernig *et al.*, 1992). It has been shown that heparin effectively prevents the binding of bFGF to the HSPG receptors in endothelial cells and mammary myoepithelial cells without affecting the binding of bFGF to the high-affinity receptors (Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989; Fernig *et al.*, 1992). The binding assays (Section 4.2.8) confirm that heparin prevents the binding of [^{125}I]-bFGF to the HS low-affinity receptors for bFGF on Rama 27 fibroblasts. Thus heparin allows the bFGF to remain in the culture medium, and to be removed by the simple washing method used in Method 2.2.3.3.

In the presence of heparin, the removal of EGF produces the same result as in the absence of heparin (Figs 3.3a,c) indicating that heparin does not invalidate this experimental method. In contrast, in the presence of heparin, the removal of bFGF causes a 50% decrease in the incorporation of [^{3}H]-thymidine into DNA compared to that observed in the controls (Figs 3.3b,d). This shows that bFGF, like EGF, must be present for longer than 9 h if it is fully to stimulate DNA synthesis. In previous studies the removal of growth factor completely abolished the stimulation of DNA synthesis

(Chana and Smith, 1991). This may reflect the much lower concentrations used previously with 3T3 cells (Chana and Smith, 1991). Thus the differences between the two studies may be caused by high concentration of growth factor producing enough signal, perhaps because of the difficulty in effectively removing all of the growth factor in some of the cells, for these to commit themselves to DNA synthesis.

When bFGF is substituted for EGF (Figs 3.4a,c) or EGF is substituted for bFGF (Figs 3.4b,d) at high concentrations of growth factor the level of DNA synthesis is the same as in the controls. Thus bFGF can be substituted for EGF or EGF can be substituted for bFGF at 9 h without affecting the mitogenic stimulus and so the intracellular signals that operate after 9 h are interchangeable. This result is similar to that obtained previously with 3T3 cells (Chana and Smith, 1991) suggesting that it is a general property of late signals.

When a high concentration of EGF (1 ng/ml) is replaced by a low concentration (10 pg/ml), the rate of DNA synthesis is only 30% greater than the combined effect of a high concentration of EGF (1 ng/ml), removed after 9 h and not replaced, plus the stimulation caused by a low concentration of EGF over 9 h (Fig. 3.5b; Table 3.1). A similar result is seen in the corresponding experiments with bFGF (Fig. 3.5d; Table 3.1). These results suggest that it is not possible to 'prime' the cells for 9 h and then rescue the growth-stimulatory response of all the cells to the growth factors by generating a low level mitogenic signal from 9 h to 18 h. However, the growth-stimulatory response of some of the cells is rescued by generating a low level mitogenic signal after 9 h. Thus it may be possible to ‘prime’ a few, but not all, of the cells.

Increasing the concentration of EGF from a low dose (10 pg/ml) to a maximal dose (1 ng/ml) gives an enhanced response (Fig. 3.5a; Table 3.1). This enhanced response is greater than can be accounted for by the combined effect of the high dose over a 9 h period of stimulation plus the stimulation produced by the low dose. When the experiment is carried out with bFGF a marginal enhancement of the growth stimulatory response is observed, similar to that seen when high concentrations of growth factor are replaced with low concentrations of growth factor (Fig. 3.5c; Table 3.1). Therefore in

the case of EGF the lower dose has a primary effect on the cells such that some cells, which would not have divided on the initial signal from the low dose alone, are nevertheless susceptible to the increased signal given later. This suggests that the signal that is generated after 9 h by EGF may have a different dependence on the concentration of growth factor than the signal that is produced during the first 9 h. However, in the case of bFGF, such effects are less clear-cut. Moreover, initially stimulating the cells with a high concentration of growth factor and attempting to rescue the mitogenic signal with a subsequent low concentration of growth factor is not very effective. It is possible that the desensitization of the growth factor receptors (Fantl *et al.*, 1993) by a high concentration of growth factor may result in the marginal 'priming' effect seen when such concentrations are used to stimulate the cells initially (Figs 3.5b,d). However, since low initial concentrations of bFGF (Fig. 3.5c) can also only prime a minority of cells, it would seem that there may be differences in the nature of the signal(s) generated by EGF and bFGF that lead to cell division.

Since growth factors may be exchanged 9 h after their initial addition (Fig. 3.4), one way to overcome the problem of receptor desensitization would be to change the growth factor itself when the concentration change is made. One would expect that the greatest degree of receptor desensitization would occur when a high concentration of growth factor is used initially. However, when a high concentration of EGF or bFGF is replaced by low concentration of bFGF or EGF, respectively, the magnitude of the rescue of the growth-stimulatory signal (Table 3.2) is similar to that observed when a homologous exchange of growth factor is made (Table 3.1). Therefore receptor desensitization may not be an important factor in the inability of low concentrations of growth factor to rescue the growth-stimulatory signal initiated by a high concentration of growth factor. Alternatively, receptor cross-talk, which is known to occur between the FGFR and the EGF receptor tyrosine kinases (Brigstock *et al.*, 1990) may cause as much receptor desensitization during the heterologous exchange experiments (Section 3.2.5) as during the homologous exchange experiments (Section 3.2.4). Nevertheless,

the results of the heterologous exchange experiments are reasonably clear-cut and demonstrate again that at least some of the mitogenic signal generated by stimulating cells with a high concentration of EGF or bFGF for 9 h may be rescued by stimulating the cells with a low concentrations of these growth factors for the subsequent 9 h. When a low concentration of EGF or bFGF was replaced by a high concentration of bFGF or EGF, respectively, the degree of enhancement of the growth-stimulatory response was greatest when EGF was the second growth factor (Table 3.2). This result agrees with that from the homologous exchange experiments (Table 3.1) in which replacing a low concentration of EGF with a high concentration of EGF resulted in a much greater enhanced response than when the experiment was carried out with bFGF (Table 3.1). Therefore although EGF and bFGF are apparently equivalent (Fig. 3.4), these two growth factors may use different signalling pathways in the latter half of the period between G_o and S-phase DNA synthesis. Alternatively, the signalling pathways used at this time may be the same but have different sensitivities to EGF and bFGF.

Interaction of growth factors with their cognate cell-surface receptors on quiescent cells leads to DNA replication and mitosis. Relatively well-characterized early events take place during the lag period before initiation of DNA synthesis. However, the cellular response is pleiotypic, and not all of the early events (Sections 1.2.2.2.3 and 1.2.3.3) are initiated by any particular growth factor acting on a particular target cell. Furthermore the early events in themselves are not sufficient to induce DNA synthesis as synthesis does not take place if the growth factor is removed from the cells before the end of the lag period (Carpenter and Cohen, 1975; Reid and Reid, 1987; Chana and Smith, 1991; Fig. 3.3). Also conditions are known under which early events may occur, but do not induce DNA synthesis, as in the case when platelet-derived growth factor interacts with a mutated receptor (Escobedo and Williams, 1988), when bFGF interacts with a mutated FGFR (Peters *et al.*, 1992; Mohammadi *et al.*, 1992) or when a truncated form of aFGF activates FGFRs (Immamura *et al.*, 1990, 1992). Many of the early events stimulated by growth factors are analogous to those stimulated by hormones, e.g., insulin, that alter cellular metabolism (White and Kahn, 1994). Some

alterations to cellular metabolism, e.g., synthesis of ribosomes and histones, are probably prerequisites for cell division. However, it would seem reasonable to suggest that given the inconsistencies between the kinetics of the stimulation of DNA synthesis, the half-life of the mitogenic signal(s) generated by growth factors and the requirement for a prolonged stimulation by growth factors on the one hand, and the kinetics of the early events on the other hand that the early events themselves are not the actual signals that cause cell division. Thus whilst the results presented in this chapter do not identify a signal(s) that does cause cell division, they do define certain properties that such a signal(s) must possess.

These properties can be summarised as follows:

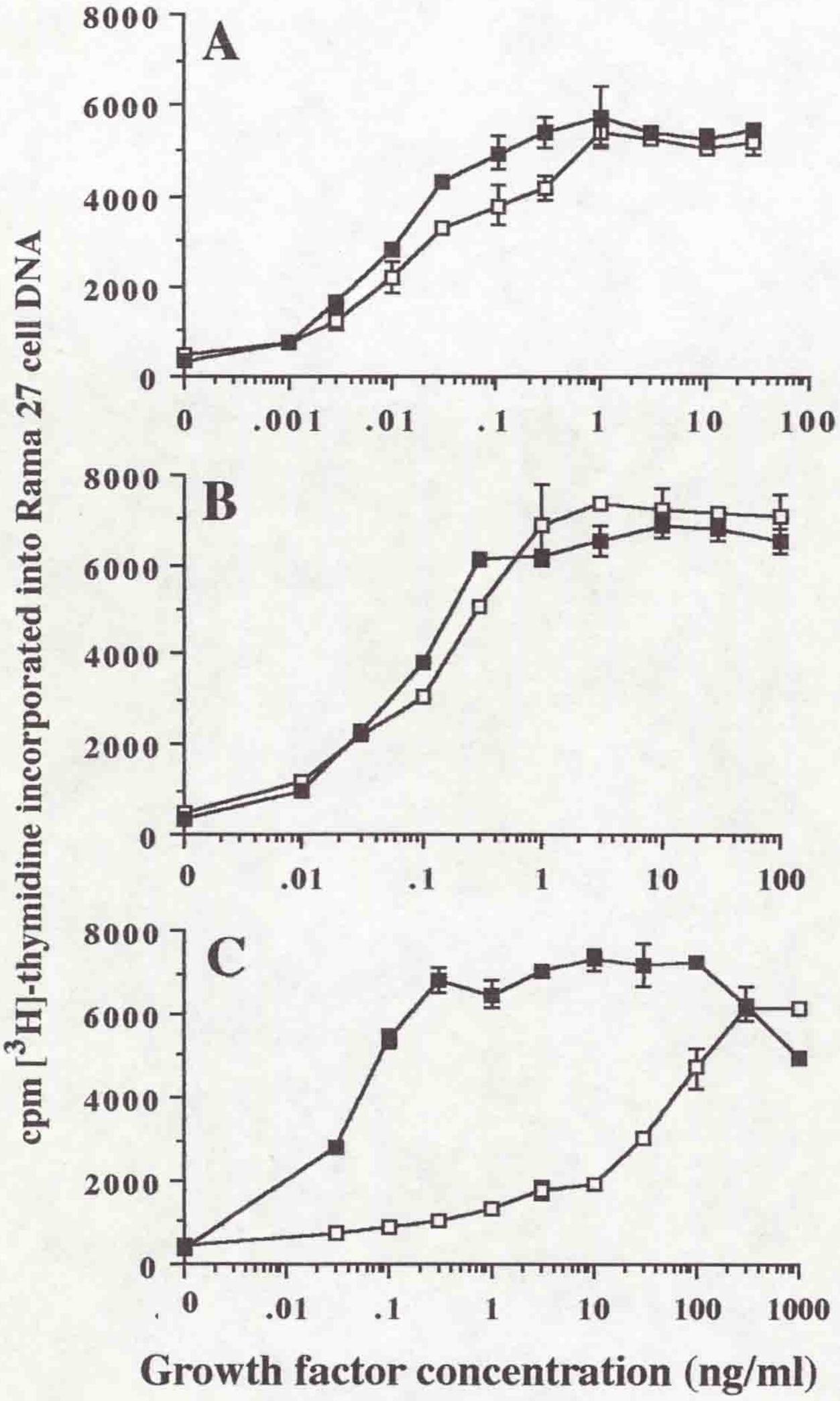
(i) A half-life of 1-2 h (Fig. 3.7).

(ii) Continuous production of the signal is necessary for DNA synthesis to occur (Fig. 3.3).

(iii) Equivalence between the signals produced by bFGF and EGF (Fig. 3.4).

(iv) Difference in the sensitivity of the signal(s) between 0-9 h and 9-18 h to growth factor concentration.

Fig. 3. 1 Concentration dependence of the stimulation of DNA synthesis in Rama 27 cells by growth factors



The experiment was carried out as described in Method 2.2.3.1. (A) EGF; (B) bFGF; (C) aFGF, in the absence (□) or presence (■) of 2 μg/ml heparin. Results are expressed as the mean cpm±SD of triplicate samples.

Fig.3.2 Kinetics of stimulation of DNA synthesis by growth factors on Rama 27 fibroblasts with and without heparin

The cells were treated as described in Method 2.2.3.2.

(A) (o) SDM; (●) 2% FCS,

(B) EGF: (o) 100pg/ml EGF, (●) 1ng/ml EGF; Inset : 1ng/ml EGF: (▫) No heparin; (■) with 2 μg/ml heparin.

(C) bFGF: (o) 100pg/ml bFGF, (●) 3ng/ml bFGF; Inset : 3ng/ml bFGF: (▫) No heparin; (■) with 2 μg/ml heparin.

(D) aFGF: (o) 10ng/ml aFGF, (●) 100ng/ml aFGF; Inset: 10ng/ml aFGF: (▫) No heparin; (■) with 2 μg/ml heparin.

Results are expressed as the mean cpm±SD.

Fig.3.2 Kinetics of stimulation of DNA synthesis by growth factors on Rama 27 fibroblasts with and without heparin

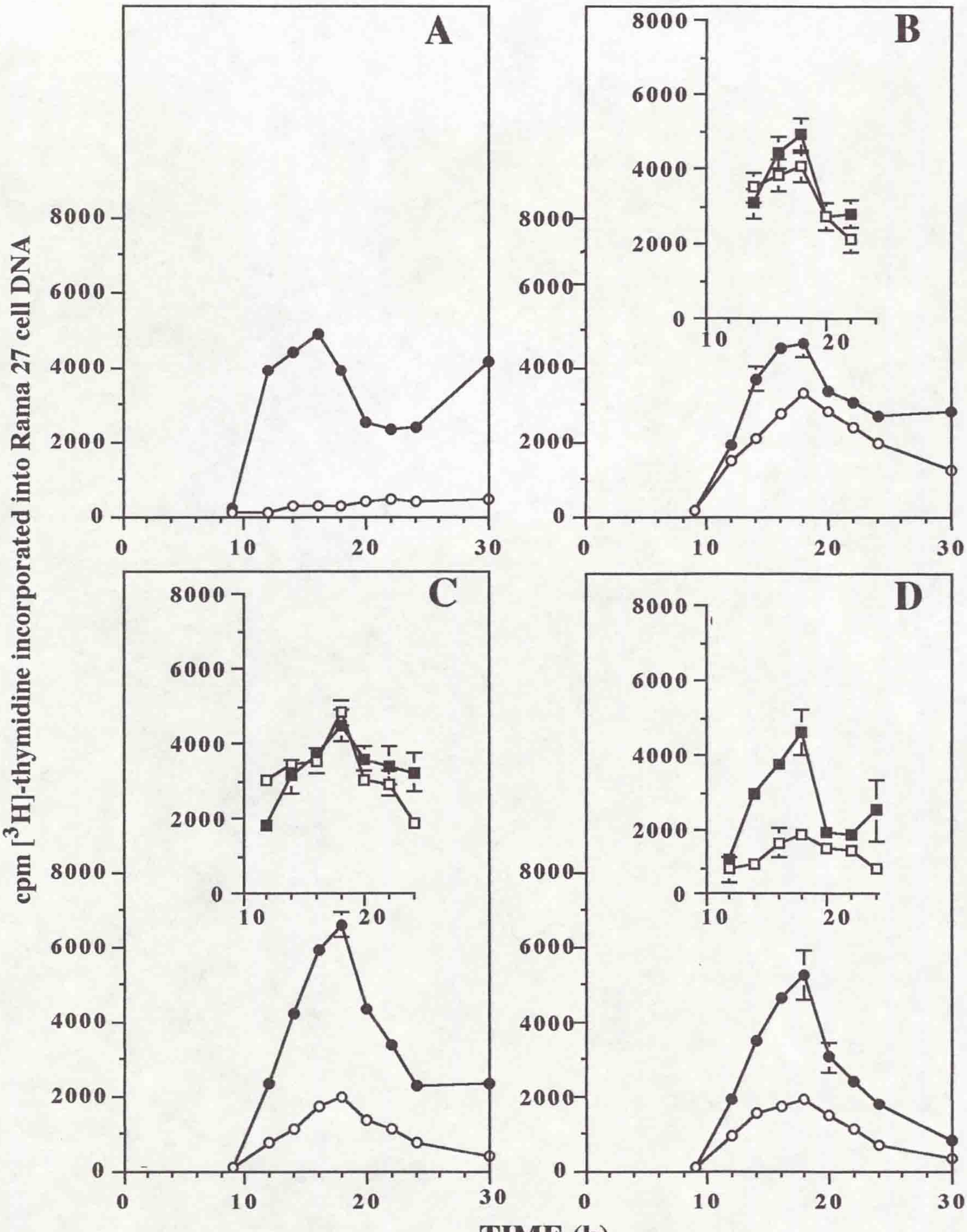


Fig. 3.3 Effect of changing the mitogenic stimulus on Rama 27 fibroblasts.

The experiment was carried out as described in Method 2.2.3.1, and all manipulations were carried out 9h after the first addition of growth factors.

Each experimental value is the mean of three datum points: Values for the controls have been pooled: BSA, no addition (mean of controls: Section 2.2.3.3, I-i and I-ii); 1% FCS (v/v) (mean of controls: Section 2.2.3.3, I-iii and I-iv); EGF and bFGF control (mean of three controls: obtained with EGF at 1 ng/ml Section 2.2.3.3, I-v, I-vi, and I-vii; bFGF at 10ng/ml Section 2.2.3.3, I-v, I-vi, and I-vii).

(A) EGF→SDM, 1ng/ml EGF replaced with SDM, no heparin; (B) bFGF→SDM, 10 ng/ml bFGF replaced with SDM, no heparin; (C) EGF→SDM, 1ng/ml EGF replaced with SDM in the presence of 2 μg/ml heparin; (D) bFGF→SDM, 10ng/ml bFGF replaced with SDM in the presence of 2 μg/ml heparin; Error bars represent the SD of three to nine datum points.

Fig. 3.3 Effect of changing the mitogenic stimulus on Rama 27 fibroblasts.

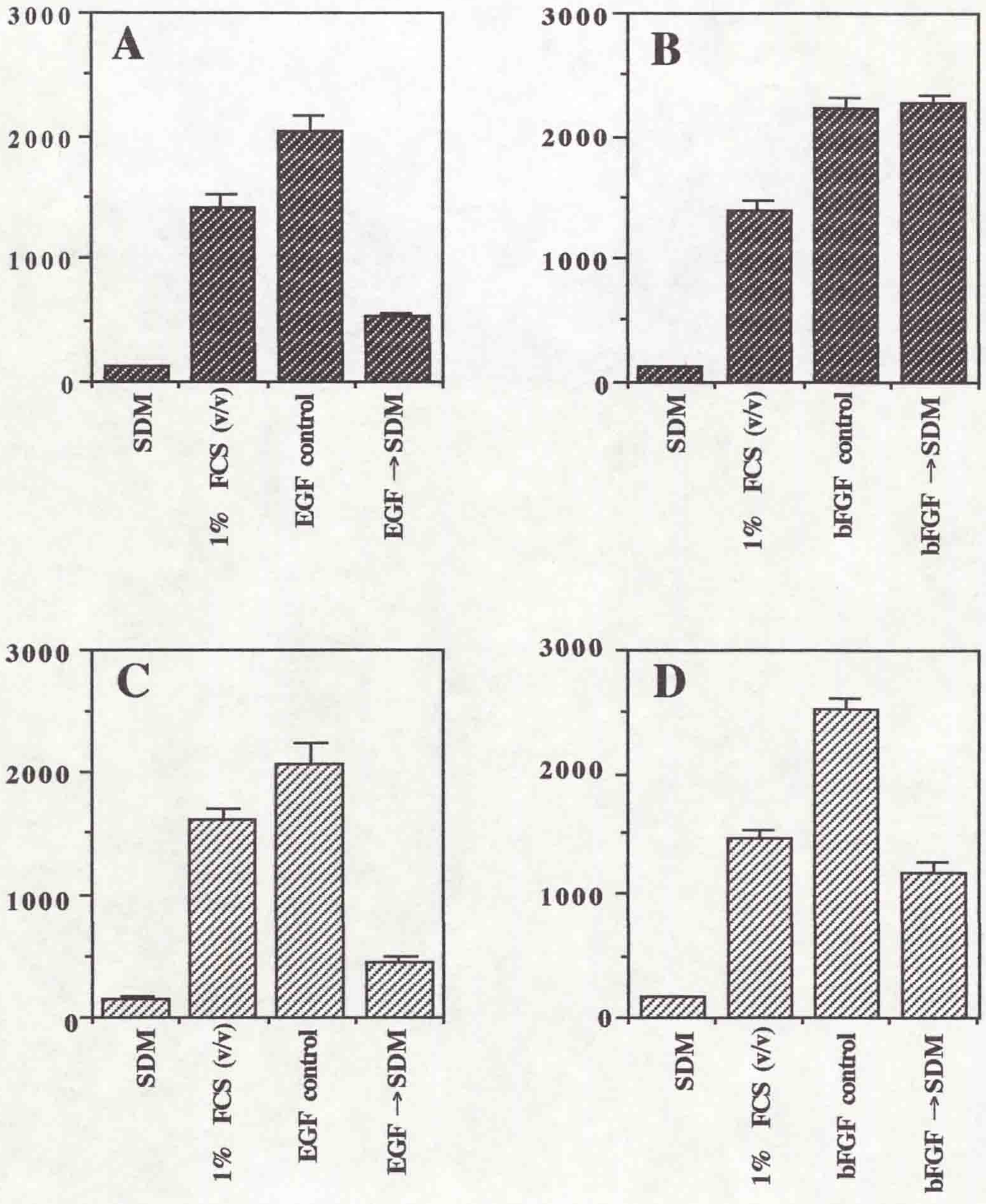


Fig. 3.4 Equivalence of late signals of EGF and bFGF on Rama 27 fibroblasts

The experiment was carried out as described in method 2.2.3.1, and all manipulations were carried out 9h after the first addition of growth factors. Each experimental value is the mean of three datum points: Values for the controls have been pooled: BSA, no addition (mean of controls: Section 2.2.3.3, I-i and I-ii); 1% FCS (v/v) (mean of controls: Section 2.2.3.3, I-iii and I-iv); EGF and bFGF control (mean of three controls: obtained with 1ng/ml EGF Section 2.2.3.3, I-v, I-vi and I-vii ; 10 ng/ml bFGF Section 2.2.3.3, I-v, I-vi, and I-vii).

(A) EGF→ bFGF, 1ng/ml EGF replaced with 10 ng/ml bFGF, no heparin; (B) bFGF→ EGF, 10ng/ml bFGF replaced with 1ng/ml EGF, no heparin; (C) EGF→ bFGF, 1ng/ml EGF replaced with 10 ng/ml bFGF, with 2 μg/ml heparin; (D) bFGF→ EGF, 10ng/ml bFGF replaced with 1ng/ml EGF, with 2 μg/ml heparin. Error bars represent the SD of three to nine datum points.

Fig. 3.4 Equivalence of late signals of EGF and bFGF on Rama 27 fibroblasts

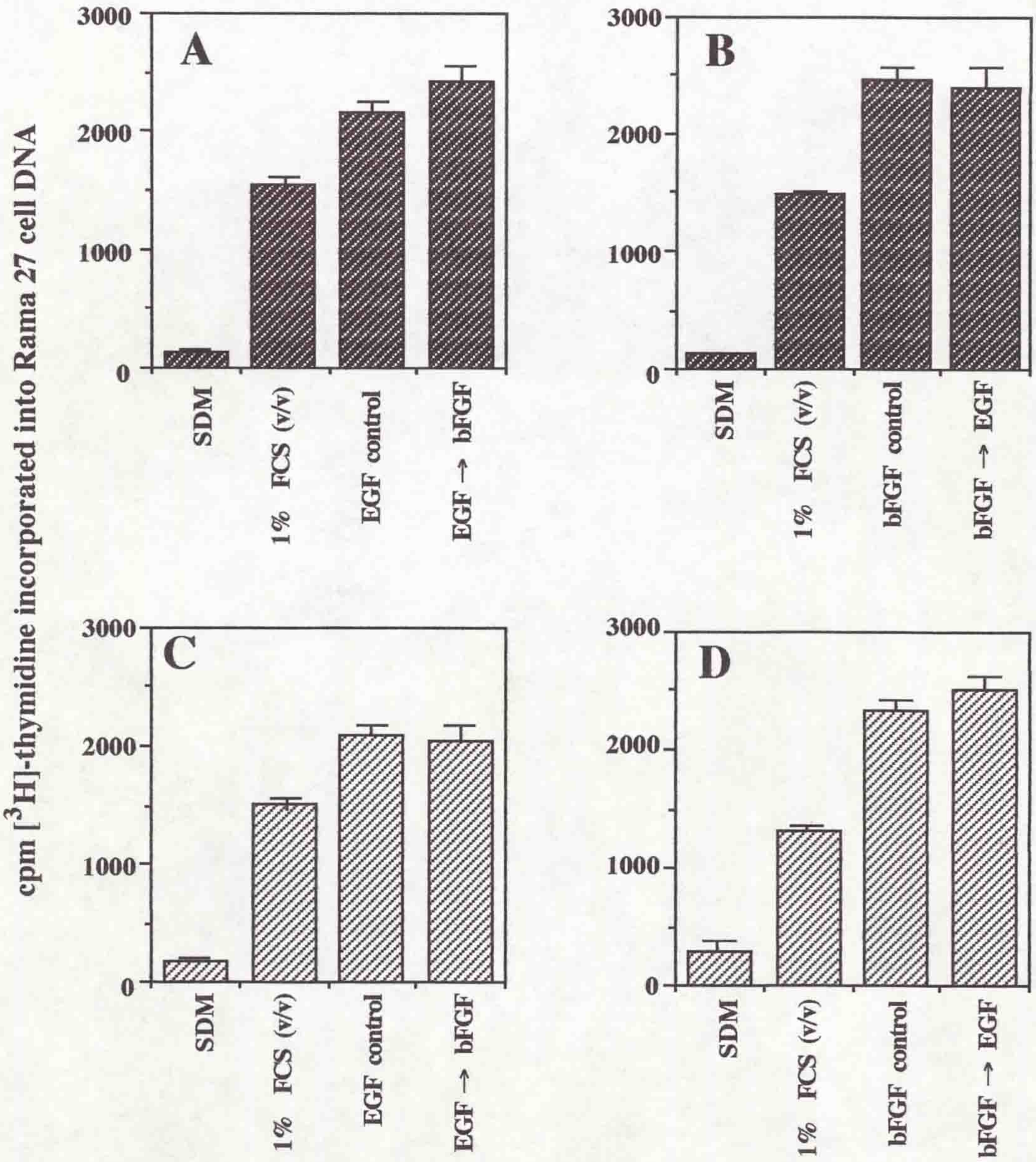


Fig. 3.5 Effect of changing the concentration of growth factor 9 h after initial stimulation of Rama 27 fibroblasts with growth factor

The experiment was carried out in a manner identical to that in Fig. 3.4. (A, B) EGF; (C, D) bFGF.

Control for baseline DNA synthesis in all panels: SDM, no addition (mean of control values: Section 2.2.3.3, II-A-i and II-B-i);

Controls for the stimulation of the cells from 9h to the addition of [^{3}H]-thymidine at 18 h by a high (A, C) or a low (B, D) concentration of growth factor: (A) SDM → EGF, SDM replaced by SDM containing 1 ng/ml EGF: Section 2.2.3.3, II- A- ii; (B) SDM→EGF, SDM replaced by SDM containing 10 pg/ml EGF: Section 2.2.3.3, II-B-ii; (C) SDM → bFGF, SDM replaced by SDM containing 3 ng/ml bFGF: Section 2.2.3.3, II-A-ii; (D) SDM→ bFGF, SDM replaced by SDM containing 100 pg/ml bFGF: Section 2.2.3.3, II- B-ii;

Control for the residual stimulation of DNA synthesis after removal of a high concentration of growth factor 9 h after the initial addition of growth factor: (B) EGF → SDM, EGF (1ng/ml) replaced by SDM: Section 2.2.3.3, II-B-vii; (D) bFGF → SDM, bFGF (3 ng/ml) replaced by SDM: Section 2.2.3.3, II-B-vii;

Control for stimulation with a low concentration of growth factors ([A]10 pg/ml EGF; [C] 10 pg/ml bFGF; Section 2.2.3.3, II- A-iii and II- A-iv ; II-B-iii and II-B-iv); control for stimulation with a high concentration of growth factors ([A, B]: 1 ng/ml EGF; [C, D]: 3 ng/ml bFGF; Section 2.2.3.3, II-A-v and II-A-vi ; II-B-v and II-B-vi).

The experiment data: (A) EGF 10 pg→ EGF 1ng, a low concentration of EGF (10 pg/ml) at 9 h is replaced by a high concentration of EGF (1 ng/ml); (B) EGF 1 ng → 10 pg, a high -concentration of EGF (1 ng/ml) is replaced by a low concentration of EGF (10 pg/ml); (C) bFGF 10 pg → bFGF 3 ng, a low concentration of bFGF (10 pg/ml) is replaced by a high concentration of bFGF (3 ng/ml); (D) bFGF 3ng → 10 pg, a high concentration of bFGF (3 ng/ml) is replaced by a low concentration of bFGF (10 pg/ml). Experimental values are the mean±SD of three or six datum points.

Fig. 3.5 Effect of changing the concentration of growth factor 9 h after initial stimulation of Rama 27 fibroblasts with growth factor

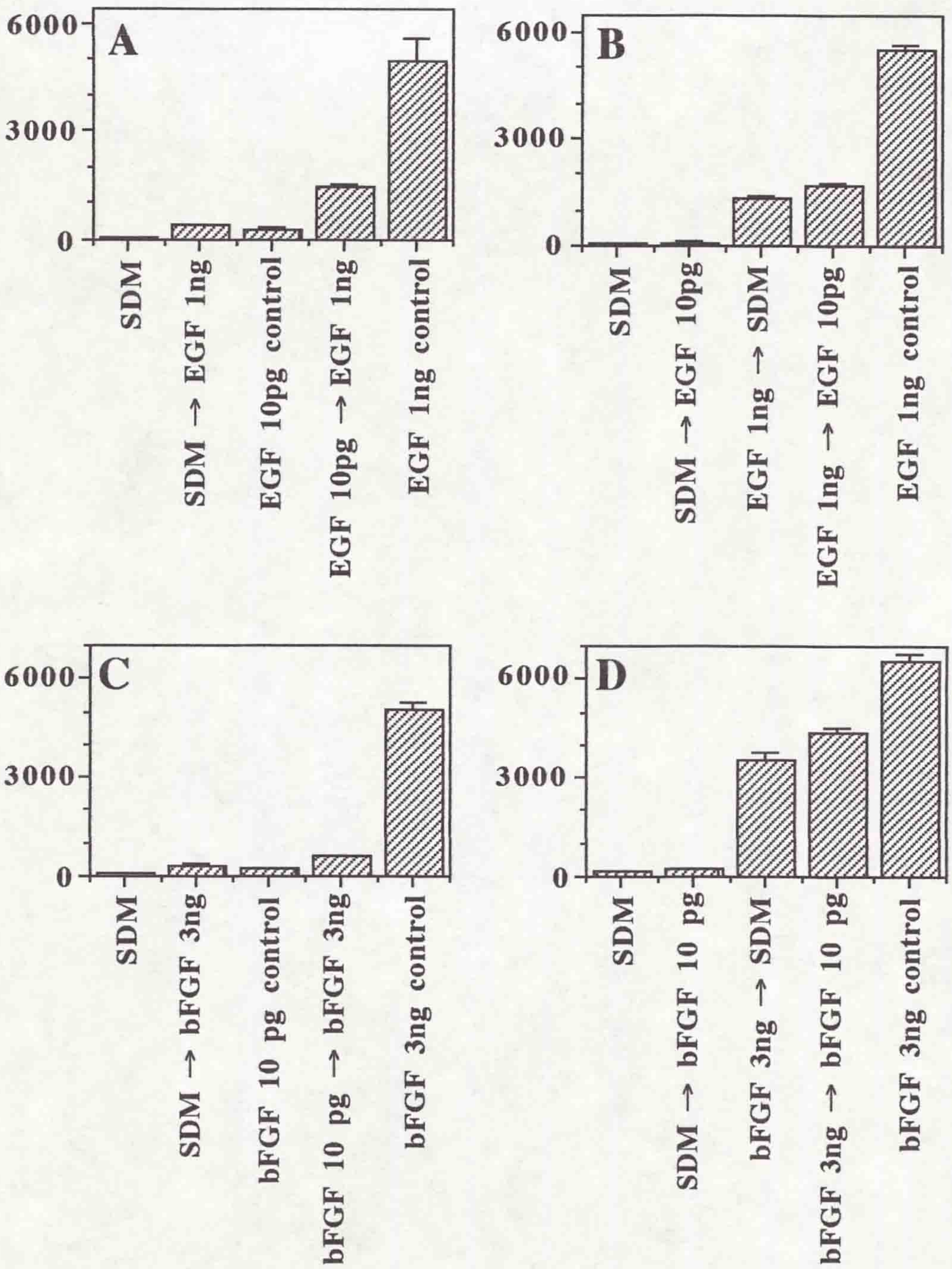


Table 3.1 Changing the concentration of growth factor 9 h after the initial stimulus results in a greater than expected level of DNA synthesis

	EGF low to high[a]	EGF high to low [b]	bFGF low to high [c]	bFGF high to low [d]
Expected value	617±44[e]	1335±95[f]	434±41[g]	3598±169[h]
Observed value	1916±16	1709±40	581±31	4352±139
ratio of observed/ expected[i]	3.1±0.2	1.3±0.1	1.3±0.1	1.2±0.1

[a] Results from Fig.3.5a;
[b] Results from Fig.3.5b;
[c] Results from Fig.3.5c;
[d] Results from Fig.3.5d;
[e] Sum of: SDM-EGF 1 ng/ml + EGF 10 pg/ml control;
[f] Sum of: SDM-EGF 10 pg/ml + EGF 1 ng/ml-SDM;
[g] Sum of: SDM-bFGF 3 ng/ml + bFGF 10 pg/ml-control;
[h] Sum of: SDM-bFGF 10 pg/ml + bFGF 3 ng/ml-SDM.
[i] Errors are the sum of the SE of the respective volues in Fig. 3.5.

Fig. 3.6 Effect of changing both the concentration of growth factor and the growth factor

The experiment was carried out in the presence of 2 μg/ml heparin as described in Fig. 3.5 except that at 9 h after the initial addition of a growth factor, both the concentration and the growth factor were changed.

Control for baseline DNA synthesis in all panels: SDM, no addition (mean of control values: Section 2.2.3.3, III-A-i and III-B-i);

Controls for the stimulation of the cells from 9h to the addition of [^{3}H]-thymidine at 18 h by a high (A, C) or a low (B, D) concentration of growth factor: (A)SDM→ bFGF, SDM replaced by SDM containing 3 ng/ml bFGF Section 2.2.3.3, III-A-ii; (C)SDM→ EGF, SDM replaced by SDM containing 1ng/ml EGF, Section 2.2.3.3, III-A-ii; (B) SDM→ bFGF, SDM replaced by SDM containing 10pg/ml bFGF, Section 2.2.3.3, III-B-ii; (D) SDM→EGF, SDM replaced by SDM containing 10 pg/ml EGF, Section 2.2.3.3, III-B-ii;

Control for the residual stimulation of DNA synthesis after removal of a high concentration of growth factor 9 h after the initial addition of growth factor: (B) EGF→ SDM, EGF (1 ng/ml) replaced by SDM, Section 2.2.3.3, III-B-vii; (D) bFGF→ SDM, bFGF (3 ng/ml) replaced by SDM, Section 2.2.3.3, III-B-vii;

Control for stimulation with a low concentration of growth factors (A: 10 pg/ml EGF; C: 10 pg/ml bFGF; Section 2.2.3.3, III-A-iii, III-A-iv and III-B-v, III-B-vi); control for stimulation with a high concentration of growth factors (1 ng/ml EGF, B, C; 3 ng/ml bFGF, A, D; Section 2.2.3.3, III-A-v, III-A-vi and III-B-iii, III-B-iv).

The experiment data: (A) EGF 10pg→ bFGF3 ng, low concentration of EGF (10 pg/ml) is replaced by a high concentration of bFGF (3 ng/ml); (B) EGF 1ng → bFGF 10pg, high-concentration of EGF (1 ng/ml) is replaced by a low concentration of bFGF (10 pg/ml); (C) bFGF 10pg→ EGF 1ng, low concentration of bFGF (10 pg/ml) is replaced by a high concentration of EGF (1 ng/ml); (D) bFGF 3ng →EGF 10pg, high concentration of bFGF (3 ng/ml) is replaced by a low concentration of EGF (10 pg/ml). Error bars represent the SD of three or six datum points.

Fig. 3.6 Effect of changing both the concentration of growth factor and the growth factor

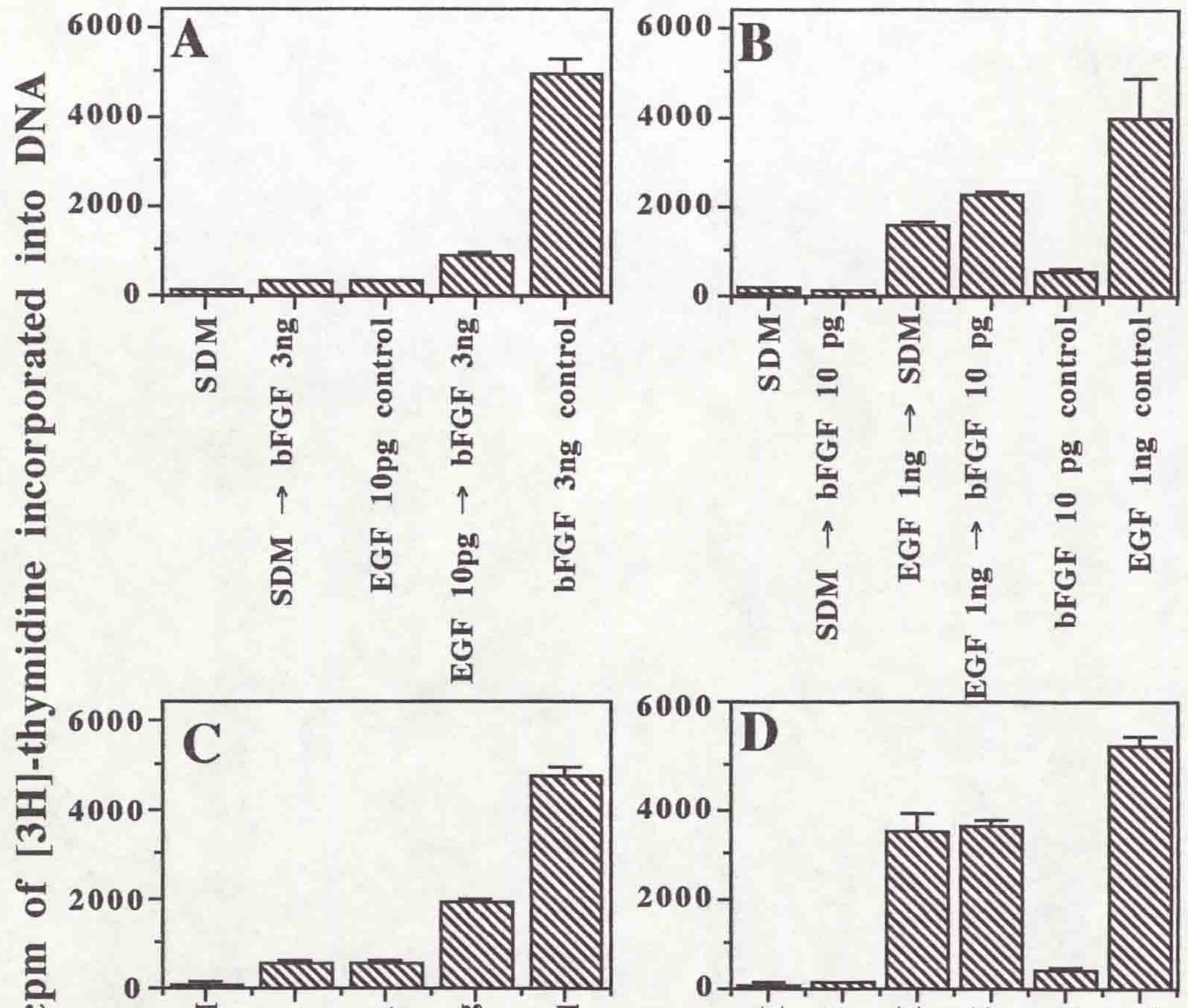


Table 3.2 Changing the concentration of growth factor 9 h after the initial stimulus results in a greater than expected level of DNA synthesis

	EGF low-bFGF high[a]	EGF high-bFGF low[b]	bFGF low-EGF high[c]	bFGF high-EGF low[d]
expected	593±23[e]	1595±84[f]	1022±74[g]	2676±182[h]
observed	893±46	2277±23	1933±48	3676±87
ratio observed/ expected[i]	1.5±0.1	1.4±0.1	1.9±0.1	1.4±0.1

[a] Results from Fig.3.6a;

[b] Results from Fig.3.6b;

[c] Results from Fig.3.6c;

[d] Results from Fig.3.6d;

[e] Sum of: SDM-bFGF 3ng/ml + EGF 10 pg/ml control;

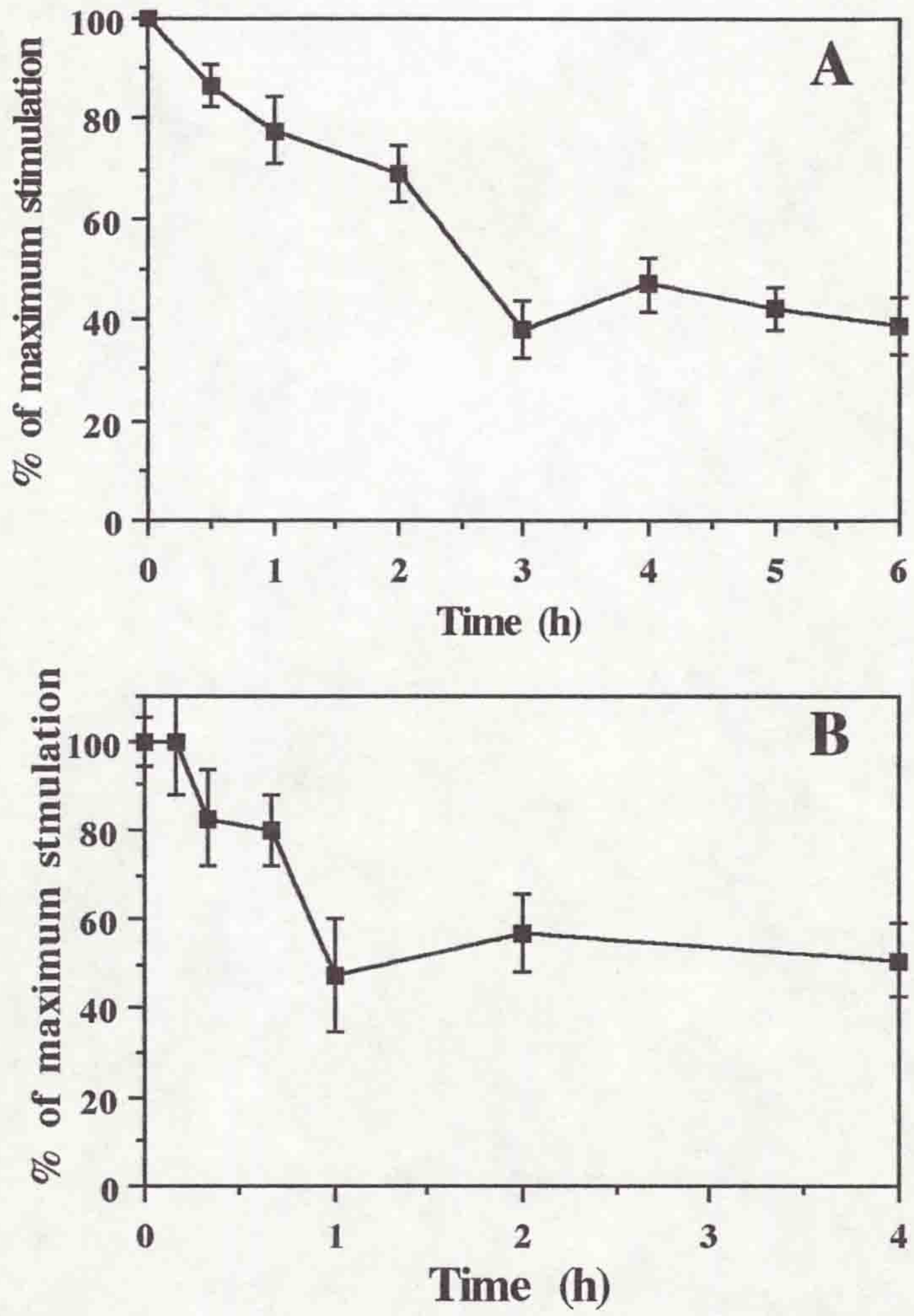
[f] Sum of: EGF 1 ng/ml-SDM+ SDM- bFGF 10 pg/ml;

[g] Sum of: SDM-EGF 1ng/ml + bFGF 10 pg/ml control;

[h] Sum of: bFGF 3 ng/ml-SDM+ SDM- EGF 10 pg/ml.

[i] Errors are the sum of the respective SE.

Fig. 3.7 Decay of mitogenic signal induced by EGF and bFGF in Rama 27 cells



Cells were treated as in Method 2.2.3.4. 9h after the initial addition of growth factor, the cells were washed and fresh SDM without growth factor was added. At various time points after the wash, growth factor was readded to the cells. (A) EGF at a concentration of 1 ng/ml in the absence of heparin (B) bFGF at a concentration of 1 ng/ml in the presence of 2 μg/ml heparin. % maximum stimulation is the mean cpm [^{3}H]-thymidine incorporated into the DNA of Rama 27 cells as a percentage of the mean incorporated in the absence of any manipulations. Results are the mean ± SD of triplicate samples.

Chapter Four

Heparan Sulphate-Dependence of FGF Growth - Stimulatory Activity

Chapter Four

Heparan Sulphate-Dependence of FGF Growth-Stimulatory Activity

4.1 Introduction

Chlorate has been shown to be an *in vitro* inhibitor of ATP-sulphurylase, the first enzyme in 3'-phosphoadenosine 5'-phosphosulphate (PAPS) biosynthesis (Burnell and Roy, 1978; Farley *et al.*, 1978; Lipmann, 1958). PAPS is the active sulphate donor for sulphotransferases (Lipmann, 1958). Subsequently chlorate has been shown in cultured cells to inhibit the sulphation of tyrosine in various proteins as well as the sulphation of the carbohydrate moiety of glycoproteins without inhibiting cell growth or protein synthesis (Baeuerle and Huttner, 1986).

As described in Section 1.2.3.3, the mitogenic activity of bFGF is believed to derive from its specific interaction with its dual receptor system. In order to investigate the dependence on HSPGs of the growth-stimulatory activity of FGFs, the specificity of chlorate as an inhibitor of heparan sulphation was established. The characteristics of the restoration of the growth-stimulatory activity of aFGF and bFGF by heparin in HS-deficient Rama 27 fibroblasts were then determined. Finally, the interaction between bFGF and its cellular receptors was examined in a series of binding assays.

4.2 Results

4.2.1 Validation of the S-free culture system containing chlorate

To select a concentration of chlorate which can inhibit the sulphation of the sugars of HS chains without inhibiting cell growth, the stimulation of DNA synthesis in Rama 27 fibroblasts maintained in normal medium (Section 2.2.1.2) by growth factors was measured in the presence of increasing concentrations of chlorate. In normal medium,

chlorate cannot inhibit ATP-sulphurylase because of the relatively high concentrations of sulphate (0.8 mM). Under these conditions, at concentrations of chlorate less than 10 mM, chlorate does not inhibit the stimulation of DNA synthesis by EGF or bFGF (Fig. 4.1). Some inhibition of DNA synthesis stimulated by EGF or bFGF is observed at 30 mM chlorate (Fig. 4.1). At higher concentrations, such as 100 mM, chlorate clearly inhibits the stimulation of DNA synthesis by growth factors, and thus is likely to be exerting a variety of non-specific effects on the cells (Fig. 4.1).

In order to validate the S-free culture conditions, the effects of a variety of reduced concentrations of cysteine and methionine on the stimulation of DNA synthesis in Rama 27 cells were tested. In Rama 27 cells grown in the medium without SO_4^{2-} containing 10% of the normal levels of cysteine and methionine, the stimulation of DNA synthesis by 1 ng/ml EGF or 3 ng/ml bFGF was identical to that observed in medium containing a normal concentration of cysteine and methionine (Fig. 4.2).

Therefore, the absence of SO_4^{2-}, the reduction of the concentration of cysteine and methionine and 15 mM chlorate have no detectable effects on the stimulation of DNA synthesis in Rama 27 cells by growth factors. Thus the S-free medium (Section 2.1.2.2) does not in itself interfere with DNA synthesis.

4.2.2 Inhibition of the synthesis of glycosaminoglycans by culture of Rama 27 cells in S-free medium

HS chains were purified from the culture medium, the cell trypsinate and the U/T cell residue of Rama 27 fibroblasts grown in normal medium or S-free medium (Section 2.2.7).

In Rama 27 fibroblasts grown in normal medium, the largest amount of labelled proteoglycans and HS was recovered from the culture medium (Fig. 4.3a and Table 4.1). Thus Rama 27 fibroblasts secrete or shed a large proportion of the HS synthesized over the 72 h labelling period. The trypsinate fraction contained considerably less labelled proteoglycans and HS, whilst the U/T fraction contained the least proteoglycans and HS (Figs 4.3b,c and Table 4.1). When Rama 27 cells were grown in S-free

medium, the incorporation of [^{3}H]-glucosamine and [^{35}S]-sulphate into both secreted and cellular proteoglycans and HS was reduced dramatically (Figs 4.3d-f and Table 4.1). Thus the incorporation of [^{3}H]-glucosamine and [^{35}S]-sulphate into the HS purified from the culture medium was reduced by 59- and 25-fold, respectively (Table 4.1). The reduction of incorporation of [^{3}H]-glucosamine and [^{35}S]-sulphate into the HS in the trypsinate and the U/T fractions could not be determined since the amount of radioactivity recovered in the cells grown in S-free RM was indistinguishable from the background of 20 dpm (Table 4.1). Overall, culture of Rama 27 cells in S-free RM (Section 2.2.2) achieves a reduction in synthesis of HS, represented by the amount of [^{3}H]-glucosamine, of 75-fold and a reduction in the sulphation of HS, represented by the amount of [^{35}S]-sulphur, of 42-fold (Table 4.1). Therefore the culture of Rama 27 cells in S-free medium according to the protocol described in Section 2.2.7 produces Rama 27 cells that are HS-deficient.

4.2.3 Heparin-dependence of the growth-stimulatory effects of FGFs in HS-deficient Rama 27 fibroblasts

The stimulation of DNA synthesis in Rama 27 cells by 1 ng/ml EGF is not affected by culture in the S-free medium. However, in S-free medium, 30 ng/ml aFGF and 3 ng/ml bFGF are unable to stimulate DNA synthesis (Fig. 4.4).

At low concentrations of heparin (1 ng/ml) some of the growth-stimulatory activity of 3 ng/ml bFGF was restored, and at 30 ng/ml heparin the full activity of 3 ng/ml bFGF was restored. In contrast 30 ng/ml aFGF required much higher concentrations of heparin to restore its activity. At 30 ng/ml heparin, only a slight growth-stimulatory effect of 30 ng/ml aFGF was observed and 1 μg/ml heparin was required to restore the full activity of the aFGF (Fig. 4.4). This result indicates that growing Rama 27 cells in the S-free medium inhibits the growth-stimulatory effects of FGFs, but not those of EGF. Rama 27 fibroblasts grown in S-free medium are HS-deficient (Fig. 4.3) and therefore the loss of the growth-stimulatory effects of FGFs under these conditions is

likely to be related to loss of cellular HS.

4.2.4 Reversibility of the effects of HS-deficiency on the stimulation of DNA synthesis by bFGF and aFGF in Rama 27 fibroblasts

To determine whether the effects of the inhibition of HS synthesis by chlorate are reversible, the cells were first incubated in S-free SDM for 24 h (Section 2.2.2) and then changed back to normal SDM or normal SDM containing 15 mM chlorate for 1 h before the addition of bFGF and aFGF. The stimulation of DNA synthesis by bFGF and aFGF was largely restored by returning the cells to SDM or SDM containing 15 mM chlorate (Fig. 4.5). However, perhaps due to the absence of a recovery period to allow the synthesis of a full complement of cellular HS, returning the cells to SDM or SDM containing 15 mM chlorate does result in a slight reduction of potency of the growth factors (Fig. 4.5).

4.2.5 Kinetics of the stimulation of DNA synthesis by FGFs in HS-deficient Rama 27 fibroblasts

The kinetics of DNA synthesis stimulated by bFGF and aFGF in HS-deficient cells was measured at specific times after the addition of bFGF or aFGF in the presence of heparin, as described in Sections 2.2.2, 2.2.3.1 and 2.2.3.2. No stimulation above baseline was observed until 9 h after the addition of the growth factors and DNA synthesis reached its maximum at 18 h (Fig. 4.6). The kinetics of the stimulation of DNA synthesis in HS-deficient Rama 27 cells was not dependent on the concentration of growth factors (Fig. 4.6). Therefore, at least as far as the timing of the cell cycle is concerned, HS-deficient Rama 27 cells behave in an identical manner to normal cells (Section 3.2.1.2).

4.2.6 Potency of aFGF and bFGF in HS-deficient Rama 27 fibroblasts

The stimulation of DNA synthesis by bFGF in Rama 27 cells grown in normal

medium is hardly affected by 2 μg/ml heparin (Fig. 4.7a and Section 3.2.1.1). In the HS-deficient Rama 27 cells bFGF is still able to stimulate DNA synthesis, although the potency of bFGF is reduced 100-fold (Fig. 4.7b). In the HS-deficient Rama 27 cells, in the presence of heparin at a concentration (30 ng/ml) that is able to restore the growth-stimulatory effects of bFGF (Fig. 4.4, Section 4.2.3), bFGF begins to stimulate DNA synthesis at a concentration of 30 pg/ml, half maximal stimulation occurs at 1 ng/ml, and maximal stimulation is at 10 ng/ml to 30 ng/ml (Fig. 4.7b). Thus under these conditions, the potency of bFGF, though about 100-fold greater than in the absence of heparin, is still 10-fold less than in control cells. The differences in the maximal stimulations seen in the control and HS-deficient cells are due to differences in the specific activity of the [^{3}H]-thymidine added to the cells.

aFGF is considerably less potent than bFGF (Fig. 4.8, Section 3.2.1.1). In control cells 2 μg/ml heparin enhances the activity of aFGF by about 1000-fold (Fig. 4.8a, Section 3.2.1.1). In HS-deficient cells, aFGF, even at concentrations as high as 10 μg/ml, has only a marginal growth-stimulatory effect (Fig. 4.8b). In the presence of 1 μg/ml heparin, a concentration sufficient to restore the growth-stimulatory effects of aFGF (Fig. 4.4) the potency of aFGF is 100-fold greater than that observed in control cells in the absence of heparin, but still 10-fold lower than that observed in control cells in the presence of 2 μg/ml heparin (Fig. 4.8).

4.2.7 Stability of FGFs in SDM and S-free SDM

In order to find out if there was a trivial explanation for the loss of potency of aFGF and bFGF in the S-free culture medium in the absence or presence of heparin, the stability of growth factors in the different SDM was measured. To measure the growth-stimulatory activity of FGFs requires an 18 h incubation (Section 3.2.2). Therefore the 20-fold reduction in the potency of bFGF preincubated for 18 h in SDM compared to bFGF that was not preincubated (Table 4.2) reflects the deactivation of the bFGF over a 36 h period. Similarly, the complete loss of the growth-stimulatory-activity of aFGF under these conditions also reflects the deactivation of aFGF over 36 h (Table 4.2).

Hence to determine the potential deactivation effects of S-free SDM, the comparison must be made between the potency of the FGFs preincubated for 18 h in SDM and S-free SDM.

Preincubation of bFGF in S-free SDM in the absence of heparin for 18 h reduced the potency of bFGF by 6-fold compared to bFGF preincubated in SDM (Fig. 4.9a; Table 4.2). However, the preincubation of aFGF in either S-free SDM or SDM for 18 h resulted in an almost complete loss of growth-stimulatory activity in the absence of the heparin (Fig. 4.9b; Table 4.2), precluding the calculation of a loss in potency.

When bFGF was preincubated in the presence of heparin in S-free-SDM for 18 h without cells the potency of the bFGF was reduced by about 3.3-fold compared bFGF incubated in SDM (Fig. 4.10a and Table 4.3). In the case of aFGF, in the presence of heparin the potency of aFGF preincubated in S-free SDM was reduced about 2-fold compared to aFGF preincubated in SDM for 18 h (Fig. 4.10b and Table 4.3).

4.2.8 Receptors for bFGF on Rama 27 fibroblasts

Rama 27 fibroblasts possess 26,000 high-affinity receptors for bFGF with a Kd of 33 pM (Table 4.4). A large number, $3x10^6$, of lower affinity receptors are also present on the Rama 27 cells (Table 4.4). When binding assays are carried out in the presence of 1 μg/ml heparin there is no evidence for the presence of low-affinity receptors (Table 4.4). Whilst heparin (1 μg/ml) does not alter the Kd of the high-affinity receptors, it does apparently reduce the number of these receptors to 4800 (Table 4.4). In HS-deficient cells, there is also no evidence for a low-affinity receptor. The Kd of the high-affinity receptor for bFGF is essentially unchanged from that observed in control cells (Table 4.4). The number of high-affinity receptors in HS-deficient Rama 27 cells is slightly lower than that seen in control cells (Table 4.4) but the inclusion of 30 ng/ml heparin in the binding medium of HS-deficient cells only increases the Kd, ie: decreases the affinity, of the high-affinity receptor by about 3-fold compared to control cells. Although the number of high-affinity receptors is significantly different between the

control and the HS-deficient cells, the existence of a continuum of numbers of receptors between control cells, HS-deficient plus heparin and HS-deficient suggests that these differences may only be marginally significant.

4.3 Discussion

The presence of sulphate substituents in the polysaccharide moiety of proteoglycans makes these substances extremely negatively charged (Section 1.2.2.3.2). Moreover, the binding sites of proteins in HS are probably mediated, in at least in part, by the strong negative charge that these substituents impart (Section 1.2.3.1).

To study the possible functions of the HSPG receptors for FGFs, the synthesis of these receptors was inhibited. Five steps were taken to reduce the level of sulphation of endogenous HS by Rama 27 cells: (i) culture in sulphate-free medium in order to deplete the supply of sulphate to the cells; (ii) the addition of chlorate to the medium in order to inhibit the activity of ATP sulphurylase (Section 4.1); (iii) the reduction of the concentration of methionine and cysteine in the medium in order to minimize the intracellular generation of sulphate by oxidation of amino acid sulphur; (iv) the trypsinization of the cell surface to remove cell-surface HSPGs; (v) the dialysis of FCS to remove SO_4^{2-}.

The three fractions of Rama 27 fibroblasts from which HS chains were purified represent HSPGs from three distinct cellular compartments: the culture medium contains secreted/shed HSPGs; the cell trypsinate contains plasma membrane-associated HSPGs; the U/T fraction extracted after trypsin treatment, contains intracellular HSPGs and extracellular matrix HSPGs (Turnbull and Gallagher, 1988, 1991a,b). The incorporation of $[^3H]$-glucosamine and $[^{35}S]$-SO_4^{2-} into glycosaminoglycans (GAGs) is a measure of their level of synthesis and sulphation, respectively. When Rama 27 cells were grown in the S-free RM the incorporation of $[^3H]$-glucosamine into proteoglycans from the trypsinate and the culture medium was reduced by 44- and 15-fold, respectively, whilst only a 2-fold reduction was observed for the proteoglycans

recovered from the U/T fraction (Table 4.1). Culture of Rama 27 cells in S-free RM reduced the incorporation of $[^{35}S]$-SO_4^{2-} into proteoglycans to a much greater extent than the incorporation of $[^3H]$-glucosamine (Table 4.1). Culture of Rama 27 fibroblasts in the S-free RM also reduced the level of synthesis and the level of sulphation of a specific GAG, HS. Thus the incorporation of $[^3H]$-glucosamine and $[^{35}S]$-SO_4^{2-} into HS was reduced 59- and 25-fold, respectively, for the HS recovered from the culture medium. The amount of radioactivity in the HS recovered from the trypsinate and the U/T fractions was similar to the background and thus could not be determined with any degree of certainty (Table 4.1). Therefore the reduction in the incorporation of $[^3H]$-glucosamine into the HS recovered from the trypsinate and the U/T extract is at least 2- and 100-fold, respectively, and of $[^{35}S]$-SO_4^{2-} at least 38- and 78-fold, respectively (Table 4.1).

It must be remembered that since RM contains 0.8 mM SO_4^{2-}, the specific activity of the $[^{35}S]$-SO_4^{2-} used to label the control cells is 10^5-fold lower than for cells cultured in S-free RM. Hence the above comparisons of the level of sulphation of GAGs and HS purified from cells grown in RM and S-free RM represent a 'worst case' in terms of the reduction in sulphation achieved by the S-free RM. Therefore the culture of Rama 27 fibroblasts in S-free RM reduces the level of both synthesis and sulphation of total cellular sulphated GAGs, including HS, to an extent that is at least comparable to that observed in other systems. Thus in Rapraeger's experimental system (Rapraeger *et al.*, 1991), chlorate, a reduced concentration of cysteine and the trypsinization of the cells achieved an 80% reduction in the sulphation of HS on Swiss 3T3 fibroblasts. When this Group studied the repression of myogenic differentiation in MM14 myoblasts by aFGF, bFGF and K-FGF, only chlorate was used and an 89% reduction in the sulphation of newly synthesized GAGs was observed (Olwin and Rapraeger, 1992). Another similar experimental system using trypsinized 3T3 fibroblasts grown in S-free DMEM, containing dialyzed donor calf serum and chlorate achieved a 95% reduction in the level of sulphation of HS (Walker *et al.*, 1994). By comparison, the present

experimental system, S-free medium containing chlorate, a reduced concentration of cysteine and methionine, dialyzed FCS and trypsinization have been used to deplete the HS of Rama 27 fibroblasts by 75-fold (Table 4.1). These results suggest that this experimental system is more stringent insofar as the reduction in cellular HS is concerned.

Chlorate is tolerated by the Rama 27 cells at concentrations up to 30 mM (Fig. 4.1). Reducing the concentration of cysteine and methionine in the medium to 10% (Section 2.1.2.2) does not inhibit the stimulation of DNA synthesis in the cells by growth factors (Fig. 4.2). Therefore the perturbations observed when Rama 27 cells are grown in S-free RM or S-free SDM are likely to be due to the specific inhibition of the synthesis and sulphation of HS. Moreover, when quiescent HS-deficient Rama 27 fibroblasts are returned to SDM prior to the addition of growth factors, only small differences in the potencies of the growth-stimulatory effects of bFGF and aFGF are observed (Fig. 4.5). Therefore the S-free medium (Section 2.1.2.2) is not toxic to the Rama 27 cells and its effects are readily reversible by including 0.8 mM SO_4^{2-} in medium (Fig. 4.5).

The HS-deficient Rama 27 fibroblasts are able to mount a growth-stimulatory response to EGF in a manner similar to that observed in untreated Rama 27 cells (Fig. 3.1; Fernig *et al.*, 1990b; Smith *et al.*, 1984). However, aFGF and bFGF are unable to stimulate DNA synthesis in HS-deficient Rama 27 fibroblasts at concentrations that normally elicit a response (Figs. 4.4, 4.7b and 4.8b). The addition of heparin restores the growth-stimulatory activity of the FGFs as has been observed in other HS-deficient cell systems (e.g., Olwin and Rapraeger, 1992; Rapraeger *et al.*, 1991). A concentration of 30 ng/ml heparin is able to restore the growth-stimulatory effects of bFGF (Fig. 4.4), which is similar to that required in other cellular systems, including those that depend on chlorate and/or heparinase treatment to inhibit HS synthesis (Olwin and Rapraeger, 1992; Rapraeger *et al.*, 1991; Walker *et al.*, 1994) and those that fail to express HS but express a defined FGFR as a consequence of transfection (Li and Bernard, 1992; Mansukhani *et al.*, 1992; Yayon *et al.*, 1991). Since the effects of the

S-free medium are reversible and wholly dependent upon the sulphation and synthesis of HS, the method used to make the Rama 27 cells HS-deficient appears to affect specifically the synthesis of HS and the HS-dependent stimulation of cell growth by FGFs.

Heparin has virtually no effect on the stimulation of DNA synthesis by bFGF on Rama 27 cells in normal medium (Section 3.2.1.1 and Fig. 4.7a). In HS-deficient Rama 27 fibroblasts the growth-stimulatory effects of bFGF are still apparent, but at concentrations that are 1000-fold higher than necessary to stimulate control cells (Fig. 4.7). Heparin at 30 ng/ml, a concentration that is able to restore the growth-stimulatory effects of bFGF (Fig. 4.4) increases the potency of bFGF in HS-deficient Rama 27 cells by 100-fold (Fig. 4.7b). This result is consistent with the view that one of the functions of cellular HS, which may be mimicked by exogenously-added heparin, is to promote the interaction of bFGF with FGFRs (Ornitz *et al.*, 1992b; Yayon *et al.*, 1991) but it is not consistent with the notion that the bFGF-FGFR interaction has an absolute requirement for HS/heparin (Ornitz *et al.*, 1992b). Furthermore, in the presence of 30 ng/ml heparin the potency of bFGF is still 10-fold lower than that observed in control cells (Fig. 4.7). Therefore, when the dose-dependence of the growth-stimulatory effect of bFGF is examined, it is apparent that exogenously-added heparin is unable to restore fully the activity of bFGF. One possible reason for this is the inactivation of bFGF in S-free SDM (Fig. 4.9). It is not easy to measure the inactivation of the bFGF during the course of a DNA synthesis assay for two reasons. Firstly, inactivation can only be measured in a DNA synthesis assay. Hence, since the growth factor must be active for most of the 18 h period between G_o and S-phase DNA synthesis (Section 3.2.2), if the rate of inactivation is not linear then the estimated rate of inactivation will be erroneous since this is the sum of the inactivation occurring in the first (the "preincubation") and the second (the "assay") 18 h periods. Secondly, the Rama 27 cells consume a considerable amount of the growth factor present in the medium, both through binding to cellular receptors and through post-receptor degradation during the 18 h incubation with growth factor (result not shown). Thus it proved impossible to measure the actual

rate of inactivation of FGFs in SDM in the presence of cells since the amount of growth-stimulatory activity present in the culture medium was also reduced by the cells themselves. Therefore the approach used was to incubate bFGF in the presence of the appropriate SDM at 37 °C for 18 h without cells and then to measure the remaining growth-stimulatory activity in a DNA synthesis assay (Fig. 4.9 and Fig. 4.10). Whilst far from perfect, these measurements allow an estimate of the inactivation of bFGF. It is apparent that the S-free SDM causes a reduction in the activity of bFGF (Figs. 4.9, 10). In the presence of 30 ng/ml heparin this reduction is attenuated and is unlikely to account for more than a 3.3-fold reduction in the potency of bFGF (Fig. 4.10; Table 4.3). Therefore there is half of the 10-fold reduction in potency of bFGF (Fig. 4.7) to be accounted for.

In contrast to bFGF, aFGF is unable to cause more than a marginal stimulation of DNA synthesis in HS-deficient fibroblasts at concentrations of aFGF that are 300-fold higher than those required for half-maximal stimulation in control cells (Fig. 4.8). In addition, a much higher concentration of heparin, 1 μg/ml, is required to restore the growth-stimulatory effects of aFGF in HS-deficient fibroblasts (Fig. 4.4). This concentration of heparin is similar to that required to potentiate the activity of aFGF in Rama 27 fibroblasts (Figs 3.1, and 4.8a,b) and other cell types grown under normal conditions (Damon *et al.*, 1989). Studies on recombinant FGFR-1 have shown that heparin at relatively high concentrations (1 μg/ml) increases the affinity of aFGF for FGFR-1 by about 4-fold (Kaplow *et al.*, 1990). However, the potentiation effect of heparin on aFGF in normal cells (Fig. 4.8a; Damon *et al.*, 1989) is 250-fold greater than the observed changes in affinity. Therefore the results suggest that the potentiation effect of heparin on the stimulation of DNA synthesis by aFGF in Rama 27 cells dominates any other effects, such as an increase in affinity for the FGFRs. Moreover, this conclusion is likely to hold for both normal cells and HS-deficient cells (Fig. 4.8). Nevertheless, in the presence of 1 μg/ml heparin, the potency of aFGF in HS-deficient fibroblasts is still 100-fold lower than that observed in control cells in the presence of 2

μg/ml heparin (Fig. 4.4). Even when incubated at 37 °C in normal SDM, aFGF is far more susceptible to inactivation than bFGF (Figs 4.9, 4.10; Tables 4.2, 4.3). Heparin (1 μg/ml) is able to stabilize aFGF in SDM and S-free SDM. Thus the propensity of aFGF to inactivation may be behind the potentiation of the activity of aFGF that is observed in the presence of heparin in normal medium (Figs 3.1 and 4.8). In chlorate-containing S-free SDM, the aFGF is completely inactivated, although, as in normal medium, heparin is able to protect much of the aFGF from inactivation (Fig. 4.8; Table 4.3). The amount of aFGF inactivated in S-free SDM in the presence of heparin can account for no more than 2% of the reduction in potency of aFGF that is observed in HS-deficient cells in the presence of heparin. Therefore, as in the case of bFGF, whilst the inactivation of aFGF in S-free SDM may be responsible, in part, for the inability of heparin to fully restore the potency of aFGF in HS-deficient Rama 27 cells, some other factor seems likely to be making the major contribution.

Therefore heparin is unable to restore the potency of either bFGF or aFGF to the levels observed in control cells. Hence aFGF and bFGF stimulate cell division more efficiently when HSPG receptors are present on the cell-surface. It has been observed that, due to their large numbers, the HSPG receptors are able to concentrate bFGF at the cell-surface (Fernig *et al.*, 1992; Flaumenthaft *et al.*, 1989; Presta *et al.*, 1989). However, soluble heparin at concentrations in the μg/ml range, which prevents the binding of large amounts of FGFs to the HSPG receptors on mammary and other cells (Table 4.4; Fernig *et al.*, 1992; Kan *et al.*, 1988), potentiates the growth-stimulatory activity of aFGF (Figs 3.1 and 4.8) and has little or no effect on that of bFGF (Figs 3.1 and 4.7a). Thus it is improbable that the absence of cellular HSPG receptors is reducing the potency of the two FGFs because of a reduction in the concentration of the growth factors at the cell-surface. Therefore an alternative explanation must be sought. One is the suggestion that the role of HS in the dual receptor system is to form a bridge between the FGF and the FGFR (Guimond *et al.*, 1993; Kan *et al.*, 1993). Cell-surface HSPGs could promote this interaction more efficiently than soluble heparin. However,

there is strong evidence to contradict this view (Ornitz *et al.*, 1992b), the binding of HS to FGFRs has yet to be demonstrated (Fernig and Gallagher, 1994) and competition of cell-surface HSPG receptors with soluble heparin does not inhibit the growth-stimulatory effects of aFGF or bFGF (Figs 3.1, 4.7 and 4.8). A more likely explanation may be the direct involvement of the HSPG receptor, perhaps through the core protein, in some aspect of the intracellular cellular signalling events that lead from receptor binding to the synthesis of DNA. Two candidate post-receptor binding events are the processing of the FGFs and their subsequent translocation to the nucleus and the stimulation of the dimerization of the FGFRs by the HSPG core protein (Section 1.2.2.3.1). In view of the ability of HSPG core proteins to oligomerize (Bernfield *et al.*, 1992), the latter is perhaps the more likely.

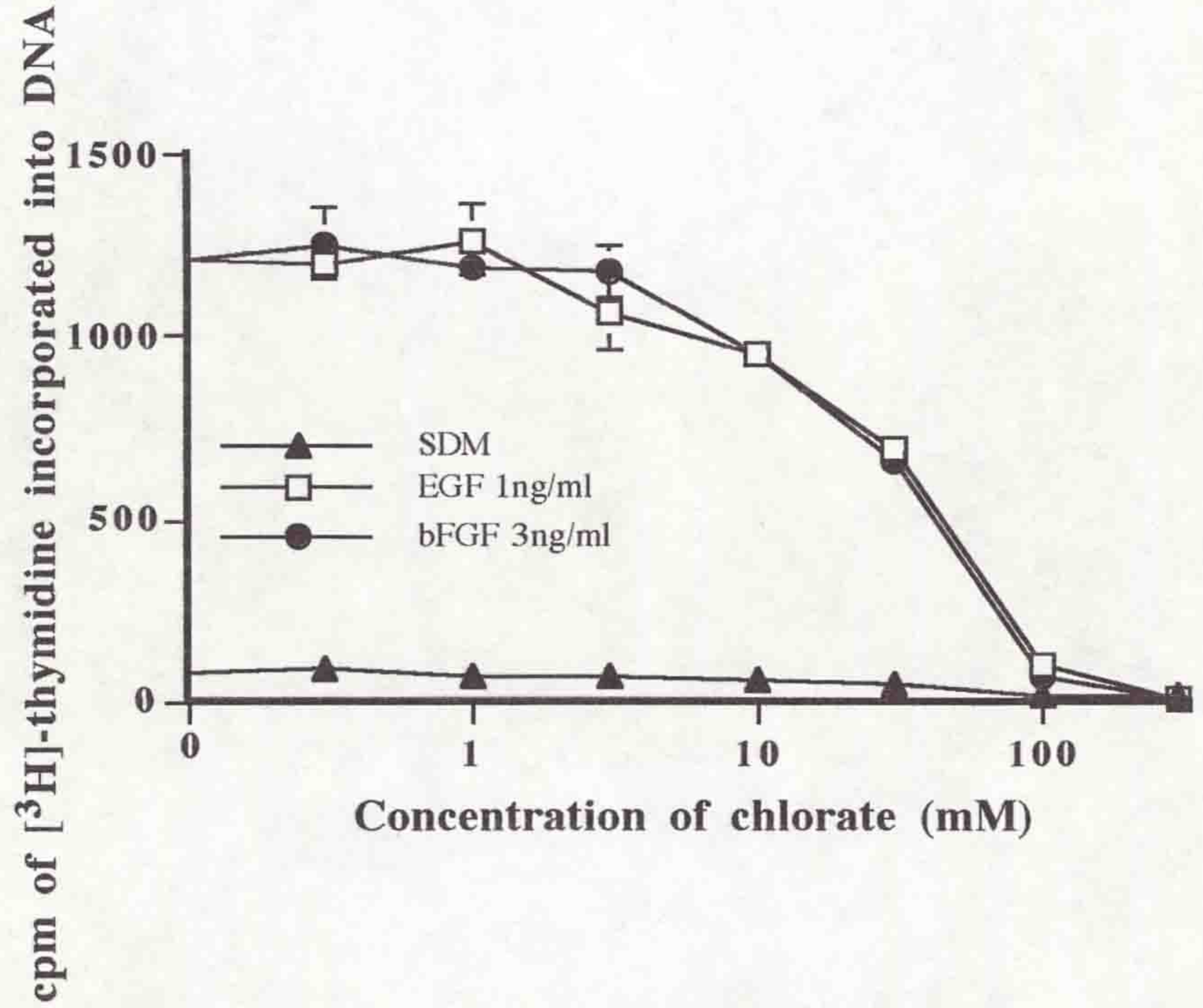
The results of the DNA synthesis assays (Figs 4.6, 4.7) are consistent with the notion that HS or heparin increases the affinity of the interaction between FGF and FGFR. However, at least in the case of bFGF, it is clear that the requirement of the bFGF-FGFR interaction for HS or heparin is not absolute (Fig. 4.7).

The results of the binding assays indicate that the large number of low-affinity receptors on Rama 27 fibroblasts are HS since this binding site is susceptible to competition by heparin and is absent from HS-deficient cells (Table 4.4). However, the binding assays also demonstrate that the high-affinity binding of bFGF is the same in control and HS-deficient cells (Table 4.4). This result contradicts all but one study of the binding of bFGF to cells possessing FGFRs but not HS. Thus Roghani *et al.* (1994) found that heparin only increased the affinity of FGFR-1 for bFGF by 2 to 4-fold in cells that were devoid of HS but that expressed a transfected FGFR-1 cDNA. This heparin-mediated increase in affinity is similar to observed for aFGF (Kaplow *et al.*, 1990). Of the other studies carried out on the HS/heparin-dependence of bFGF-FGFR interactions, the majority have simply used growth assays or qualitative binding assays (Li and Bernard, 1992; Mansukhani *et al.*, 1992; Rapraeger *et al.*, 1991; Walker *et al.*, 1994). Those studies that did examine the binding reaction between FGFs (usually bFGF) and FGFRs invariably claim a large increase in affinity for bFGF

mediated by the polysaccharide. For example, Yayon *et al.*, (1991) claim that in the absence of heparin, the Kd of bFGF for FGFR-1 is 1 nM. This group has obtained similar, though qualitative, results using a recombinant chimera of the extracellular domain of FGFR-1 and alkaline phosphatase (Ornitz *et al.*, 1992b). Other groups have also obtained analogous results in their cell systems (Maccarana *et al.*, 1993; Hou *et al.* 1993; McKeehan and Kan, 1994); This discrepancy between the results in Table 4.4 and those in the above studies could arise for number of reasons. Firstly, all the other studies use Scatchard analysis (Scatchard, 1948) to determine the Kd and number of receptors from the binding data. However, Scatchard plots of FGF-binding data are invariably curvilinear, and none of the above studies has made an objective, mathematically correct, attempt at extracting one or more straight lines from their Scatchard plots. Therefore the results of such analyses should be treated with a great deal of caution. Secondly, the binding of polypeptide ligands to cell-surface receptors is often not saturable, as revealed by a plot of the concentration of bound ligand against the log of the concentration of free ligand (Klotz, 1982; Fernig *et al.*, 1992). This observation is paradoxical since the concentration of free ligand in binding assays is often two or more orders of magnitude higher than the Kd. Nonetheless, non-saturating binding data cannot be analysed by the method of Scatchard (Scatchard, 1948; Klotz, 1982) and other techniques must be used. Thirdly, with the exception of McKeehan's group (Hou *et al.*, 1993, Kan *et al.*, 1988), all of the above studies, including those of Moscatelli and co-workers (Roghani *et al.*, 1994), use a high salt wash (2 M NaCl) to remove HS-bound bFGF and then an acid wash (pH 4.5) to remove FGFR-bound bFGF. However, the rationale behind the use of this technique to distinguish between the two binding sites is severely weakened when the claim is made that the binding of bFGF to HS/heparin is required for binding to the FGFRs (e.g, Yayon *et al.*, 1994; Spivakkroizman *et al.*, 1994). Moreover, it has been known for some time that sequential salt and acid washes do not distinguish between the low- and high-affinity binding sites for bFGF (Fernig *et al.*, 1992; Kan *et al.*, 1988).

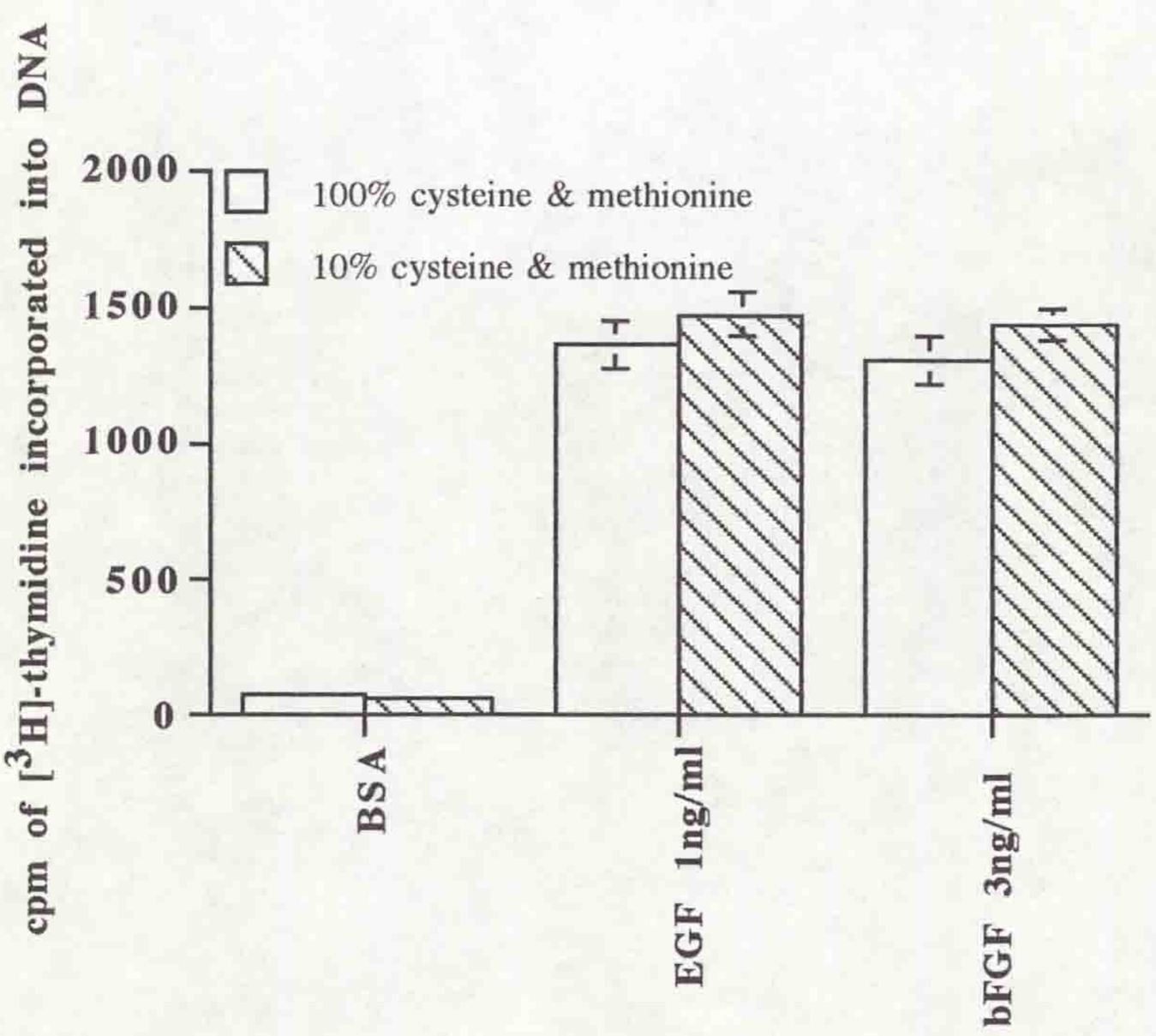
Therefore it is perhaps not surprising that the use of non-linear curve fitting to analyze bFGF-binding data yields a different result to most of the other studies that have investigated FGFR-bFGF-HS interactions. Whilst bFGF is still able to interact with high-affinity with the FGFR in HS-deficient cells (Table 4.4) under these conditions, bFGF is unable to stimulate growth efficiently (Fig. 4.7). Moreover, in the presence of 30 ng/ml heparin, the affinity of bFGF for the high-affinity receptor is slightly decreased, yet the growth-stimulatory activity of bFGF is largely, but not entirely restored under these conditions. Therefore, it would seem reasonable to suggest that the role of the HS receptor in the dual receptor system is to generate intracellular signals rather than to promote the binding of bFGF to FGFRs. Thus it is possible that both the bFGF-binding HS chains and the core protein of the HSPG receptors are involved with the FGFRs in generating the signal(s) that ultimately lead to cell division.

Fig. 4.1. Stimulation of DNA synthesis in Rama 27 fibroblasts by growth factors in the presence of increasing concentrations of chlorate



Chlorate was added immediately before the addition of the growth factors. (▲) SDM; (□) 1 ng/ml EGF; (●) 3 ng/ml bFGF. Results are expressed as the mean cpm±SD of triplicate samples.

Fig. 4.2 Stimulation of DNA synthesis by EGF and bFGF in Rama 27 fibroblasts maintained in SO_4^{2-}-free medium with a reduced concentration of cysteine and methionine



Rama 27 fibroblasts were grown in S-free-medium with (□) a normal concentration of cysteine and methionine or with (▨) 10% of the normal concentration of cysteine and methionine before the addition of growth factors. Results are expressed as the mean cpm±SD of triplicate samples.

Fig. 4.3 Effect of S-free RM on the production of secreted, plasma membrane, and extracellular matrix glycosaminoglycans in Rama 27 fibroblasts.

Proteoglycans prepared from Rama 27 fibroblasts ($2x10^6$) grown for 72 h in the presence of [^{3}H]-glucosamine and [^{35}S]-sulphate were separated by anion exchange chromatography and the radioactivity in each fraction determined as described in Section 2.2.7.3.

Left hand panels (A-C) show proteoglycans purified from Rama 27 fibroblasts grown in normal medium and the right hand panels (D-E) show proteoglycans purified from Rama 27 cells grown in S-free RM (Section 2.2.7). Panels A and D show the amount of glucosamine and sulphate incorporated into proteoglycans secreted/shed by Rama 27 fibroblasts into the culture medium. Panels B and E show proteoglycans released from R27 cells by the action of trypsin. Panels C and F show proteoglycans extracted with U/T which corresponds to extracellular matrix and intracellular proteoglycans. (—) solid line represents [^{3}H]-glucosamine incorporation; (····) short dotted line represents [^{35}S]-sulphur incorporation; (---) long dotted line represents the NaCl gradient (0.15 M to 2 M).

Fig. 4.3 Effect of S-free RM on the production of secreted, plasma membrane, and extracellular matrix GAGs in Rama 27 fibroblasts.

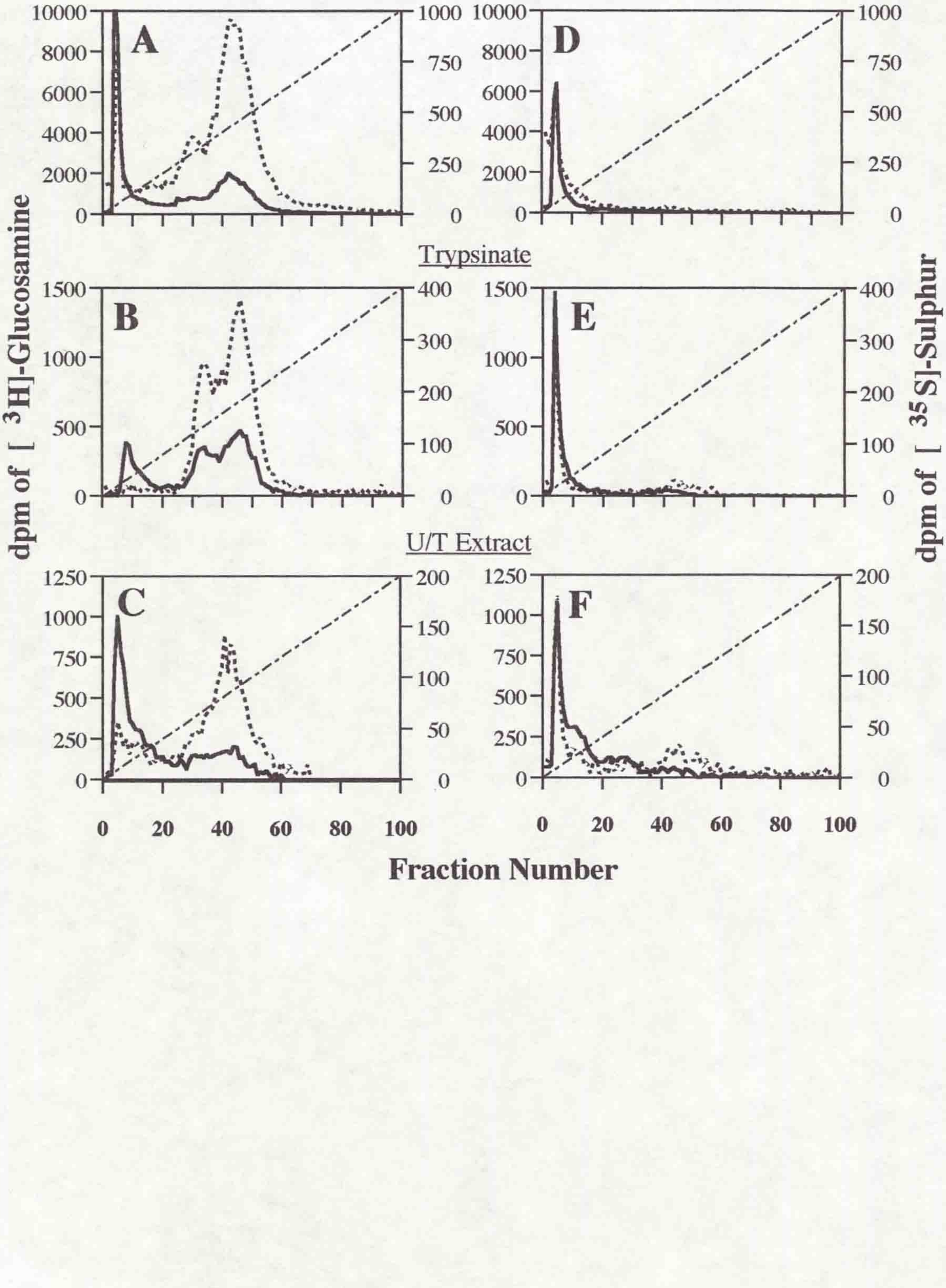


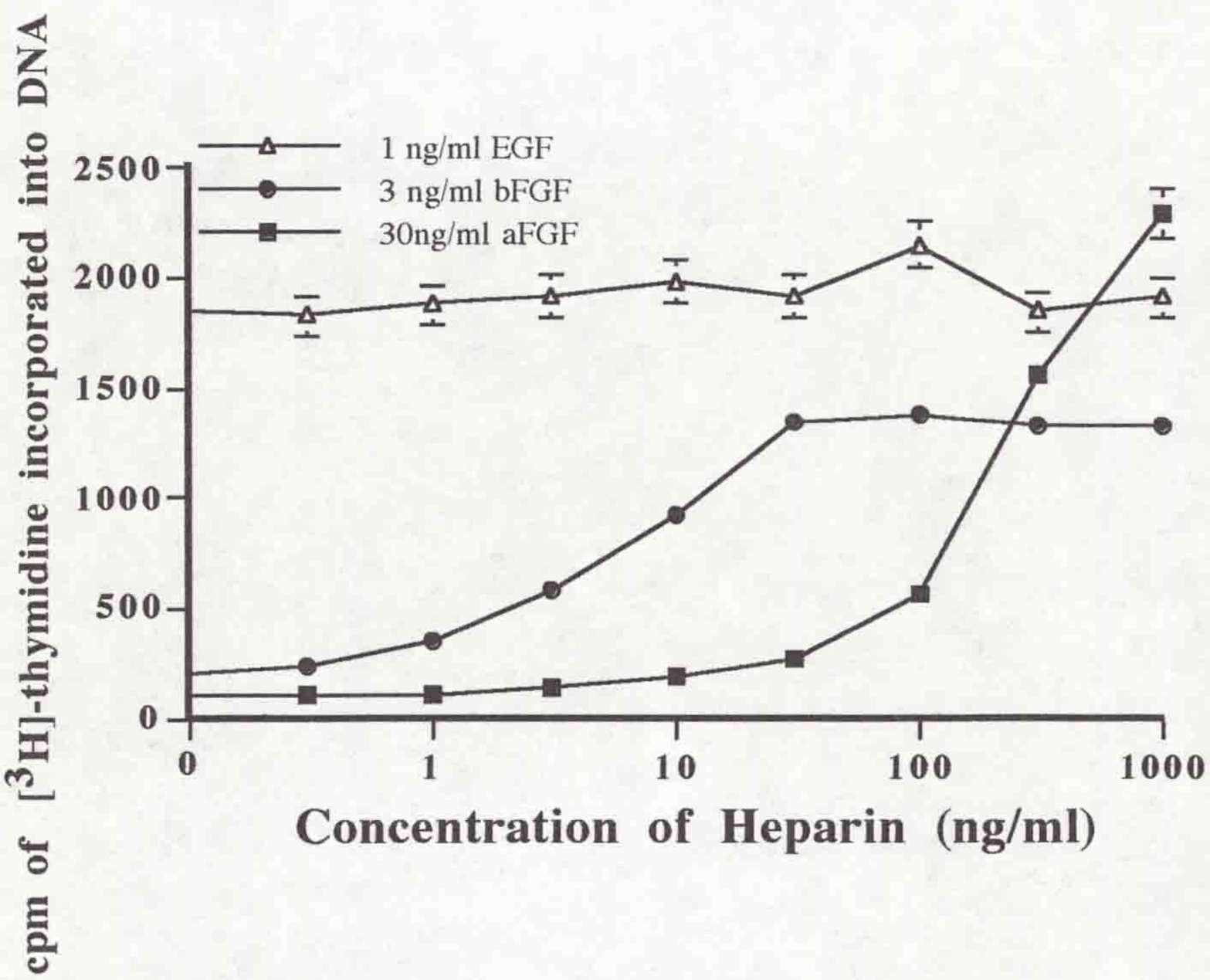
Table 4.1 Production of GAGs and HS by Rama 27 fibroblasts grown in RM and S-free RM

Cellular Fraction	dpm of ^{3}[H]-glucosamine			dpm of [^{35}S]-SO_4		
	Cells		Ratio control/	Cells		Ratio control/
	control	low-sulphur	low-sulphur	control	low-sulphur	low-sulphur
Sulphated GAGs						
Culture medium	746680	50760	15	353220	1880	187
Trypsinate	157700	3580	44	111060	560	198
U/T	73060	29940	2.4	28240	820	34
HS						
Culture medium	175480	3000	59	69240	2730	25
Trypsinate	40480	-*	-	29280	-*	-
U/T	8520	-*	-	15440	-*	-
Total HS	224480	3000	75	113960	2730	42

* The amount of radioactivity in the chromatography fractions was indistinguishable from the background, 20 dpm.

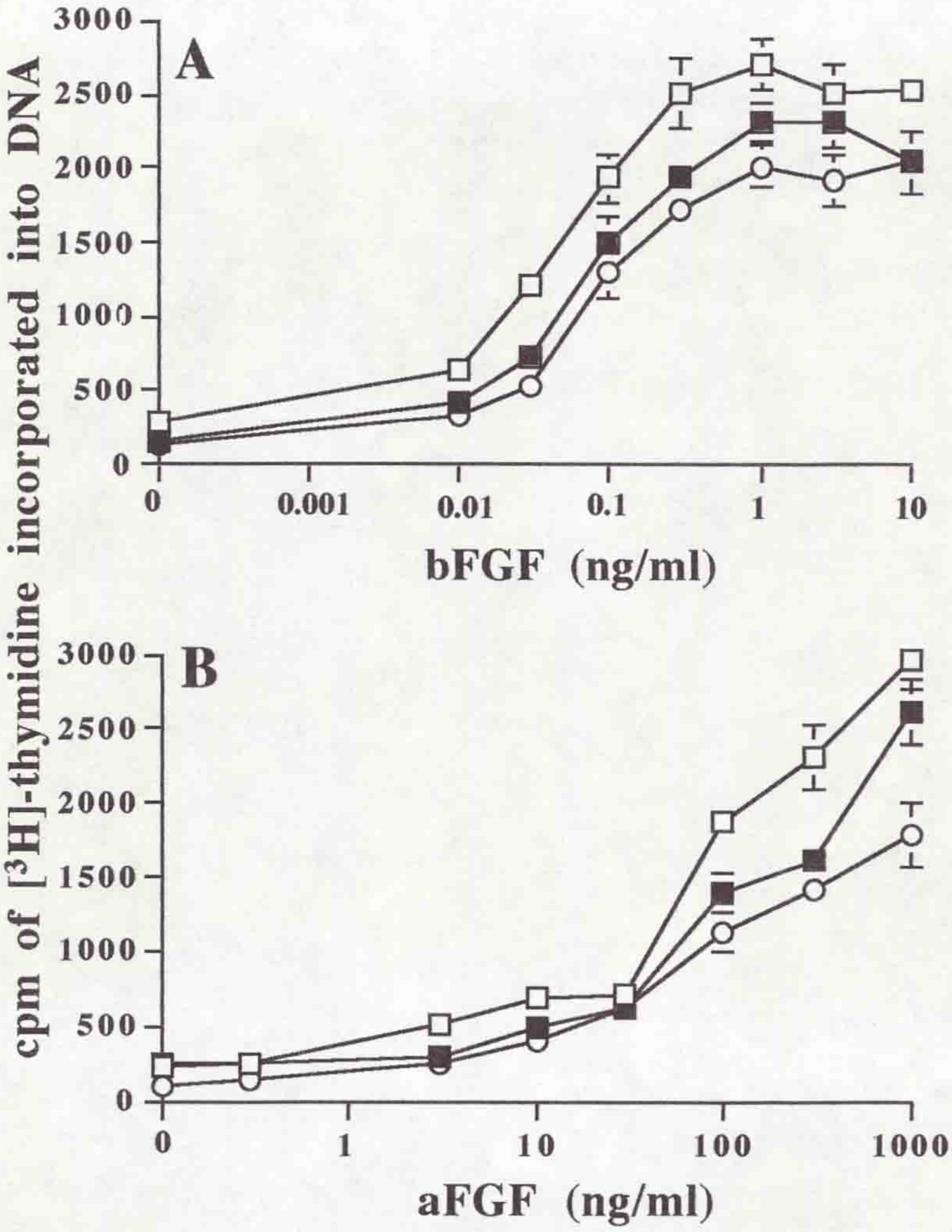
Glycosaminoglycans and HS were purified from three fractions of Rama 27 fibroblasts, grown under control or low-sulphur conditions, as described in Section 2.2.1.2 and 2.2.2. The dpm of [^{3}H]-glucosamine and [^{35}S]-sulphur present in each of the 2 ml chromatography fractions containing GAGs from the first (approximately 0.5-0.7 M NaCl; Fractions 30-55) (Fig. 4.3), and HS from the second (approximately 0.5-0.7 M NaCl; Fractions 5-15), anion-exchange chromatography steps were summed to calculate the total radioactivity present in each cellular fraction.

Fig. 4.4 Effects of heparin on the stimulation of DNA synthesis by growth factors in quiescent HS-deficient Rama 27 fibroblasts



Increasing concentrations of heparin were added with the growth factors to the quiescent HS-deficient Rama 27 fibroblasts to ascertain the dependence of DNA synthesis on cellular HS. (△) EGF; (●) bFGF; (■) aFGF. Results are expressed as the mean cpm±SD of triplicate samples.

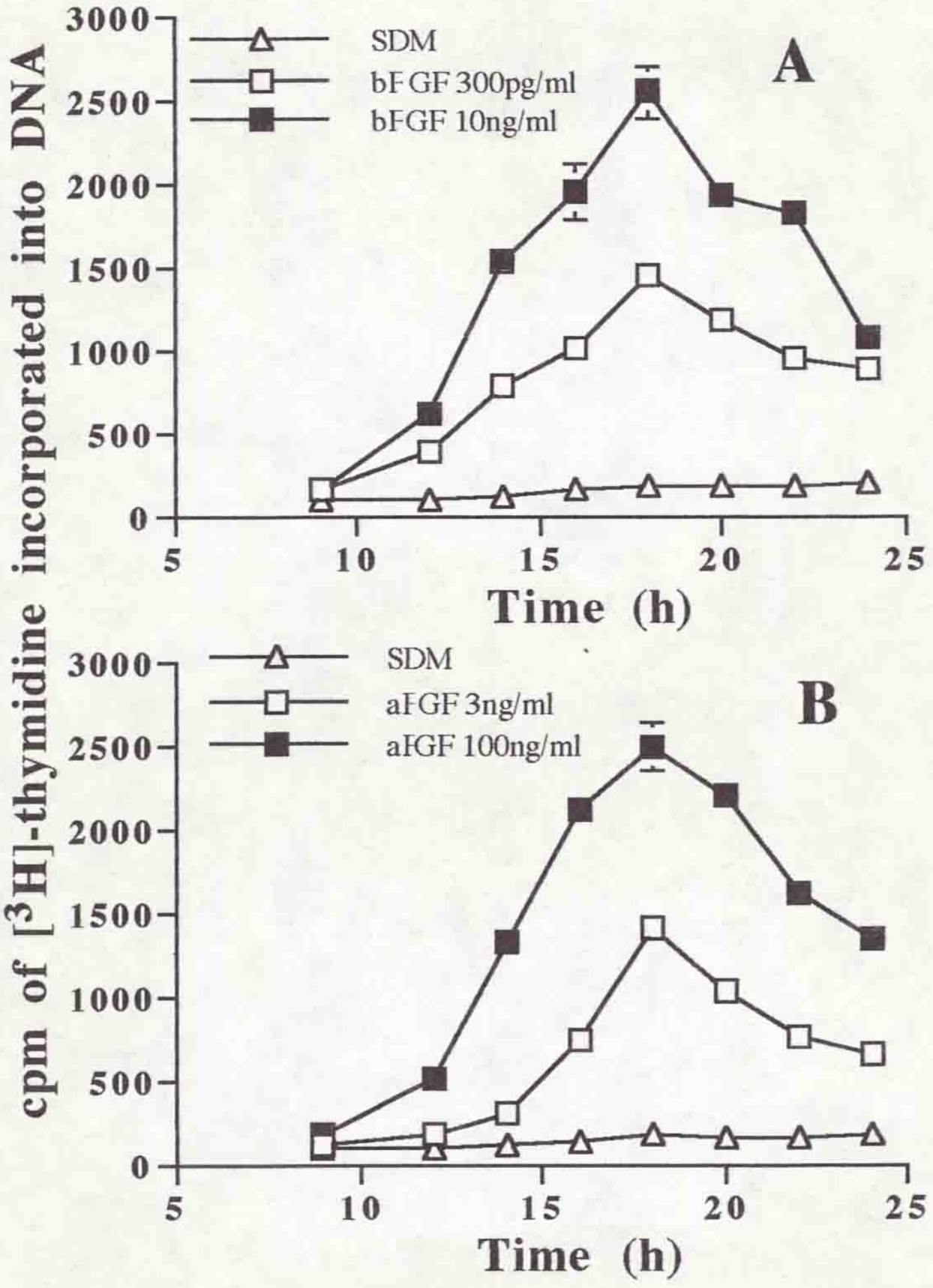
Fig. 4.5 Reversibility of the effects of S-free medium on the stimulation of DNA synthesis by bFGF and aFGF in Rama 27 fibroblasts



Hs-deficient Rama 27 cells were originally incubated in S-free SDM for 24 h and then changed back to normal SDM 1 h before the addition of bFGF and aFGF. The incorporation of [^{3}H]-thymidine into DNA of Rama 27 cells used the method described in Method 2.2.3.1.

(A) bFGF; (B) aFGF. (□) Rama 27 fibroblasts maintained in normal SDM; (■) Rama 27 fibroblasts maintained in S-free-SDM for 24 h and then normal SDM was added prior to the addition of growth factors; (○) Rama 27 fibroblasts were grown in S-free-SDM for 24 h and then SDM containing 15 mM ClO_3 was added prior to the addition of growth factors . Results are expressed as the mean cpm±SD of triplicate samples.

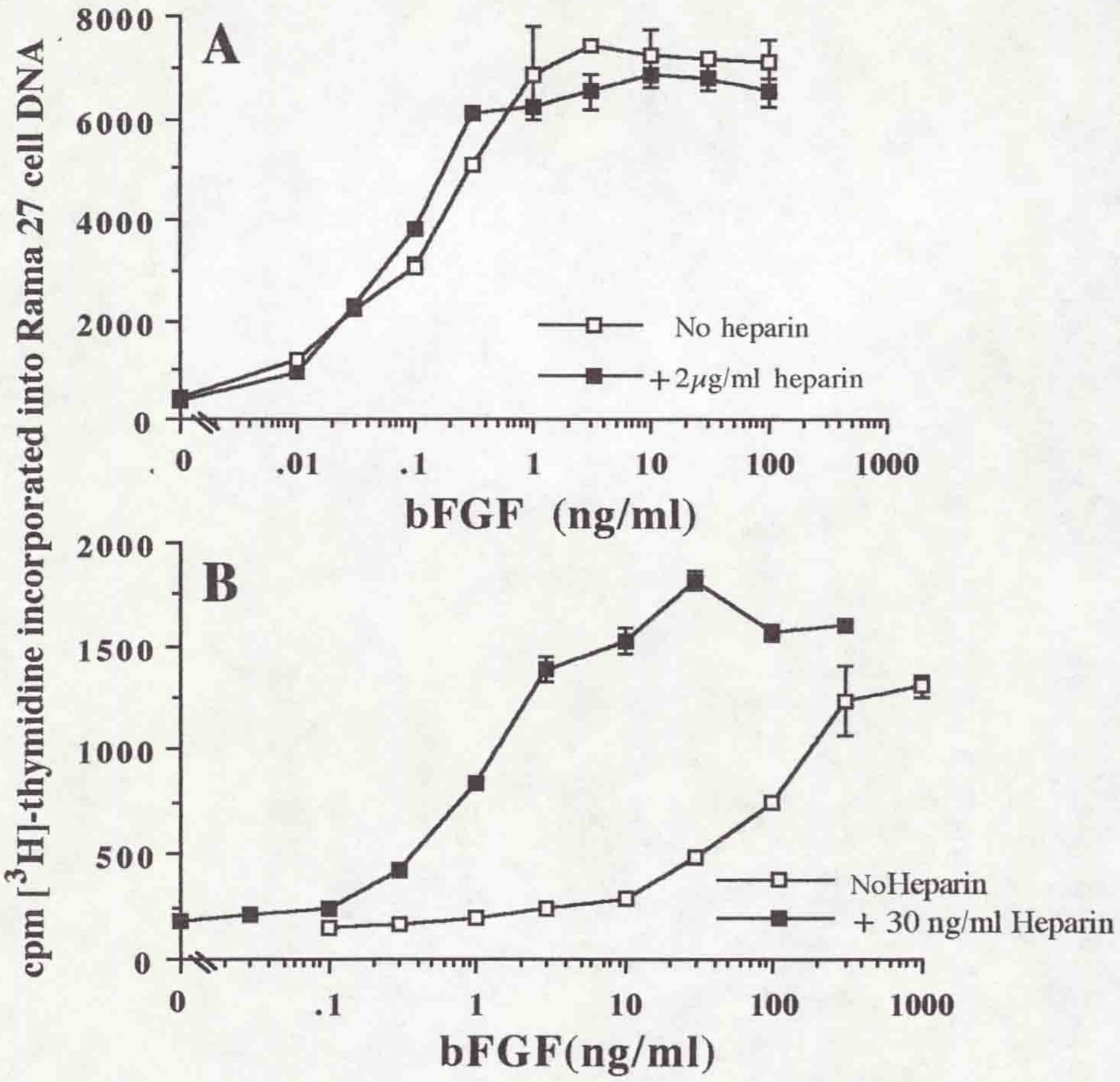
Fig. 4.6 Kinetics of the stimulation of DNA synthesis by bFGF and aFGF in Rama 27 cells



HS-deficient Rama 27 fibroblasts were maintained in S-free SDM (Section 2.2.2) with 30 ng/ml heparin for bFGF and 1 μg/ml heparin for aFGF. After addition of bFGF and aFGF at different times, DNA synthesis was measured as described in Section 2.2.3.2.

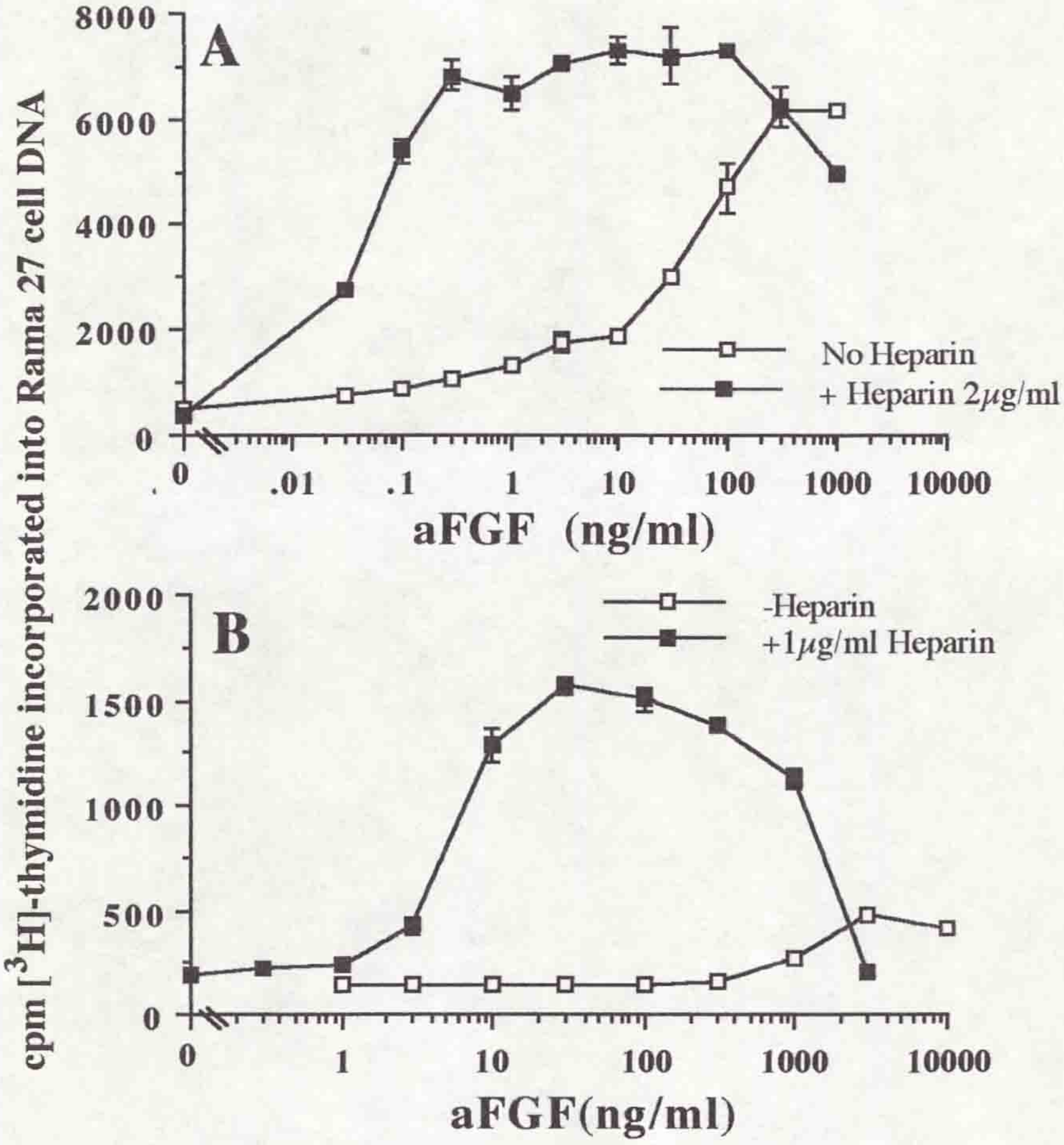
(A) bFGF; (B) aFGF. (Δ) SDM; (□) low concentration of bFGF (300 pg/ml) and aFGF (3 ng/ml); (■) high concentration of bFGF (10 ng/ml) and aFGF (100 ng/ml). Results are expressed as the mean cpm±SD of triplicate samples.

Fig. 4.7 Comparison of DNA synthesis stimulated by bFGF in control and HS-deficient Rama 27 fibroblasts



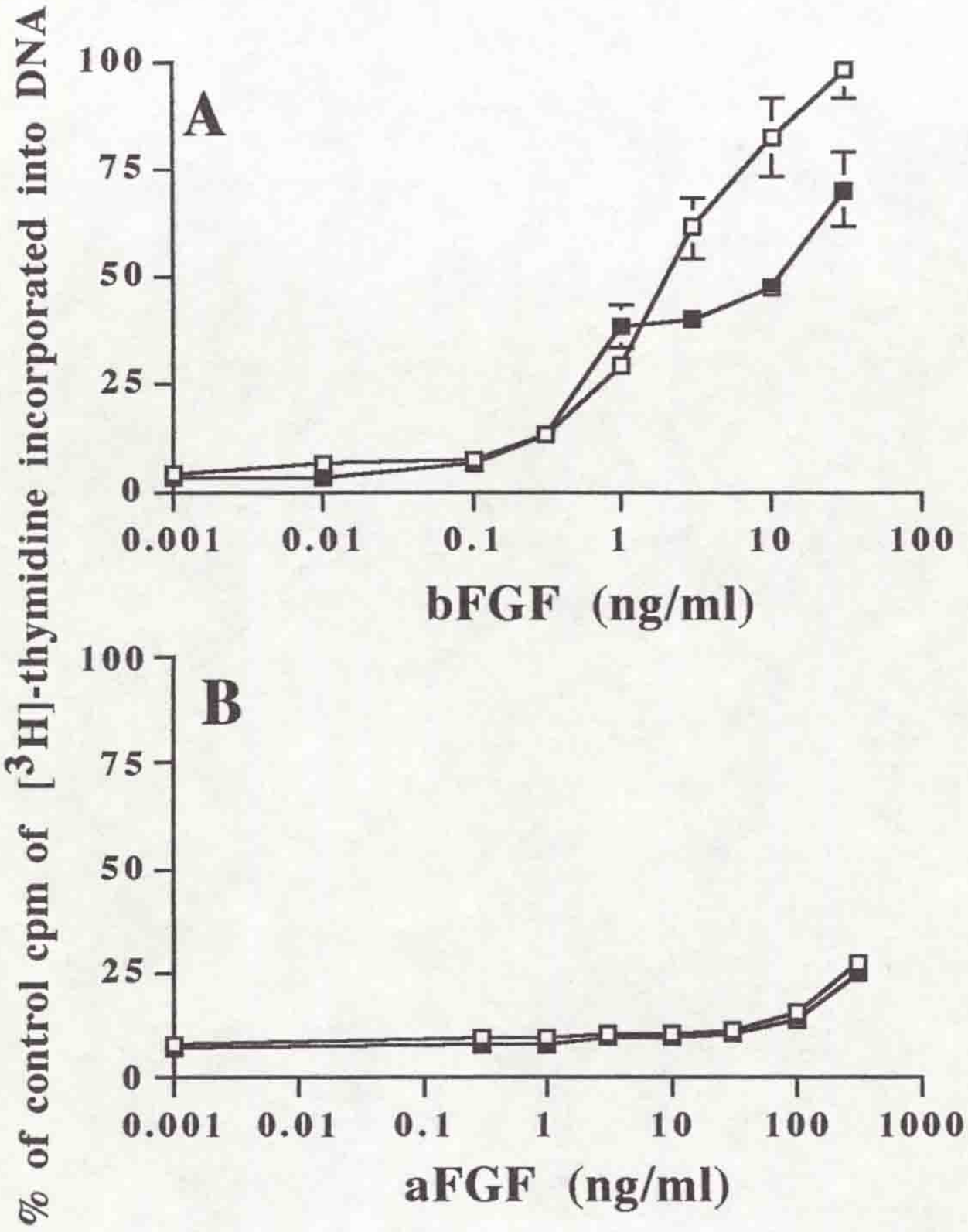
(A) Rama 27 cells in control medium with and without 2μg/ml heparin.
(B) HS-deficient Rama 27 cells with and without 30 ng/ml heparin.
(□) without heparin; (■) with heparin. Results are expressed as the mean cpm±SD of triplicate samples.

Fig. 4.8 Comparison of DNA synthesis stimulated by aFGF in control and HS-deficient Rama 27 fibroblasts



(A) Rama 27 cells in control medium with and without 2μg/ml heparin.
(B) HS-deficient Rama 27 cells with and without 1 μg/ml heparin. (□) without heparin; (■) with heparin. Results are expressed as the mean ± SD of triplicate samples.

Fig. 4.9 Stimulation of DNA synthesis in Rama 27 fibroblasts by aFGF and bFGF preincubated in SDM or S-free SDM without heparin



aFGF and bFGF were preincubated in SDM or S-free SDM for 18 h at 37°C without cells and then added to quiescent Rama 27 cells in SDM.
(A) bFGF; (B) aFGF. (□) aFGF and bFGF preincubated in normal SDM; (■) aFGF and bFGF preincubated in S-free SDM. Results are expressed as the percentage of the stimulation of incorporation of [^{3}H]-thymidine into DNA of control ± SD. The control values were the maximum level of incorporation 3 ng/ml bFGF (4320±115 cpm), and 100 ng/ml aFGF (2317±118 cpm) were added directly to the cells without preincubation.

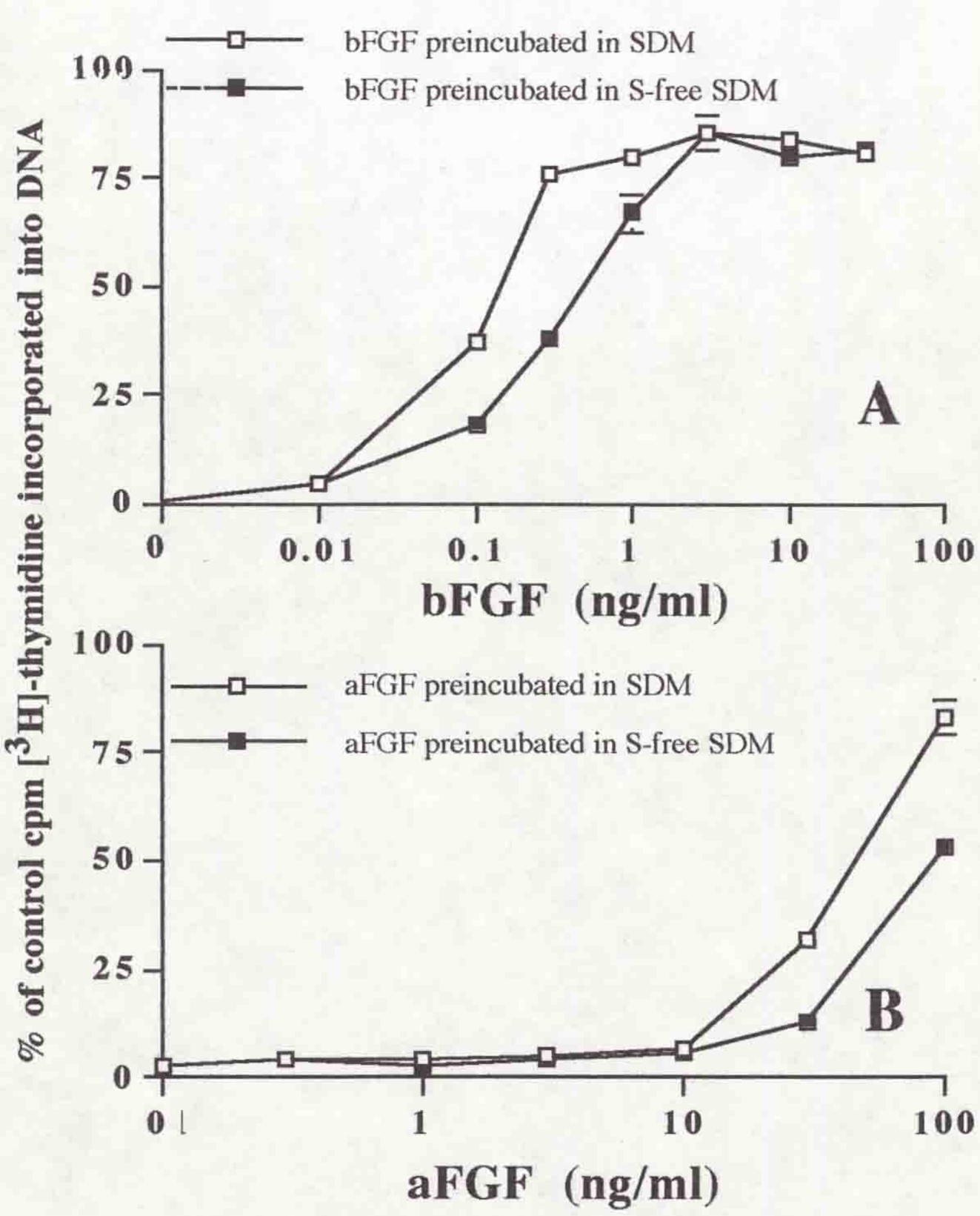
Table 4.2 Inactivation of bFGF and aFGF by preincubation in SDM and S-free-SDM for 18 h at 37°C without heparin

	Concentration required for half-maximum stimulation of DNA synthesis (ng/ml)				
	control (a)*	preincubated (b)‡			ratio of (a)/(b)
		normal SDM (c)	S-free SDM (d)	ratio of (c)/(d)	
bFGF	0.1	2	12	1 : 6	1 : 20 and 1 : 120
aFGF	30	-	-	-	

* Data are from Fig. 3.1

‡ Data are from Fig. 4.9

Fig. 4.10 Stimulation of DNA synthesis in Rama 27 fibroblasts by aFGF and bFGF preincubated in SDM or S-free SDM with heparin



bFGF and aFGF were preincubated in normal SDM or S-free SDM in the presence of heparin for 18 h without cells, and then added to quiescent Rama 27 fibroblasts.

(A) bFGF with 30 ng/ml heparin; (B) aFGF with 1 μg/ml heparin. (□) aFGF and bFGF preincubated in SDM; (■) aFGF and bFGF preincubated in S-free SDM with 30ng/ml or 1μg/ml heparin, respectively. Results are expressed as the percentage of the stimulation of incorporation of [^{3}H]-thymidine into DNA of control ± SD. The control values were the maximum level of incorporation of [^{3}H]-thymidine observed when 3 ng/ml bFGF (4220±78.7 cpm), and 30 ng/ml aFGF (5939±119 cpm) were added directly to the cells withput preincubation.

Table 4.3 Inactivation of bFGF and aFGF by incubation in SDM and S-free-SDM for 18 h at 37°C with heparin

	Concentration required for half-maximum stimulation of DNA synthesis (ng/ml)				
	control(a)*	preincubated(b)‡			ratio of (a)/(b)
		normal SDM (c)	S-free SDM (d)	ratio of (c)/(d)	
bFGF[1]	0.1	0.15	0.5	1 : 3.3	1 : 1.5 and 1 : 5
aFGF[2]	30	45	90	1 : 2	1 : 1.5 and 1 : 3

* Data are from Fig. 3.1.

‡ Dataare from Fig. 4.9

[1]In the presence of 30 ng/ml heparin.

[2]In the presence of 1 μg/ml heparin.

Table 4.4 Receptors for [^{125}I]-bFGF on Rama 27 fibroblasts grown under various conditions

Condition	High-affinity receptor		Low-affinity receptor	
	Kd[1] (pM)	Number of receptors[1] (10^3 per cell)	Kd[1] (nM)	Number of receptors[1] (10^6 per cell)
Control[2]	33±20	26±16	8±6	3±2
Control + 1 μg/ml heparin	32±20	4.8±1.3		ne[3]
HS-deficient	24±9	14±2		ne[3]
HS-deficient + 30 ng/ml heparin	109±26	19±3		ne[3]

[1] Mean±SE calculated from data pooled from four independent experiments.

[2] Analysis of the binding data for control Rama 27 cells with the LIGAND program (Section 2.2.6) indicated that a two-site model was superior to a one-site model. Thus a two-site model yielded an improved runs test and a reduced mean square (P = 0.005), whilst the other measures of goodness of fit were unchanged.

[3] ne, no evidence. When a two-site model was used to fit the data from the control+1 μg/ml heparin and the HS-deficient experiments, regardless of the starting values of the binding parameters, the model would not converge. The data from the HS-deficient + 30 ng/ml heparin experiments did yield a two-site model that converged. The two-site model had a reduced mean square (P = 0.008) compared to the one-site model. However, the runs test for two of four experiments indicated a non-random distribution of data points around the model (2 runs out of 12 points for each data set), the errors in some of the parameters increased to over 100% of the mean, and the non-specific binding for the lower affinity sites was equal to the amount of specific binding. For these reasons, low-affinity binding sites are not detectable on the surface of Rama 27 cells in the presence of 1 μg/ml heparin, or on HS-deficient Rama 27 cells.

Chapter Five

Characterization of Mammary HS

Chapter Five
Characterization of Mammary HS

5.1 Introduction

There is considerable evidence for a link between the expression of bFGF and its receptors and the differentiation pathway that leads to the myoepithelial cell in the mammary gland. *In vivo,* it would appear that the expression of a high capacity HS sink for bFGF is associated with non-growing structures in the mammary gland (Section 1.2.5.3). Moreover, the receptors for bFGF, and sometimes bFGF itself are expressed ectopically by malignant mammary epithelial cells (Section 1.2.5). In particular, it has become apparent that the malignant mammary epithelial cells may express HS that is paradoxically able to participate in the dual receptor system, but unable to act as a storage site for bFGF (Section 1.2.5.3). As a first step towards understanding how the cells of the mammary gland may control the activity of bFGF by expressing HS receptors with the bFGF storage/sink and bFGF activation functions (Section 1.2.3.1), HS was first purified from a panel of mammary cells representative of mammary development and cancer. These cells were: Rama 27 fibroblasts, Rama 37 epithelial cells, Rama 401 myoepithelial cells, and Rama 800 malignant epithelial cells (Table 2.1). HS was isolated from three fractions of the cultured cells: (1) the cells' culture medium, representing secreted/shed HS; (2) the trypsinate, representing plasma membrane HS; (3) a U/T extract, representing extracellular matrix HS and intracellular HS. Then the ability of the purified HS to activate bFGF in HS-depleted Rama 27 fibroblasts was determined.

5.2 Results

5.2.1 Cell-derived HS can restore the growth-stimulatory activity of bFGF

The different types of HS purified from the mammary cells were not equivalent in their ability to restore the activity of bFGF in HS-deficient Rama 27 fibroblasts. The HS purified from the culture medium of Rama 27 fibroblasts was hardly able to restore the activity of bFGF, whilst the HS purified from the trypsinate and the U/T fractions was able to restore the activity of bFGF (Fig. 5.1). In contrast, the HS purified from all three fractions of Rama 401 myoepithelial cells was able to restore partially the activity of bFGF in HS-deficient Rama 27 fibroblasts (Fig. 5.1). The culture medium and U/T fractions of Rama 37 epithelial cells contained HS that was able to activate bFGF in HS-deficient Rama 27 fibroblasts (Fig. 5.2). However, the HS that was recovered from the trypsinate was unable to activate bFGF in this assay (Fig. 5.2). In contrast the culture medium and the trypsinate of the malignant epithelial Rama 800 cells contained HS that activated bFGF whilst the U/T fraction was only marginally able to do so (Fig. 5.2).

The amounts of HS purified from the mammary cells were too low to quantify directly. However, since the number of cells from which the HS was prepared was known, it was possible to quantify the bFGF-activating activity of the HS in terms of cell number and the response observed with 30 ng/ml heparin (Table 5.1). This method of quantification has the advantage of allowing a comparison with receptor numbers, which are conventionally expressed as "number of receptors per cell".

It is clear that the samples of mammary HS fall into three classes: those with little or no activity (Rama 27 culture medium; Rama 37 trypsinate; Rama 800 U/T); those with a high level of activity (18-33, Table 5.1); the two most active samples, the U/T fraction of Rama 27 cells and the culture medium of Rama 401 cells.

5.2.2 Production of GAGs and HS by mammary cells

The Rama 401 myoepithelial cells produced large amounts of GAGs in all cellular

fractions (Fig. 5.3; Table 5.2). The culture medium contained the most GAGs, although the amount present in the U/T and trypsinate fractions was of the same order of magnitude (Fig. 5.3; Table 5.2). The three cellular fractions of Rama 401 myoepithelial cells also contained similar amounts of HS (Fig. 5.3; Table 5.2). However, a comparison of the amounts of GAGs and HS suggests that the trypsinate and to a lesser extent the U/T fraction of Rama 401 cells are enriched in HS and depleted in other GAGs whilst the culture medium fraction is enriched in GAGs other than HS (Table 5.2).

The culture medium and U/T fractions of Rama 37 epithelial cells contained the largest amount of GAGs , indicating that these cells shed/secrete HS into the culture medium HS on a large scale and deposit a substantial amount of GAGs into the extracellular matrix (Fig. 5.4 a-c; Table 5.3). However, more HS was recovered from the culture medium and the trypsinate fractions, suggesting that the U/T fraction, representing intracellular and extracellular matrix GAGs is relatively enriched in GAGs other than HS (Table 5.3). In addition, the HS recovered from the trypsinate would appear to be relatively undersulphated, since [^{35}S]-radioactivity was not detectable in this HS.

Most of the GAGs produced by the malignant epithelial Rama 800 cells are recovered in the culture medium (Fig. 5.5; Table 5.4). The Rama 800 cells contained only a relatively small amount of GAGs in the trypsinate and U/T fractions (Fig. 5.5b,c; Table 5.4). Similarly, most of the HS produced by the malignant epithelial Rama 800 cells is in the culture medium, with only small amounts being recovered from the trypsinate and U/T fractions (Fig. 5.5; Table 5.4). Therefore Rama 800 cells shed/secrete most of their GAGs, including HS.

5.3 Discussion

HS was purified in sufficient amounts from a series of rat mammary cell lines that represent the different cellular compartments of the mammary gland (Table 2.1) to allow

a preliminary determination of the ability of such HS to activate bFGF.

Most of the HS-activating properties of the panel of mammary cells used for this study correlate closely with the bFGF-binding and extracellular matrix synthesis properties of the cells. HS purified from the U/T (extracellular matrix and intracellular HS) of Rama 27 fibroblasts, Rama 37 epithelial cells and Rama 401 myoepithelial cells was able to restore the activity of bFGF (Figs. 4.1; 5.1, and 5.2). All of these cells are known to deposit extracellular matrix (Tables 2.1 and 5.1), so it is not surprising that this fraction contains considerable HS. Moreover, it is unlikely that the extracellular matrix is "seen" by [^{125}I]-bFGF in a binding assay, since the extracellular matrix is between the cell monolayer and the plastic substratum. Therefore, in the case of Rama 37 epithelial cells that do not possess receptors for bFGF (Fernig *et al.*, 1993), such extracellular matrix receptors (Fig. 5.2) would be inaccessible to exogenously-added bFGF. Similarly, the amount of HS isolated from the U/T fraction of the Rama 800 cells was very low, in accordance with the inability of such malignant mammary epithelial cells to synthesise and deposit extracellular matrix (Dunnington *et al.*, 1983). The low level of HS recovered from the extracellular matrix of the Rama 800 cells would therefore explain the inability of this HS to activate bFGF (Figs. 5.2 and Table 5.4).

The activation activity of the HS purified from the culture medium of the cells is slightly puzzling. HS in the culture medium will represent HSPGs that have been shed from the cell surface, in the form of syndecans, through the action of a trypsin-like enzyme (Section 1.2.2.3.1) and glypican through the action of a phosphatidylinositol-specific phospholipase C (Section 1.2.2.3.1). It would be expected that such HS would therefore be identical in bFGF-activating properties to the HS isolated from the trypsinate. Whilst this is the case for the Rama 401 myoepithelial cells (Fig. 5.3), and for the Rama 800 cells (Fig. 5.5), it is clearly not true for the Rama 27 fibroblasts and the Rama 37 epithelial cells (Fig. 5.3 and Fig. 5.4). Thus the culture medium, but not the cell surface, of the Rama 37 epithelial cells contains HS that can activate bFGF whilst the Rama 27 fibroblasts possess HS that is able to activate bFGF on their cell-

surface, but the HS in the culture medium is largely inactive (Table 5.1). One explanation for these results is that the cells are secreting HSPG that possess HS chains with different bFGF-activating characteristics to the HSPGs of the plasma membrane. However, the evidence on the source of HSPGs found in the culture medium of cells, particularly mammary cells, indicates that these HSPGs are not secreted, but are instead shed from the plasma membrane (Bernfield *et al.*, 1992; Elenius and Jalkanen, 1994; Rapraeger *et al.*,1986). Moreover, there is considerable evidence that *in vivo* HSPGs are also shed from the cell-surface (Jalkanen *et al.*, 1988). Thus it would appear unlikely that the HSPGs in the culture medium are not directly related to those on the cell-surface.

An alternative explanation for these observations is that there is a mechanism whereby the activity of the enzymes responsible for the shedding of HSPGs into the culture medium is somehow controlled by the sequences found on the HS chains. Hence HSPGs that can activate bFGF are preferentially lost from the surface of Rama 37 cells and in Rama 27 cells HSPGs that are unable to activate bFGF are preferentially lost from the cell-surface. It could be argued that the source of the HS in the medium in the Rama 37 cells could be the extracellular matrix since HS isolated from the U/T fraction of these cells is able to activate bFGF (Fig. 5.2). However, since both the extracellular matrix and the plasma membrane fractions of HS isolated from Rama 27 cells are able to activate bFGF, there is no source of HS in these cells that could supply the HS in the culture medium without some type of selectivity operating when HSPGs are shed into the culture medium.

The trypsinate fraction is likely to be reasonably representative of the plasma membrane HSPGs. Thus the syndecans possess a trypsin sensitive site between the plasma membrane and the first GAG attachment site (Section 1.2.2.3.1). The syndecans are the major HSPG core proteins found on the plasma membranes of many epithelium-derived cells, including those of the mammary gland (Section 1.2.2.3.1). In addition, whilst trypsin is a relatively specific protease, it seems reasonable to assume

that at least some of the other HSPG core proteins (Section 1.2.2.3.1) that may be found on mammary cell-surfaces will also be degraded, at least in part, by trypsin. Therefore it would be expected that the bFGF-activating characteristics of the HS isolated from the trypsinate would correlate with the known bFGF-binding properties of the cells. Thus the Rama 27 fibroblasts and the Rama 401 myoepithelial cells both possess large number of HS receptors for bFGF (Table 5.1; Fernig et al., 1990b; 1992) and the HS isolated from the trypsinate of these cells is able to activate bFGF in HS-deficient Rama 27 fibroblasts (Figs. 4.3 and 5.1). Similarly, the Rama 37 epithelial cells do not possess receptors for bFGF (Fernig *et al.*, 1993) and the HS isolated from the trypsinate of these cells has no detectable activation effect on bFGF (Figs. 5.2 and 5.4) However, the HS isolated from the trypsinate of the Rama 800 malignant epithelial cells is able to restore the activity of bFGF in HS-deficient Rama 27 fibroblasts, yet Rama 800 cells, like other malignant mammary epithelial cells do not possess low-affinity receptors for bFGF (Fernig *et al.*, 1993; Peyrat *et al.*, 1992). The bulk of the HS receptors for bFGF on mammary (Fernig *et al.*, 1992; 1993) and other cells are the so-called low-affinity binding sites, of which there 10^6 or more per cell. The absence of low-affinity receptors for bFGF (the HS receptor) on Rama 800 cells could be accounted for by the relatively low amount of HS present in the trypsinate of these cells compared to that recovered from the Rama 27 fibroblasts (Figs. 4.3, 5.5 and Tables 4.1 and 5.4). However, the small amount of HS in the trypsinate of the Rama 800 cells is able to activate bFGF, on a per cell basis, as efficiently as the much larger amount of HS recovered from the trypsinate of the Rama 27 fibroblasts (Table 5.1). One explanation of these results is that only a low level of bFGF-binding HS receptor is necessary to activate bFGF, and that such a low level is not detectable in binding assays. However, since the binding assays can detect as little as 10^3 receptors per cell (Fernig *et al.*, 1993) this seems rather unlikely in view of the fact that ng/ml concentrations of heparin are required to restore the activity of bFGF in HS-deficient cells (Fig. 4.4). A more interesting possibility is that the structure in HS responsible for the low-affinity receptor may be a different structure from that responsible for the bFGF-activating property.

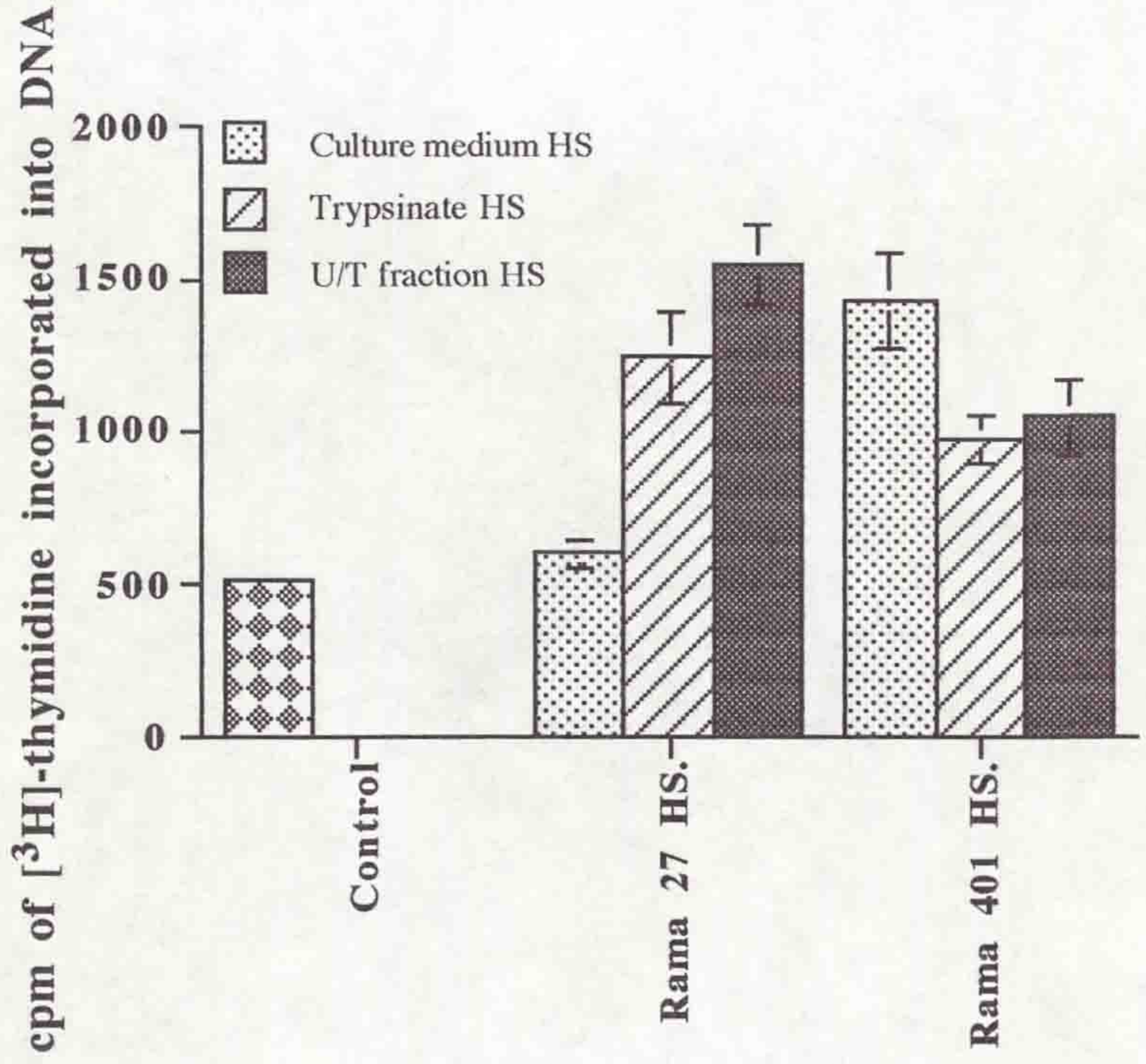
There are data to support this contention. For example, syndecan-1 was isolated by expression cloning as a low-affinity receptor for bFGF (Kiefer *et al.*, 1990), yet it is claimed that syndecan-1 activates bFGF poorly in HS-deficient cells (Aviezer *et al.*, 1994). However, such supporting data are considerably weakened by the observation that the same core protein (syndecan-1) can carry, in two clonal cell lines derived from the same parent, HS chains with different protein-binding characteristics (Sanderson *et al.*, 1994). The latter result strongly suggests that the information that dictates the structure of the HS chains on a core protein is not encoded in the sequence of the core protein itself. Hence the syndecan-1 that activated bFGF poorly (Aviezer *et al.*, 1994), may not have possessed bFGF-binding HS chains. Thus until the fine structure of the HS from the trypsinate of Rama 800 cells and, e.g., Rama 27 fibroblasts is resolved, the distinction, at the structural level, of the bFGF-binding and the bFGF-activating properties of HS can only remain a hypothesis, albeit an exciting one.

Table 5.1 Quantification of bFGF-activating activity of HS purified from rat mammary cells

	bFGF-activating activity of HS[a]		
Cell	culture medium	trypsinate	U/T fraction
Rama 27	4	33	46
Rama 401	41	21	24
Rama 37	18	0	29
Rama 800	32	37	4

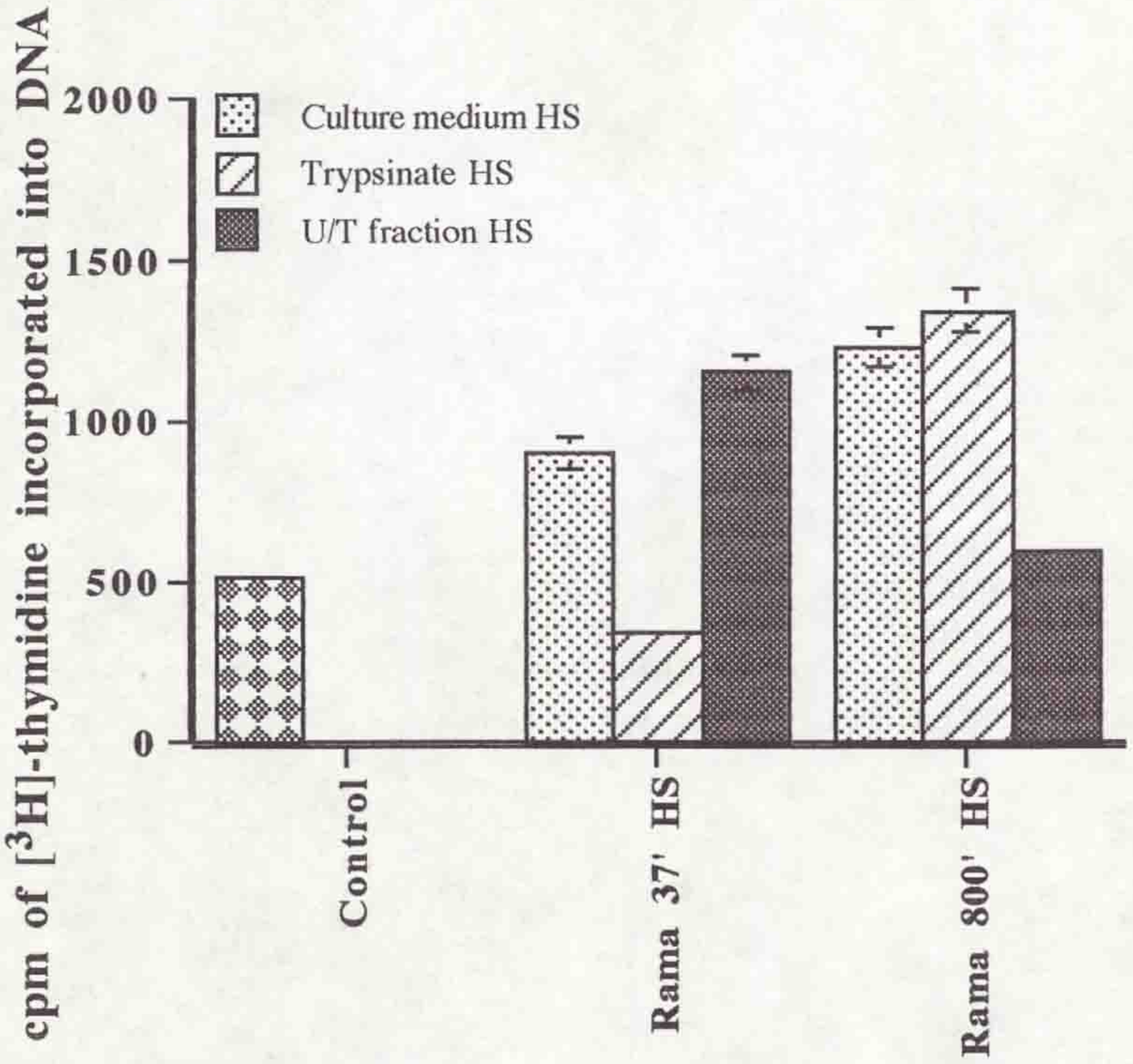
a The bFGF-activating activity of the HS purified from the three fractions of rat mammary cells was quantified in terms of the activity (cpm of [^{3}H]-thymidine incorporated into Rama 27 cells DNA) of the HS produced by 10^3 cells as a percent of the activity of 30 ng/ml heparin. Data are from Figs. 5.1 and 5.2.

Fig. 5.1 Partial restoration of the growth -stimulatory activity of bFGF in HS-deficient Rama 27 cells by HS purified from Rama 27 and Rama 401 cells .



DNA synthesis in HS-deficient Rama 27 fibroblasts was stimulated by 3 ng/ml bFGF. Control, no HS added. 30 μl/ml of each sample of cellular HS was added. The stimulation of DNA synthesis observed with 30 ng/ml heparin was 1500 ± 133 cpm . Results are the mean±SD of triplicate determinations.

Fig. 5.2 Partial restoration of the growth -stimulatory activity of bFGF in HS-deficient Rama 27 cells by HS purified from Rama 37 and Rama 800 cells



DNA synthesis in HS-deficient Rama 27 fibroblasts was stimulated by 3 ng/ml bFGF. Control, no HS added. 30 μl/ml of each sample of cellular HS was added. The stimulation of DNA synthesis observed with 30 ng/ml heparin was 1500 ± 133 cpm . Results are the mean±SD of triplicate determinations.

Fig. 5.3 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 401 myoepithelial cells

Rama 401 myoepithelial cells ($2x10^6$) were grown for 72 h in RM in the presence of [^{3}H]-glucosamine and [^{35}S]-sulphur and proteoglycans were separated by anion exchange chromatography and the radioactivity in each fration determined as described in Section 2.2.7.3.

Panel A shows the amount of glucosamine and sulphate incorporated into proteoglycans secreted/shed by Rama 401 myoepithelial cells into the culture medium. Panel B shows proteoglycans released from Rama 401 myoepithelial cells by the action of trypsin. Panel C shows proteoglycans extracted with U/T which corresponds to extracellular matrix and intracellular proteoglycans. (—) solid line represents [^{3}H]-glucosamine incorporation; (····) short dotted line represents [^{35}S]-sulphur incorporation; (---) long dotted line represents the NaCl gradient (0.15 M to 2 M).

Fig. 5.3 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 401 myoepithelial cells

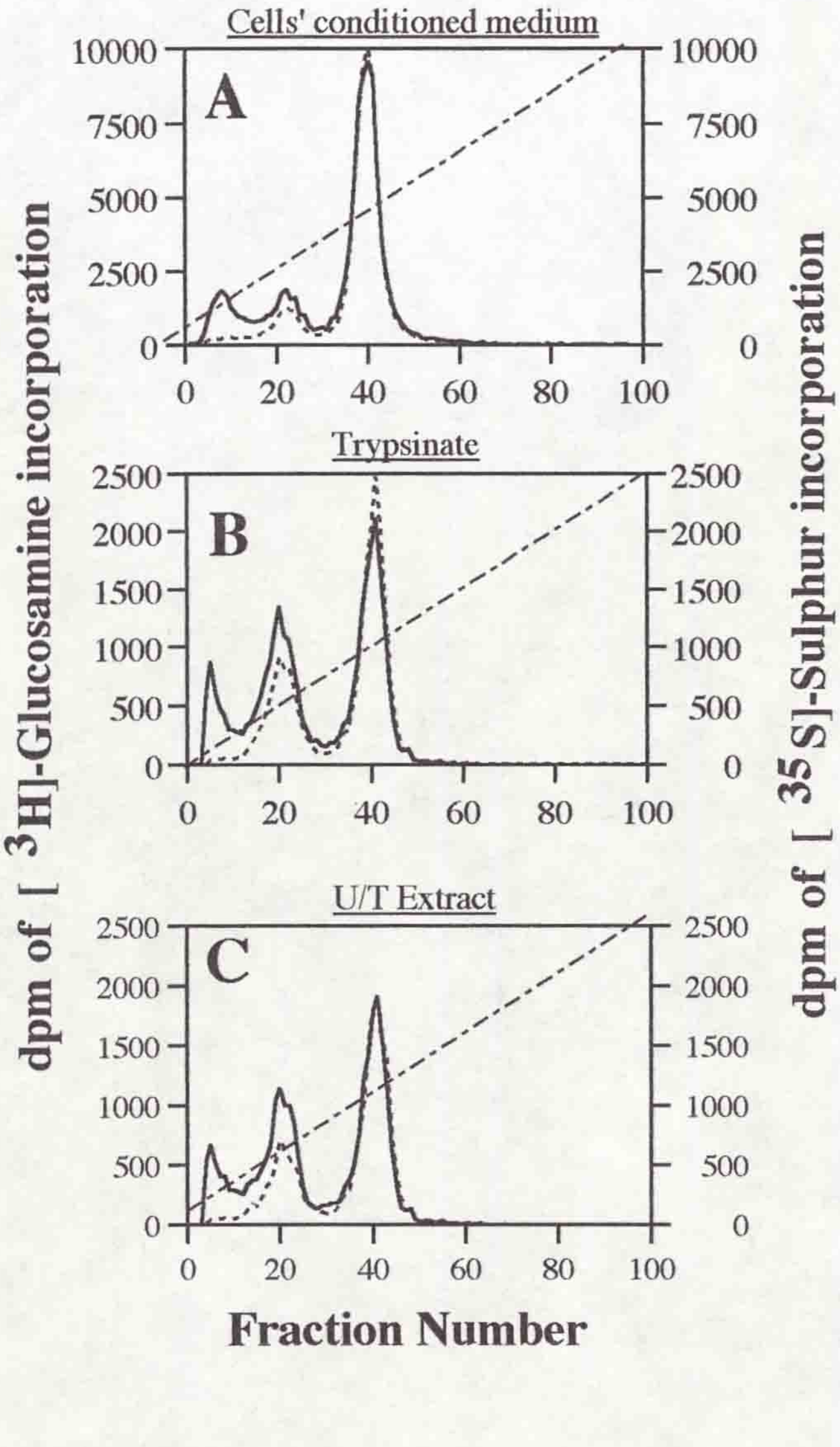


Table 5.2 Production of GAGs by Rama 401 myoepithelial cells grown in RM

Cellular Fraction	dpm [^{3}H]-glucosamine	dpm [^{35}S]-sulphur
Sulphated GAGs		
Culture medium	8518212	7547491
Trypsinate	2347920	2098220
U/T	2092720	1743220
HS		
Culture medium	456120	129210
Trypsinate	315780	95900
U/T	145780	78395

Fig. 5.4 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 37 epithelial cells

Rama 37 epithelial cells ($2x10^6$) were grown for 72 h in RM in the presence of [^{3}H]-glucosamine and [^{35}S]-sulphur and proteoglycans were separated by anion exchange chromatography and the radioactivity in each fration determined as described in Section 2.2.7.3.

Panel A shows the amount of glucosamine and sulphate incorporated into proteoglycans secreted/shed by Rama 37 epithelial cells into the culture medium. Panel B shows proteoglycans released from Rama 37 epithelial cells by the action of trypsin. Panel C shows proteoglycans extracted with U/T which corresponds to extracellular matrix and intracellular proteoglycans. (—) solid line represents [^{3}H]-glucosamine incorporation; (····) short dotted line represents [^{35}S]-sulphur incorporation; (---) long dotted line represents the NaCl gradient (0.15 M to 2 M).

Fig. 5.4 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 37 epithelial cells

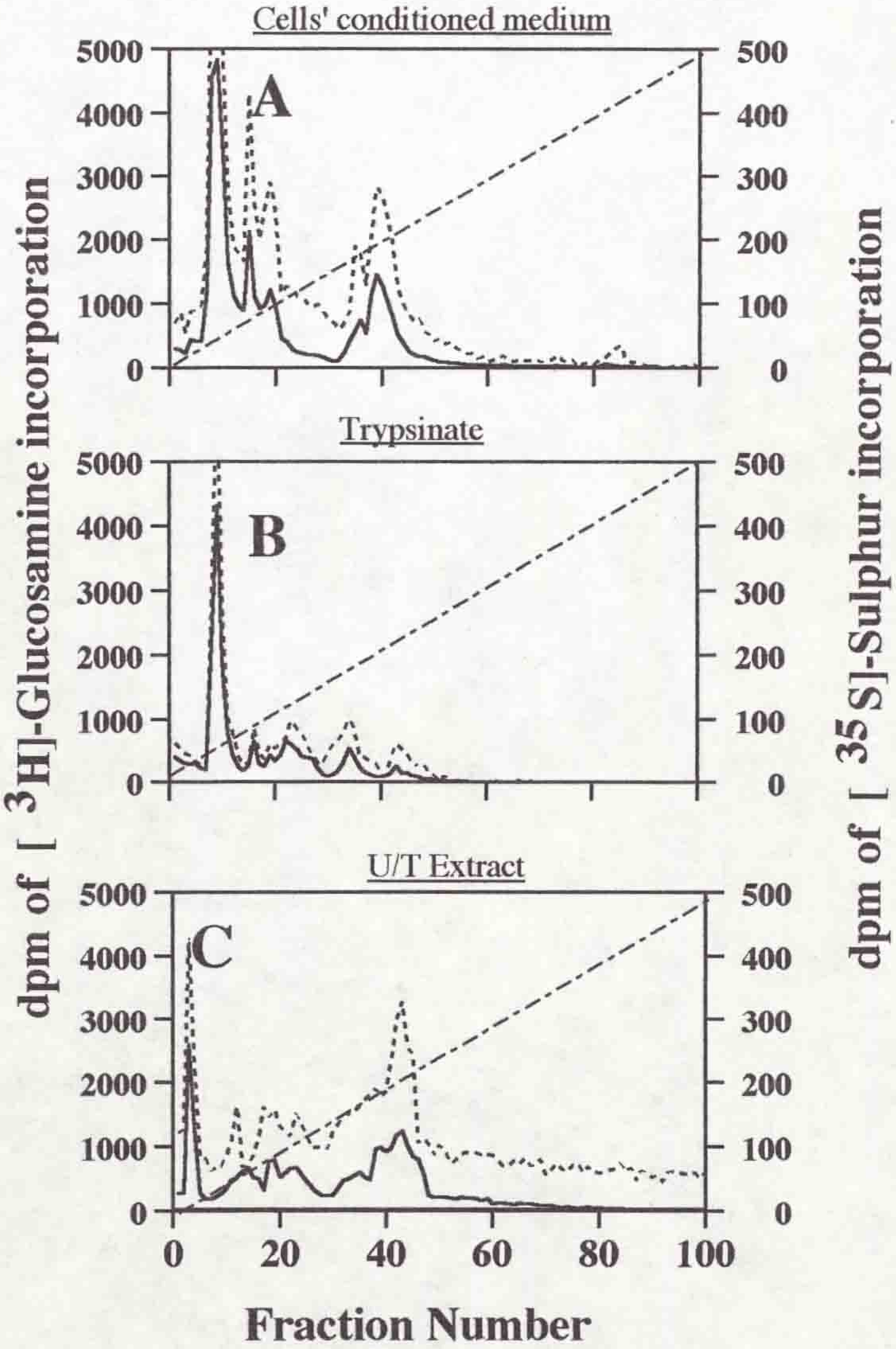


Table 5.3 Production of GAGs by Rama 37 epithelial cells grown in RM

Cellular Fraction	dpm [^{3}H]-glucosamine	dpm [^{35}S]-sulphur
Sulphated GAGs		
Culture medium	1986705	463750
Trypsinate	892850	103600
U/T	2156450	506950
HS		
Culture medium	52450	2405
Trypsinate	9035	ne*
U/T	2675	1175

* no evidence: the [^{35}S]-radioactivity in this fraction was indistinguishable from the background.

Fig. 5.5 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 800 malignant epithelial cells

Rama 800 malignant epithelial cells ($2x10^6$) were grown for 72 h in RM in the presence of [^{3}H]-glucosamine and [^{35}S]-sulphur and proteoglycan were separated by anion exchange chromatography and the radioactivity in each fraction determined as described in Section 2.2.7.3.

Panel A shows the amount of glucosamine and sulphate incorporated into proteoglycans secreted/shed by Rama 800 malignant epithelial cells into the culture medium. Panel B shows proteoglycans released from Rama 800 malignant epithelial cells by the action of trypsin. Panel C shows proteoglycans extracted with U/T which corresponds to extracellular matrix and intracellular proteoglycans. (—) solid line represents [^{3}H]-glucosamine incorporation; (····) short dotted line represents [^{35}S]-sulphur incorporation; (---) long dotted line represents the NaCl gradient (0.15 M to 2 M).

Fig. 5.5 Production of secreted/shed, plasma membrane, and extracellular matrix GAGs by Rama 800 malignant epithelial cells

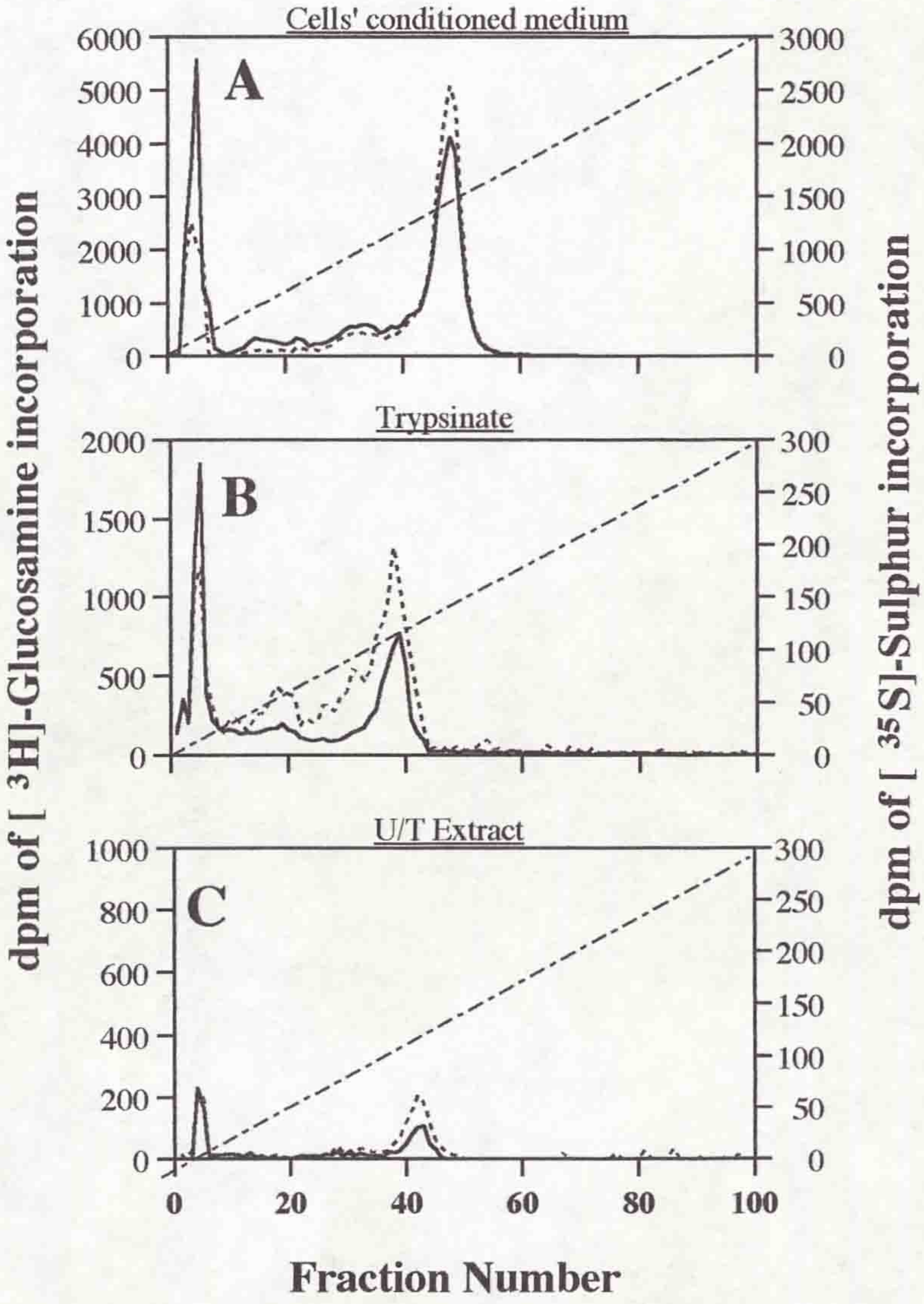


Table 5.4 Production of GAGs by Rama 800 malignant epithelial cells grown in RM

Cellular Fraction	dpm [^{3}H]-glucosamine	dpm [^{35}S]-sulphur
Sulphated GAGs		
Culture medium	3362700	1659960
Trypsinate	603600	146630
U/T	30150	13450
HS		
Culture medium	107325	9095
Trypsinate	20560	1815
U/T	6095	195

Chapter Six

Processing of [^{125}I]-bFGF in Mammary Cells

Chapter Six
Processing of [^{125}I]-bFGF in mammary cells

6.1 Introduction

A long-lasting interaction of bFGF with Rama 27 fibroblasts is required to induce cell proliferation (Section 3.2.2). It is possible that the requirement for this long-lasting interaction is related to the post-receptor processing of bFGF that has been observed in a number of different cell types (Section 1.2.3.3).

In this chapter, the post-receptor processing of [^{125}I]-bFGF and the subcellular localization of the processed [^{125}I]-bFGF was examined. Initially [^{125}I]-bFGF was added to quiescent Rama 27 fibroblasts to determine whether this interaction resulted in the processing of bFGF or, as occurs for most polypeptide hormones and growth factors, the bFGF was rapidly degraded. This experiment was also performed on other mammary cells such as Rama 401 myoepithelial-like cells and Rama 800 malignant epithelial cells (Table 2.1). Since these cells possess different cell surface-receptors for bFGF (Fernig et al., 1992, 1993), such experiments may reveal not only the dependence of the processing of [^{125}I]-bFGF on cell type but also on cell-surface receptors. The relationship between the kinetics of bFGF-stimulated DNA synthesis in Rama 27 cells and the processing of [^{125}I]-bFGF was examined to see if the processing of [^{125}I]-bFGF may be a candidate long-term signal. Then normal and HS-deficient Rama 27 fibroblasts were fractionated to identify whether the internalized and processed [^{125}I]-bFGF was located in the cytoplasm or the nucleus and to determine directly the dependence of the processing of [^{125}I]-bFGF on HSPG receptors and FGFRs.

6.2 Results

6.2.1 Processing of [^{125}I]-bFGF by mammary cells

6.2.1.1 Processing of [^{125}I]-bFGF by Rama 27 fibroblasts

Quiescent Rama 27 cells were incubated at 37°C in the presence of 20 ng/ml [^{125}I]-bFGF for different periods of times and then processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.1. After 30 min a substantial amount of [^{125}I]-bFGF was associated with the cells. Some of this was of lower apparent molecular weight (under 18 kDa) than the original [^{125}I]-bFGF. Later in the time course, at 2 h and 5 h, four bands are apparent, one of which has the same relative molecular weight (Mr) as the original bFGF (18,000) whilst the other three bands correspond to polypeptides that have a lower Mr; 16,000, 12,000 and 9,000 (Fig. 6.1). In the later part of the time course the bands become less intense (Fig. 6.1). After 14 h the intensity of the bands corresponding to polypeptides of Mr 12,000 and 9,000 was much weaker than the bands corresponding to polypeptides of Mr 18,000 and 16,000. A band corresponding to a polypeptide of Mr 36,000 is also apparent in each lane (Fig. 6.1). This is likely to be a dimer of bFGF that is produced in low amounts in *E coli* (Ke *et al.*, 1992).

The above experiment demonstrates the continuous processing of [^{125}I]-bFGF. The processing of [^{125}I]-bFGF was then examined in a pulse-chase experiment. [^{125}I]-bFGF (20 ng/ml) was "loaded" onto cellular receptors by incubating Rama 27 cells for 2 h at 4°C in the presence and in the absence of 2 μg/ml heparin. The cells were then washed with PBS and fresh SDM without [^{125}I]-bFGF was added. Following incubation of the Rama 27 cells for different time periods the processing of [^{125}I]-bFGF was analyzed by SDS-PAGE (Method 2.2.9.1).

In the absence of heparin, lower molecular weight bands corresponding to polypeptides of 16,000, 12,000 and 9,000 were apparent after 2 h incubation at 37°C. By 6 h the intensity of the bands had reached a maximum, and by 24 h the two lower molecular weight polypeptides were no longer detectable (Fig. 6.2a). However, upon

prolonged exposure of the autoradiograph, the band corresponding to a polypeptide of Mr 9,000 was still detectable at the 24 h time point (Fig. 6.2b). In the presence of 2 μg/ml heparin, the intensity of the bands was reduced (Fig. 6.2c), presumably because the heparin prevented the [^{125}I]-bFGF from binding to the large number of low-affinity HS receptors on the Rama 27 cells (Table 4.4). Prolonged exposure of the gel indicated that a similar pattern of processing of [^{125}I]-bFGF was occurring in the presence of heparin (Fig. 6.2d) as in the absence of heparin (Fig. 6.2a). Because of the high energy of the [^{125}I]-radiation, it is not clear whether the band corresponding to a polypeptide of Mr 16,000 (Figs 6.1 and 6.2) is due to the bFGF running as a doublet or the consequence of processing by the cells. However, the bands corresponding to polypeptides of Mr 12,000 and 9,000 are clearly the result of the processing of [^{125}I]-bFGF by the Rama 27 cells. Since at 0 h only the band of Mr 16,000 is apparent (Fig. 6.2), it would seem that the 16,000 band may not be produced by the cells processing the [^{125}I]-bFGF. Instead the band corresponding to a polypeptide of Mr 16,000 may be a consequence of the [^{125}I]-bFGF running as a doublet rather than due to the processing of the [^{125}I]-bFGF by the cells.

6.2.1.2 Exchange experiments

In an attempt to elucidate whether the time-dependence of the processing of bFGF corresponds to the result obtained for the kinetics of the stimulation of DNA synthesis by bFGF (Section 3.2.1.2), quiescent Rama 27 fibroblasts were incubated with 10 ng/ml EGF for 9 h in the presence of 2 μg/ml heparin. The cells were then washed with PBS and fresh SDM containing 20 ng/ml [^{125}I]-bFGF was added. Following incubation of the cells for 2-10 h at 37°C, the cells were prepared for SDS-PAGE (Section 2.2.9.1).

The exchange of 10 ng/ml EGF for 20 ng/ml [^{125}I]-bFGF, shows a typical result for these experiments. The signal-to-noise ratio in this experimental system was much greater than in the DNA synthesis assays, and the only clear-cut observation is that processing of [^{125}I]-bFGF does indeed occur under these conditions (Fig. 6.3).

6.2.1.3 Processing of [^{125}I]-bFGF by different mammary cells

The processing of [^{125}I]-bFGF by Rama 401 myoepithelial cells, Rama 600 and Rama 800 malignant epithelial cells was examined under the same conditions as for Rama 27 fibroblasts (Fig. 6.1).

In the case of the myoepithelial Rama 401 cells, the time course of the appearance of polypeptides of Mr 16,000, 12,000 and 9,000 (Fig. 6.4) was similar to that observed with the Rama 27 cells. By 2 h all three bands of lower relative molecular weight were apparent, and the intensity of these bands reached a maximum after incubating the cells for 5-11 h at 37°C (Fig. 6.4). By 19 h the two lower bands corresponding to polypeptides of relative molecular weight 12 kDa and 9 kDa were barely detectable.

The two malignant epithelial cell lines appeared to process [^{125}I]-bFGF somewhat differently. In the Rama 800 cells, whilst the bands corresponding to polypeptides of Mr 16,000 and 9,000 were apparent, the presence of the 12,000 polypeptide was not clear (Fig. 6.5). Moreover, the intensity of the lower molecular weight band, of Mr 9,000, was already greatly reduced by 5 h.

In contrast, by 2 h the Rama 600 malignant epithelial cells not only processed [^{125}I]-bFGF to polypeptides of Mr 16,000, 12,000 and 9,000, but the levels of these polypeptides are maintained over 11 h, and by 19 h they are still apparent (Fig. 6.6).

6.2.2 Processing of [^{125}I]-bFGF in fractionated Rama 27 fibroblasts

In order to investigate further the receptor dependence and subcellular localization of the processing [^{125}I]-bFGF by Rama 27 fibroblasts, normal and HS-deficient Rama 27 fibroblasts were incubated for 18 h with [^{125}I]-bFGF at 37°C and then fractionated into a cytoplasmic and a nuclear fraction (Section 2.2.5.2). Homogenization controls were performed to ensure that the proteolytic cleavage of [^{125}I]-bFGF and the nuclear uptake of [^{125}I]-bFGF polypeptides were the result of cellular processes occurring during the 18 h incubation period rather than the consequence of homogenization. These controls consisted of adding [^{125}I]-bFGF, at the same concentrations as in the culture medium

(30 ng/ml) to the cells in MKT buffer at 4°C prior to homogenization. The cells were then homogenized and fractionated (Section 2.2.5.2).

6.2.2.1 Nuclear fraction of Rama 27 fibroblasts

In nuclei of quiescent Rama 27 fibroblasts that were incubated for 18 h in SDM without heparin, four bands were observed, two of which were at 18,000 and 16,000 respectively whereas the other two bands exhibited lower apparent molecular weights of about 12,000 and 9,000 (Fig. 6.7). This result appears to be identical to that observed in whole cells (Figs 6.1 and 6.2) and indicates that all four bFGF-polypeptides may be recovered in the nucleus following subfractionation of the cells. When Rama 27 fibroblasts are incubated with 30 ng/ml [^{125}I]-bFGF and 30 ng/ml heparin, the pattern of [^{125}I]-polypeptides in the nuclear fraction is similar to that observed in the cells grown in the absence of heparin (Fig. 6.8 lanes 1 and 3). In HS-deficient Rama fibroblasts, two bands are observed, corresponding to polypeptides of relative molecular weight 18,000 and 16,000 (Fig. 6.7 lane 2). No lower molecular weight bands were observed, suggesting that the processing of [^{125}I]-bFGF to polypeptides of Mr 12,000 and 9,000 and translocation of these polypeptides to the nucleus is wholly dependent upon cellular HSPG receptors. In HS-deficient Rama 27 fibroblasts incubated with 30 ng/ml of [^{125}I]-bFGF and 30 ng/ml heparin, two bands are observed, corresponding to polypeptides of Mr 18,000 and 16,000 (Fig. 6.7 lane 4). These results appear to suggest that the translocation of the [^{125}I]-bFGF-derived polypeptides of Mr 12,000 and 9,000 to the nucleus is entirely dependent upon cellular HSPG receptors and cannot be restored in HS-deficient cells by the inclusion of 30 ng/ml heparin. The two homogenization controls (Fig. 6.7 lanes 5 and 6) indicate that the processing of [^{125}I]-bFGF to polypeptides of Mr 12,000 and 9,000 and the nuclear localization of these polypeptides (cf: Fig. 6.7 lanes 4 and 5,6) is not an artefact of homogenization. However, these controls demonstrate that [^{125}I]-bFGF itself is able, during homogenization, to associate with the nucleus. Moreover the presence of the 16,000

polypeptide in the controls indicates that the [^{125}I]-bFGF is running as a doublet. Indeed, when 10 μg bFGF from the same batch used to prepare the [^{125}I]-bFGF was fractionated by SDS-PAGE and the gel was fixed and stained (Section 2.2.9.2.1) two bands were apparent of Mr 18,000 and 16,000 (result not shown).

6.2.2.2 Cytoplasmic fraction of Rama 27 cells

The overall pattern of [^{125}I]-bFGF polypeptides in the cytoplasmic fraction is similar to that observed in the nuclear fraction. The cytoplasm of quiescent Rama 27 cells incubated in SDM with 30 ng/ml [^{125}I]-bFGF in the absence (Fig. 6.8, lane 1) or presence (Fig. 6.8, lane 3) of 30 ng/ml heparin contained four bFGF-polypeptides, two of which were of Mr 18,000 and 16,000 whereas two had lower apparent molecular weights of 12,000 and 9,000. The cytoplasm of HS-deficient Rama 27 fibroblasts contained just two bFGF polypeptides of Mr 18,000 and 16,000. However, in the cytoplasmic fraction of HS-deficient Rama 27 fibroblasts incubated with 30 ng/ml bFGF in the presence of 30 ng/ml heparin, four bands were observed, corresponding to polypeptides of Mr 18,000 and 16,000, 12,000 and 9,000.

The homogenization controls were identical to those of the nuclear fraction, indicating that the production of the 12,000 and 9,000 [^{125}I]-bFGF polypeptides depends on the interaction of [^{125}I]-bFGF with intact cells (Fig. 6.8 lanes 5 and 6), whilst the 16,000 polypeptide is the consequence of the [^{125}I]-bFGF running as a doublet.

6.2.2.3 Culture medium of Rama 27 fibroblasts

To see whether the processed [^{125}I]-bFGF polypeptides were released into the culture medium, or whether the processing itself occurred in the culture medium, samples of the culture medium were analyzed by SDS-PAGE. It is clear that neither the 12,000 nor the 9,000 polypeptide was present in the culture medium of Rama 27 cells or HS-deficient Rama 27 cells, whether heparin was present or not (Fig. 6.9). However, both the 18,000 and the 16,000 polypeptides were present in the culture medium.

6.3 Discussion

After binding to its cognate cellular receptors, bFGF, unlike other polypeptide growth factors and hormones is processed to lower molecular weight polypeptides rather than being degraded directly to amino acids. In the Rama 27 fibroblasts, within 2 h of exposing the cells to [^{125}I]-bFGF, polypeptides of Mr 16,000, 12,000 and 9,000 were apparent (Fig. 6.1). It seems clear that the 16,000 polypeptide is in fact a constituent of the recombinant bFGF (Figs 6.7 and 6.8) and therefore not a consequence of cellular events. However, the production of the polypeptides of 12,000 and 9,000 would appear to be due to the interaction of [^{125}I]-bFGF with the cells. The time-dependence of the production of these polypeptides in quiescent Rama 27 fibroblasts, over 19 h, is intriguingly close to the more general observations made on the kinetics of DNA synthesis (Section 3.2.1.2). Thus there is a lag before the 12,000 and 9,000 polypeptides become apparent (Fig. 6.1) and these polypeptides are still present 14 h after the initial addition of the [^{125}I]-bFGF (Fig. 6.1). This result therefore agrees with the idea that the processing of bFGF to smaller polypeptides is somehow involved in the growth-stimulatory response of cells (Bouche, 1987; Baldin *et al.,* 1990; Amalric *et al.,* 1994).

The inability of the exchange experiments to yield a clear-cut result is perhaps not surprising. The DNA synthesis assays have a signal-to-noise ratio of 10 or 20 to 1. However, when 10 ng/ml bFGF is removed from quiescent Rama 27 cells, only a 2-fold drop in the incorporation of [^{3}H]-thymidine into DNA is observed (Fig. 3.3). The analogous experiment could not be carried out with [^{125}I]-bFGF to examine the processing of bFGF, since at the end point of the DNA synthesis assays (19 h Fig. 3.2) the levels of the products of bFGF-processing were reduced. When [^{125}I]-bFGF replaced the EGF used to stimulate the quiescent Rama 27 cells for an initial 9 h period (analogous to the experiment in Fig. 3.4) it was evident that the bFGF was being processed (Fig. 6.3). Moreover the time-dependence of the processing of the [^{125}I]-

bFGF was identical to that observed when the [^{125}I]-bFGF was added first (Fig. 6.1). However, these results do not determine whether the processing of bFGF is part of the mechanism whereby bFGF engenders a growth-stimulatory response.

The Rama 401 myoepithelial cells are similar to the Rama 27 fibroblasts in terms of their complement of receptors for bFGF and their growth-stimulatory response to bFGF (Table 4.4; Fernig *et al.*, 1992). Therefore it is not surprising that bFGF is processed by Rama 401 myoepithelial cells in a similar manner to that observed with Rama 27 fibroblasts (Figs. 6.1 and 6.4).

The malignant Rama 600 cells possess both low-affinity HSPG and high-affinity receptors for bFGF (Fernig *et al.*, 1993) and these cells are only weakly malignant (Table 2.1). Despite possessing a similar complement of receptors for bFGF to the myoepithelial 401 cells, the Rama 600 cells appear to produce the 12,000 and 9,000 polypeptides for a longer period of time (Fig. 6 6). This may be the result of the Rama 600 cells progressing through the cell cycle in a more random manner, which could either be due to difference in cell cycle timing or to the difficulty in rendering epithelial cells quiescent (Fernig *et al.*, 1990b; Smith *et al.*, 1994).

The Rama 800 cells are moderately malignant and possess high- but not low-affinity receptors for bFGF (Fernig *et al.*,1993). Moreover, the Rama 800 cells produce very little cell-associated HS (Table 5.4). The levels of the processed [^{125}I]-bFGF polypeptides are somewhat lower in the Rama 800 cells than in the other three mammary cell lines (Figs 6.1, 6.3 - 6.5). This may be the result of the absence of low-affinity HSPGs receptors on the Rama 800 cells. The lower level of production of [^{125}I]-bFGF polypeptides of Mr 12,000 and 9,000 may therefore simply be the consequence of the lower level of [^{125}I]-bFGF associated with the Rama 800 cells.

The cellular subfractionation studies show that 18,000 [^{125}I]-bFGF is able to associate with the cell nucleus during homogenization (Fig. 6.7). This result indicates that the observation of translocation of 18,000 bFGF from the cells' exterior to the cell nucleus (Amalric *et al.*, 1994; Baldin *et al.*, 1990; Hawker and Granger, 1992; Presta

et al., 1993) should be treated with caution. However, it is clear that the bFGF-derived polypeptides of Mr 12,000 and 9,000 are only found in the nucleus of the cells that possess HSPG receptors for bFGF and which have been incubated with [^{125}I]-bFGF (Fig. 6.7). The processing of [^{125}I]-bFGF to polypeptides of 12,000 and 9,000 is apparent in cells possessing HSPG receptors for bFGF and in HS-deficient cells incubated with both [^{125}I]-bFGF and 30 ng/ml heparin (Fig. 6.8). The absence of the 12,000 and 9,000 polypeptides in the nucleus of HS-deficient cells incubated with [^{125}I]-bFGF and 30 ng/ml heparin suggests the following relationships:

(1) Processing occurs in cytoplasmic compartments of the cells and is entirely dependent upon the presence of HSPG receptors; soluble polysaccharide can replace cell-surface HSPG receptors.

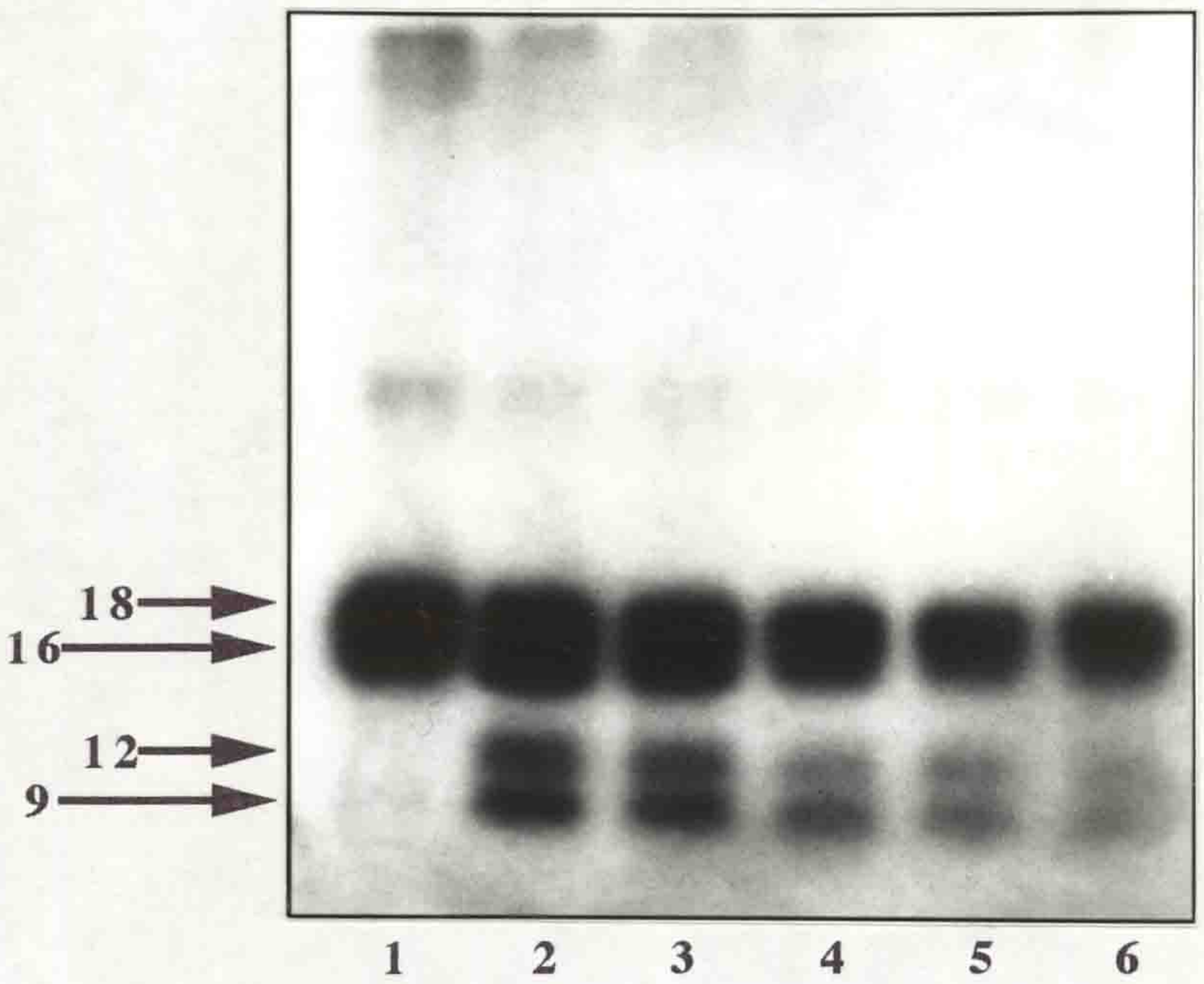
(2) The subsequent translocation of the 12,000 and 9,000 polypeptides to the nucleus only occurs if cellular HSPG receptors are present; soluble polysaccharide cannot replace cell-surface HSPG receptors.

These results raise a number of exciting possibilities as to the role of the post-receptor processing of bFGF, but do not supply a causal relationship between the processing of bFGF and the growth-stimulatory response of the cells.

The occurrence of the post-receptor processing of bFGF would appear to occur in all cell types (Amalric *et al.*, 1994; Baldin *et al.*, 1990; Hawker and Granger, 1992; Presta *et al.*, 1993), including those of the mammary gland (Figs 6.1, 6.4, 6.5 and 6.6), that can respond to bFGF. The production of bFGF-derived polypeptides is sustained over 14 h or more hours and is thus consistent with the requirement of the cells for long-term stimulation by bFGF if they are to divide. The translocation of the 12,000 and 9,000 polypeptides to the nucleus suggests that they could have a direct function there. Indeed, immunofluorescence studies on endothelial cells have suggested that bFGF immunoreactivity accumulates in the nucleoli, the site of ribosome assembly, an essential step in the cell cycle (Bouche *et al.*, 1987). The presence of the 12,000 and 9,000 polypeptides in the cell cytoplasm correlates with the ability of the cells to respond efficiently to bFGF (Figs 4.4, 4.7, 6.8 and 6.9). Moreover, the absence of the 12,000

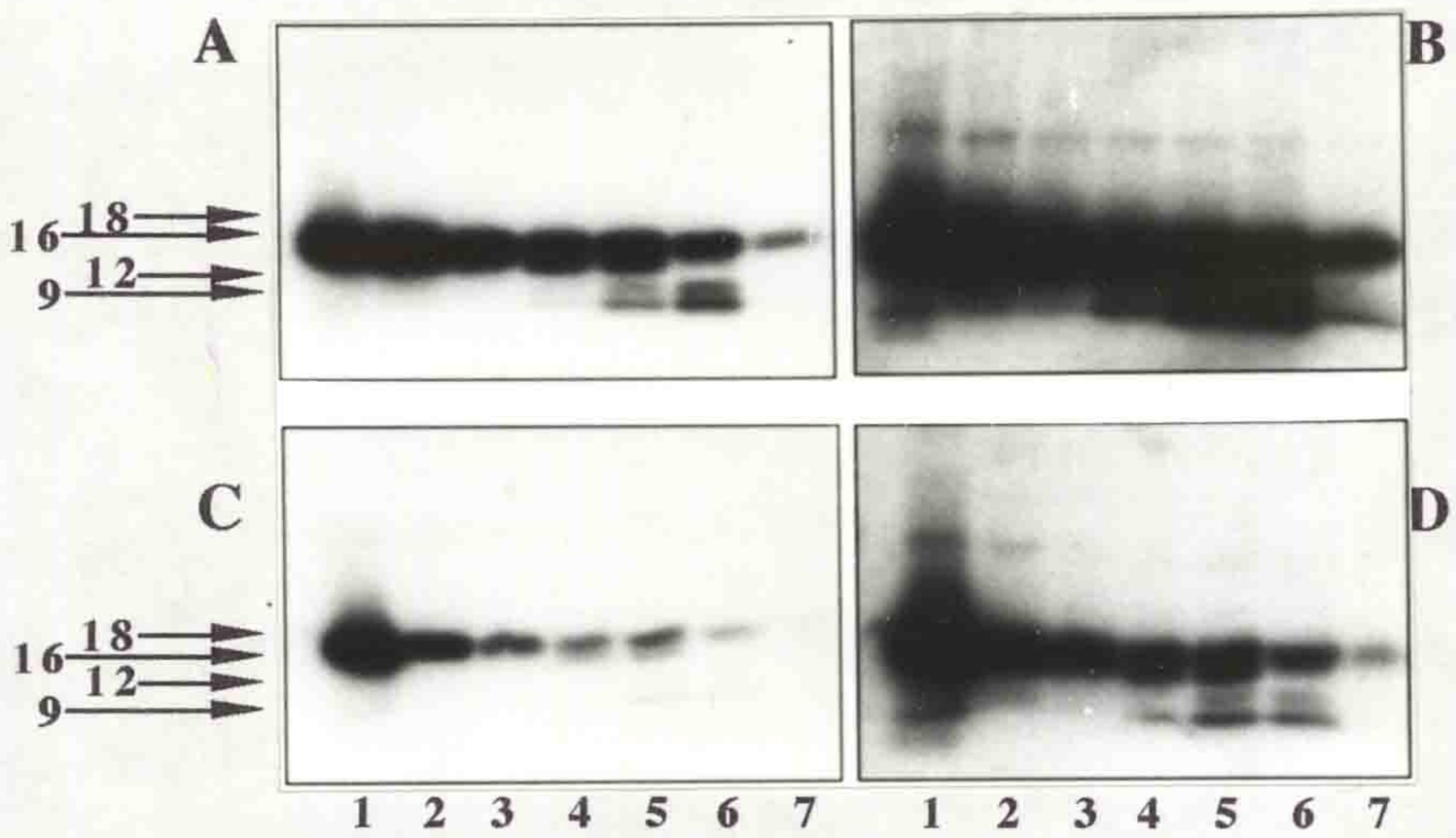
and 9,000 polypeptides from the nucleus in HS-deficient cells incubated with both [^{125}I]-bFGF and 30 ng/ml heparin (Fig. 6.7) correlates with the 10-fold loss of potency of bFGF (Fig. 4.7). Therefore, under different experimental conditions, the processing of bFGF and the subsequent translocation of the 12,000 and 9,000 polypeptides to the nucleus correlates with the potency of the bFGF in growth assays.

Fig. 6.1 Processing of [^{125}I]-bFGF by Rama 27 fibroblasts



Quiescent Rama 27 cells were incubated in SDM in the presence of [^{125}I]-bFGF (20 ng/ml) for different periods of times and then processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.
Lane 1, 30 minutes; lane 2, 2 h; lane 3, 5 h; lane 4, 11 h; lane 5, 14 h; lane 6, 19 h.
Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6. 2 Pulse-chase of [^{125}I]-bFGF processing by Rama 27 fibroblasts



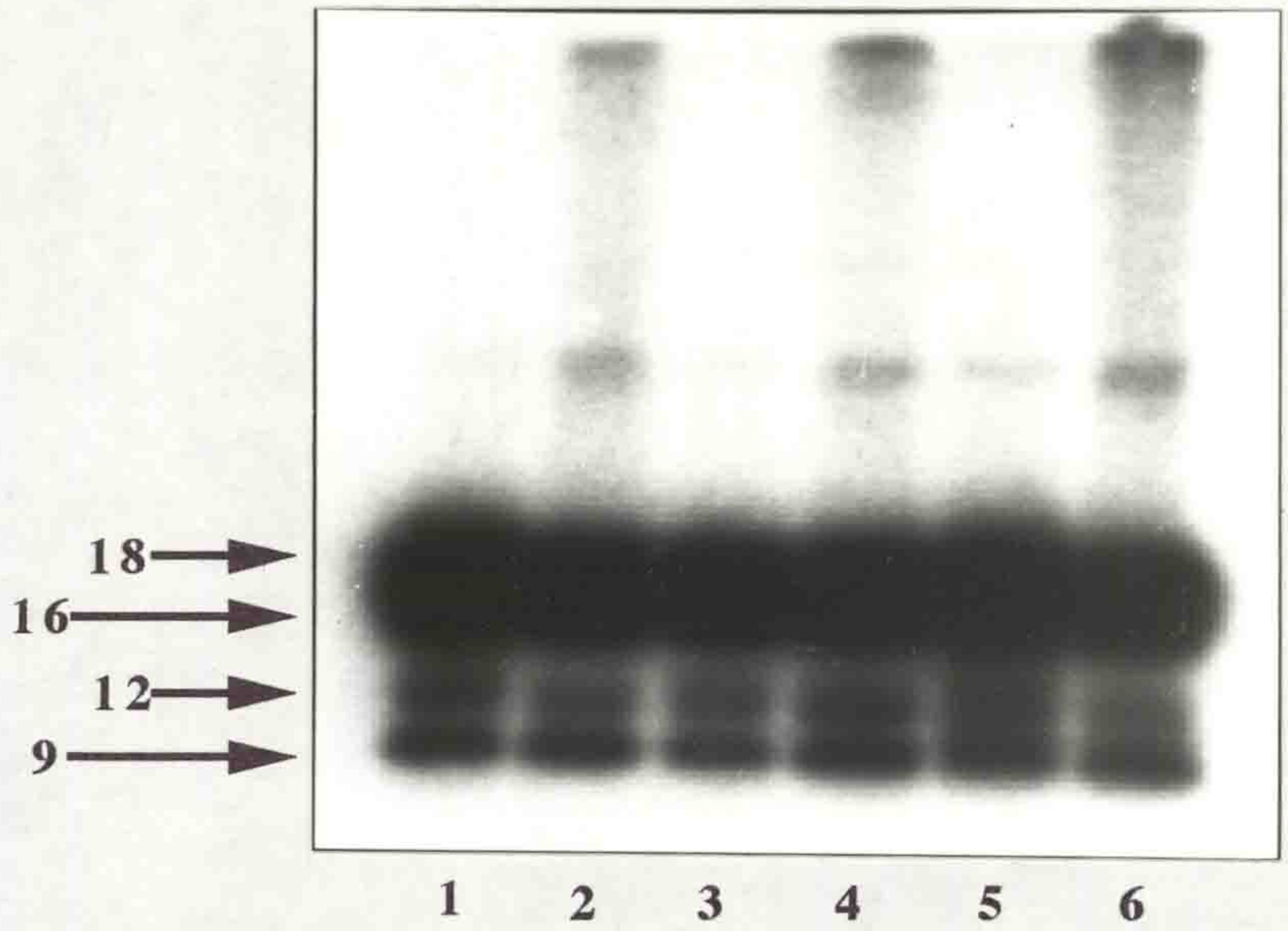
Quiescent Rama 27 cells were incubated in the presence of [^{125}I]-bFGF (20 ng/ml) for 2 h at 4°C with or without 2 μg/ml heparin and then the cells were washed and fresh SDM was added to the cells. Following incubation at 37°C for different times the cells were processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.

(a) No heparin, short exposure of autoradiograph; (b) No heparin, long exposure of autoradiograph; (c) 2 μg/ml heparin, short exposure of autoradiograph; (d) 2 μg/ml heparin, long exposure of autoradiograph.

Lane 1, 5 μg [^{125}I]-bFGF in sample buffer for control; Lane 2, 0, lane 3, 20 min; lane 4, 1 h; lane 5, 2 h; lane 6, 6 h; lane 7, 24 h.

Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6. 3 Processing of [^{125}I]-bFGF by quiescent Rama 27 cells following a 9 h stimulation with EGF

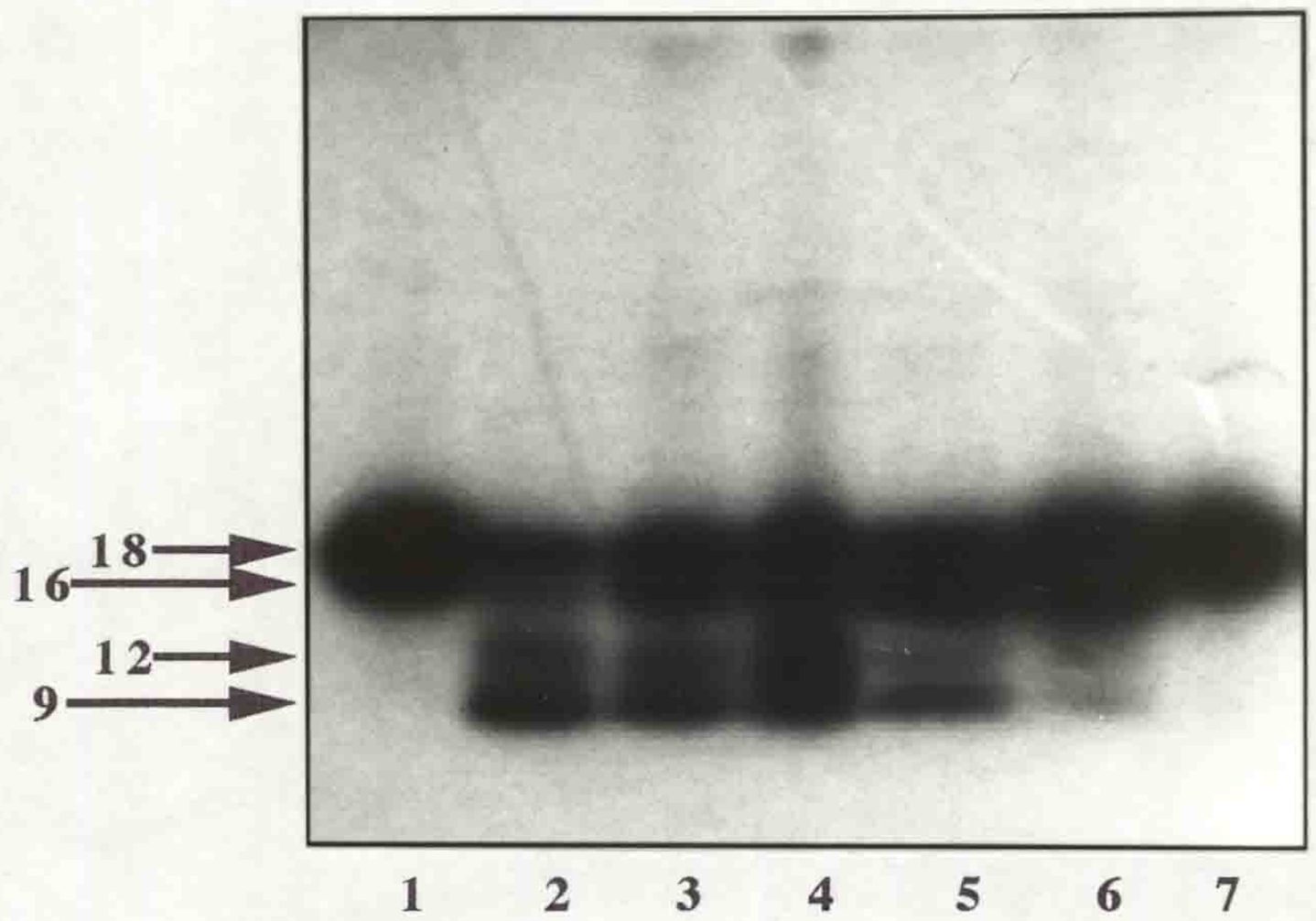


Quiescent Rama 27 cells were incubated in the presence of 10 ng/ml EGF for 9 h, and then the cells were washed twice with PBS and fresh SDM was added containing 20 ng/ml [^{125}I]-bFGF with 2 μg/ml heparin. The cells were then collected at different times following a further incubation at 37°C and processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.

Lane 1 and 2, 2 h; lane 3 and 4, 5 h; lane 5 and 6, 10 h. Lanes 2, 4, and 6 samples with cross-linker DSS.

Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6.4 Processing of [^{125}I]-bFGF by Rama 401 myoepithelial cells

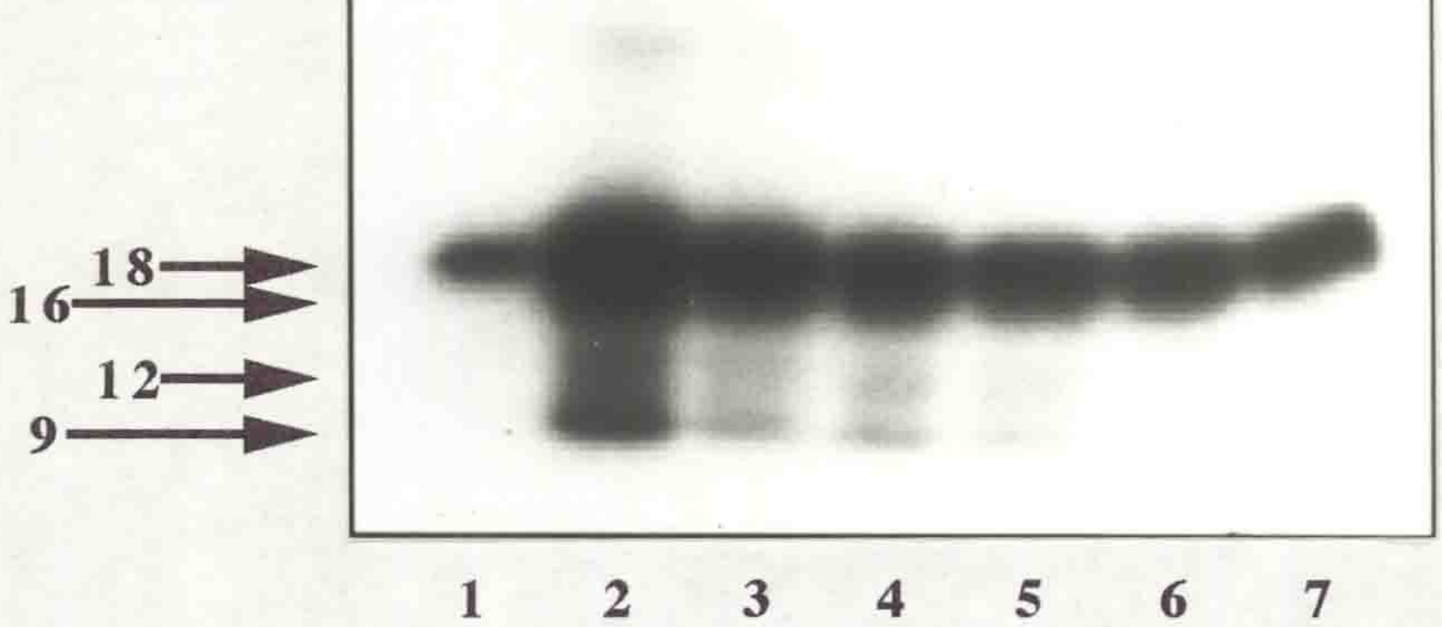


Quiescent Rama 401 cells were incubated in SDM in the presence of [^{125}I]-bFGF (20 ng/ml) for different times and then processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.

Lane 1, 30 min; lane 2, 2 h; lane 3, 5 h; lane 4, 11 h; lane 5, 14 h; lane 6, 19 h; lane 7, 0 min.

Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6.5 Processing of [^{125}I]-bFGF by Rama 800 malignant epithelial cells

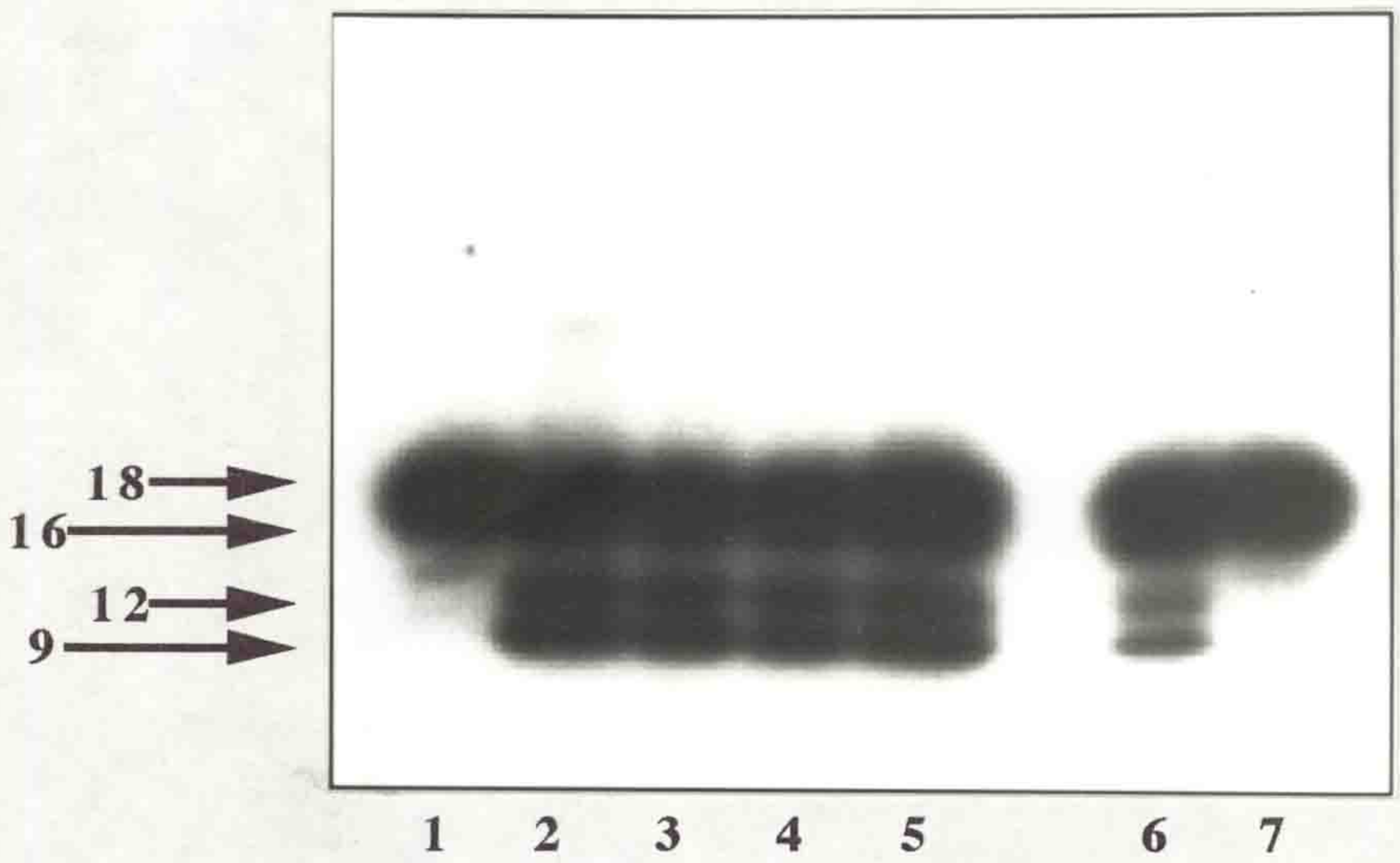


Quiescent Rama 800 cells were incubated in SDM in the presence of [^{125}I]-bFGF (20 ng/ml) for different times and then processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.

Lane 1, 30 min; lane 2, 2 h; lane 3, 5 h; lane 4, 11 h; lane 5, 14 h; lane 6, 19 h; lane 7, 0 min.

Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6.6 Processing of [^{125}I]-bFGF by Rama 600 malignant epithelial cells

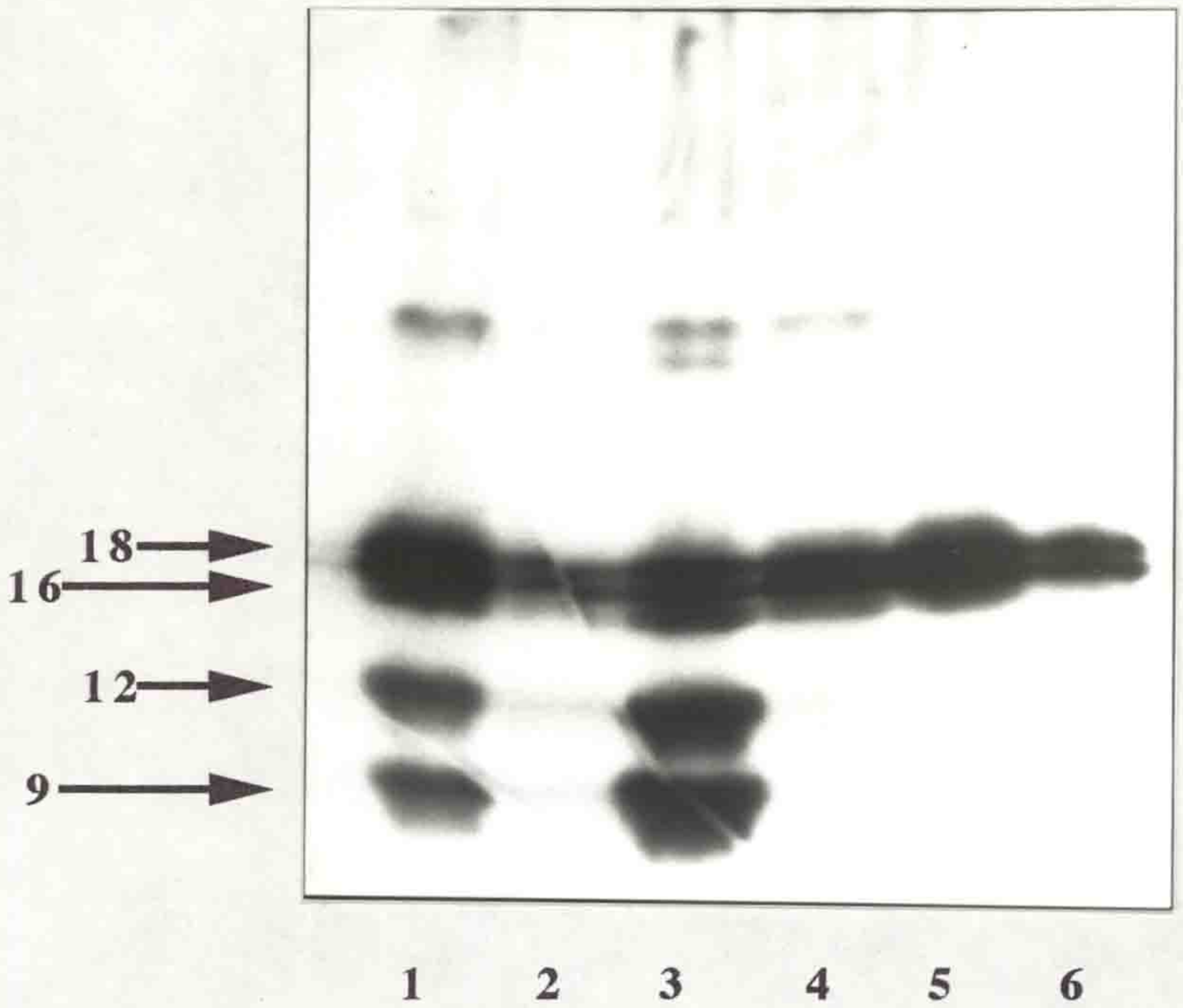


Quiescent Rama 600 cells were incubated in the presence of [^{125}I]-bFGF 20 ng/ml) for different periods of times and then processed for SDS-PAGE according to Methods 2.2.5.1 and 2.2.9.

Lane 1, 30 min; lane 2, 2 h; lane 3, 5 h; lane 4, 11 h; lane 5, 14 h; lane 6, 19 h; lane 7, 0 min.

Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6. 7 [^{125}I]-bFGF polypeptides in the nuclear fraction of Rama 27 fibroblasts



Quiescent Rama 27 cells, in some cases HS-deficient, were incubated in the presence of [^{125}I]-bFGF (30 ng/ml) in SDM or S-free SDM, respectively, with or without 30 ng/ml heparin for 18 h at 37°C. The nuclear fraction was prepared as described in Method 2.2.5.2 and then processed for SDS-PAGE according to Method 2.2.9. The culture conditions of the cells were as follows:

Lane 1, SDM, no heparin;

lane 2, S-free SDM, no heparin;

lane 3, SDM, 30 ng/ml heparin;

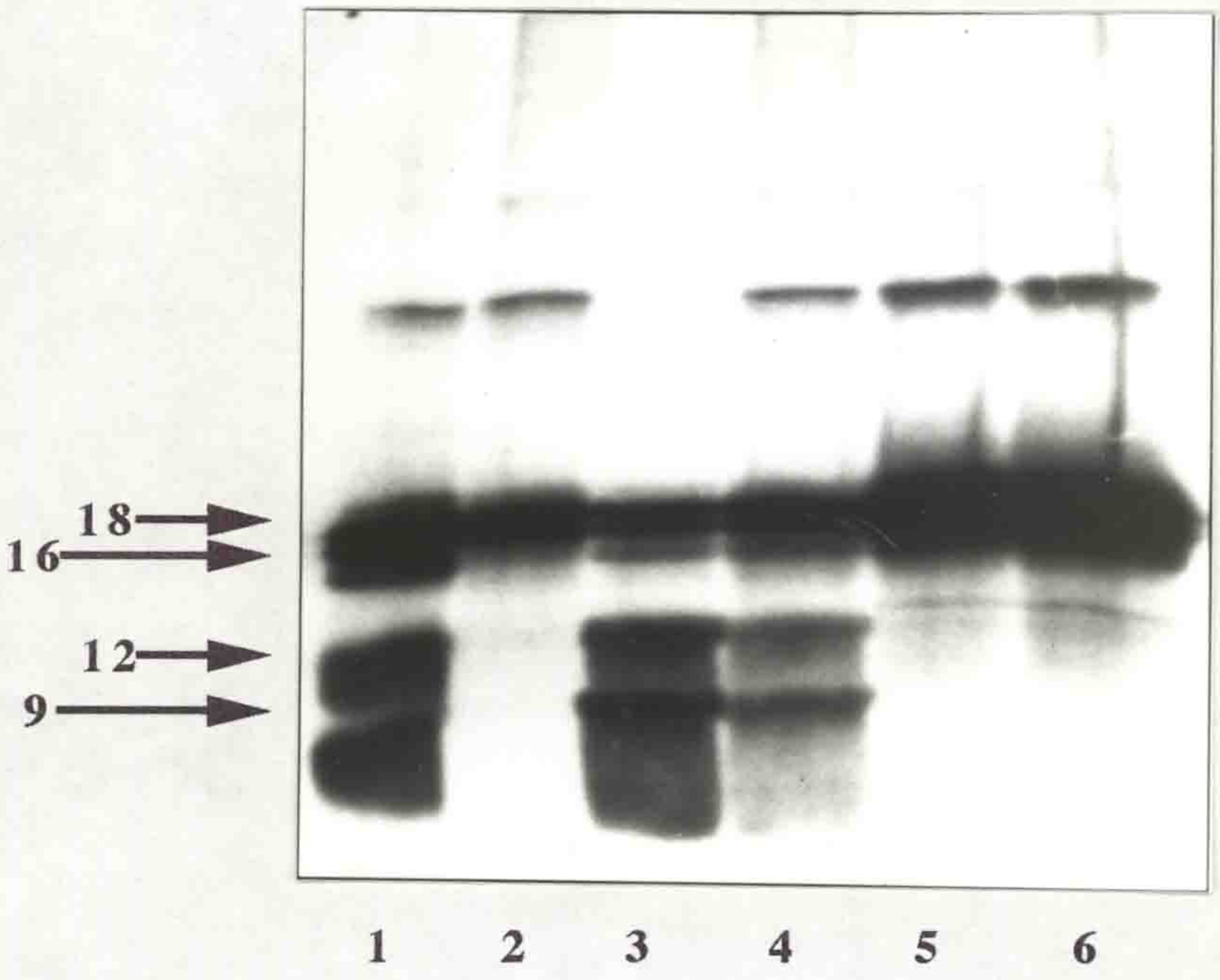
lane 4, S-free SDM, 30 ng/ml heparin;

lane 5, SDM, homogenization control;

lane 6, S-free SDM, homogenization control.

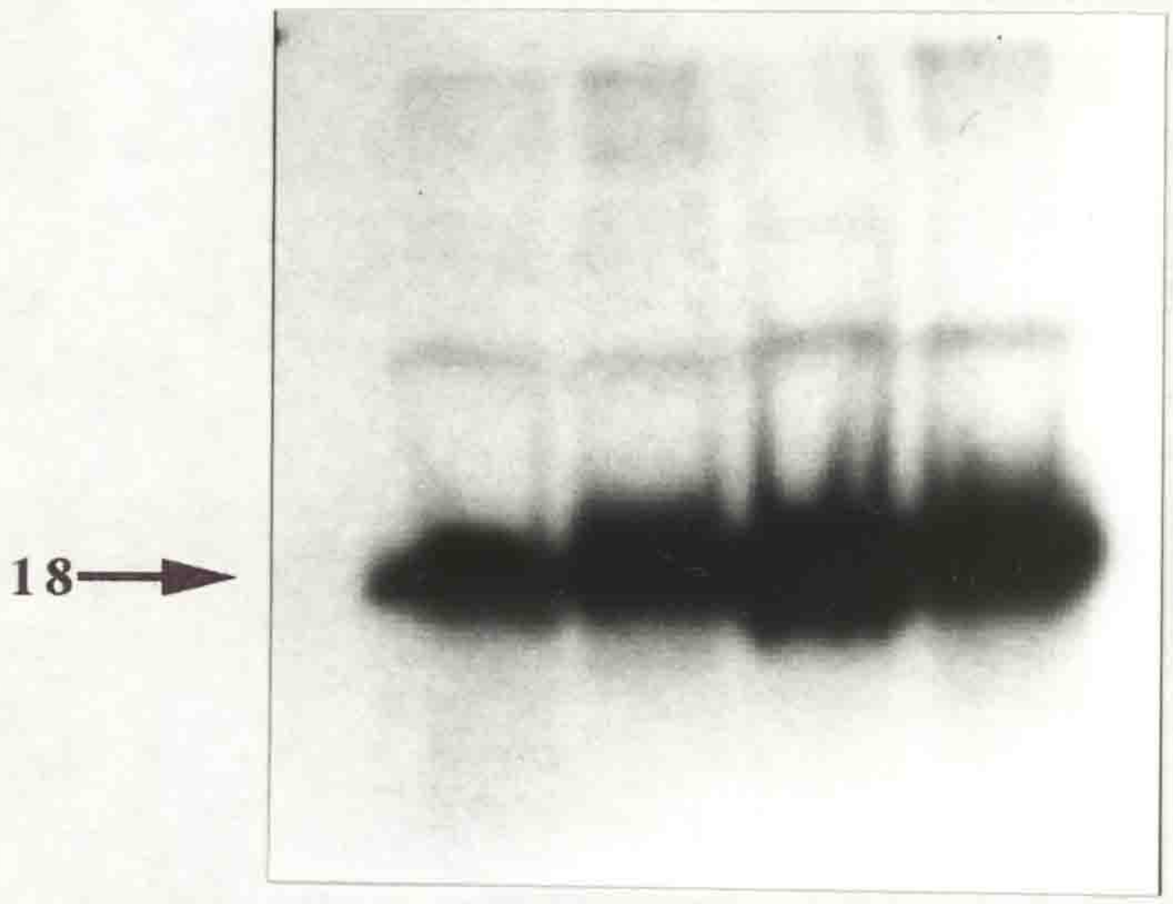
Arrows represent the Mr (x 10^3) of the bands, calculated from standards.

Fig. 6. 8 Processing of [^{125}I]-bFGF by cytoplasmic fraction of Rama 27 fibroblasts



Quiescent Rama 27 cells, in some cases HS-deficient, were incubated in the presence of [^{125}I]-bFGF (20 ng/ml) in SDM or S-free SDM respectively with or without 30 ng/ml heparin. The cytoplasmic fraction was prepared as described as Method 2.2.5.2 and then processed for SDS-PAGE according to Method 2.2.9. The culture conditions of the cells were as follows:
Lane 1, SDM, no heparin;
lane 2, S-free SDM, no heparin;
lane 3, SDM, 30 ng/ml heparin;
lane 4, S-free SDM, 30 ng/ml heparin;
lane 5, SDM, homogenization control;
lane 6, S-free SDM, homogenization control.
Arrows represent the Mr (x 10^3) of the bands, calculated from standards

Fig. 6. 9 [^{125}I]-bFGF polypeptides in the culture medium fraction of Rama 27 fibroblasts



Quiescent Rama 27 cells, in some cases HS-deficient, were incubated in the presence of [^{125}I]-bFGF (30 ng/ml) in SDM or S-free SDM respectively, with or without 30 ng/ml heparin. A sample of the cells culture medium was processed for SDS-PAGE according to Method 2.2.9. The culture conditions of the cells were as follows:

Lane 1, SDM, no heparin;

lane 2, S-free SDM, no heparin;

lane 3, SDM, 30 ng/ml heparin;

lane 4, S-free SDM, 30 ng/ml heparin;

Arrows represent the Mr (x 10^3) of the bands, calculated from standards

Chapter Seven

General Discussion and Conclusion

Chapter Seven
General Discussion and Conclusion

The regulation of tissue growth and development is controlled to a large extent by growth factors and the FGFs in particular appear to be involved in many developmental events that involve an epithelium and an underlying mesenchyme (Section 1.2.1.1). An understanding of how development is controlled will require the elucidation of the molecular mechanisms that underlie the actions of growth factors. The work described in this thesis has concentrated on one member of a family of growth factors, bFGF, and its interactions with a variety of mammary cells to define some of the properties of the mechanism(s) of action of the FGFs.

The Rama 27 fibroblasts have been used as a model to study the interactions of two FGFs, aFGF and bFGF, with mammary cells. Rama 27 fibroblasts are representative of a cell type found in the stromal compartment of the breast. These cells are known to express bFGF and its high- and low-affinity receptors (Sections 1.2.5.3 and 4.2.8). In addition Rama 27 cells and myoepithelial cells produce mRNA encoding FGFR-1 (Ke *et al.*, 1993; Barraclough and Fernig, unpublished observations). Unlike the myoepithelial cells and intermediate cells (Section 1.2.5.2) the Rama 27 fibroblasts can readily be rendered quiescent. Thus the Rama 27 fibroblasts provide a robust, though only approximate, model for the myoepithelial and intermediate cells of the mammary parenchyma.

Rama 27 cells require both the FGFR high-affinity receptor and the HSPG low-affinity receptor to mount an efficient growth-stimulatory response to aFGF or bFGF. In the absence of the HSPG receptor bFGF is a growth factor of low potency, whilst aFGF is unable to stimulate cell growth (Section 4.2.6). The cellular HSPG receptors may be replaced by soluble heparin, although only at the cost of a loss of potency of the FGFs. This suggests that although FGFs bind to the HS chains of HSPGs, soluble HS chains are not the equivalent of HSPGs and that the core protein of the proteoglycans

may have a direct role in the dual receptor system of FGFs (Section 4.3). Like heparin, HS chains isolated from cells representative of some of the different cells found in the normal mammary gland and malignant mammary tumours are able to replace cellular HSPG receptors. However, the relationship between the bFGF-binding properties of a mammary cell line and the ability of HS isolated from such cells to participate in the dual receptor system do not always correlate (Chapter 5). This result suggests that these two functions of the HSPG receptors may be accounted for by different structures within the HS chains. Whilst the above results can be accommodated by existing models of the dual receptor system (Section 1.2.3.2), the ability of bFGF to interact efficiently with its high-affinity receptors in the absence of HSPG receptors (Section 4.2.8) argues strongly that the existing models are incorrect (Section 4.3). Before alternative models can be considered, certain conditions must be met if the interactions of FGFs with the dual receptor system are to lead to cell division.

Quiescent Rama 27 cells require a prolonged exposure to growth factors if they are to replicate their genome (Sections 3.2.1.2 and 3.2.2). These observations are supported by a large, though disparate, body of evidence obtained in a variety of biological systems, e.g., *Xenopus* oocytes (Reid and Reid, 1987); 3T3 fibroblasts (Chana and Smith, 1991; Rudland and Jiminez de Asua, 1979); endothelial cells (Presta *et al.*, 1991b). The half-life of the signal(s) that cause cell division that are generated by bFGF and EGF are of the order of 1-2 h and there would appear to be an equivalence between the signals generated by EGF and bFGF since these two growth factors may be interchanged during the G_0-S-phase lag period (Section 3.2.3). However, this equivalence is not exact since during the G_0-S-phase lag period the cells exhibit a difference in sensitivity towards EGF and bFGF (Section 3.2.4 and 3.2.5). The constraints placed on the signal(s) generated by bFGF and EGF that lead to cell division (Section 3.3) are as follows:

(i) A half-life of 1-2 h (Fig. 3.7);

(ii) Continuous production of the signal is necessary for DNA synthesis to occur (Fig. 3.3);

(iii) Equivalence between the signals produced by bFGF and EGF (Fig. 3.4);

(iv) Difference in the sensitivity of the signal(s) between 0-9 h and 9-18 h to growth factor concentration.

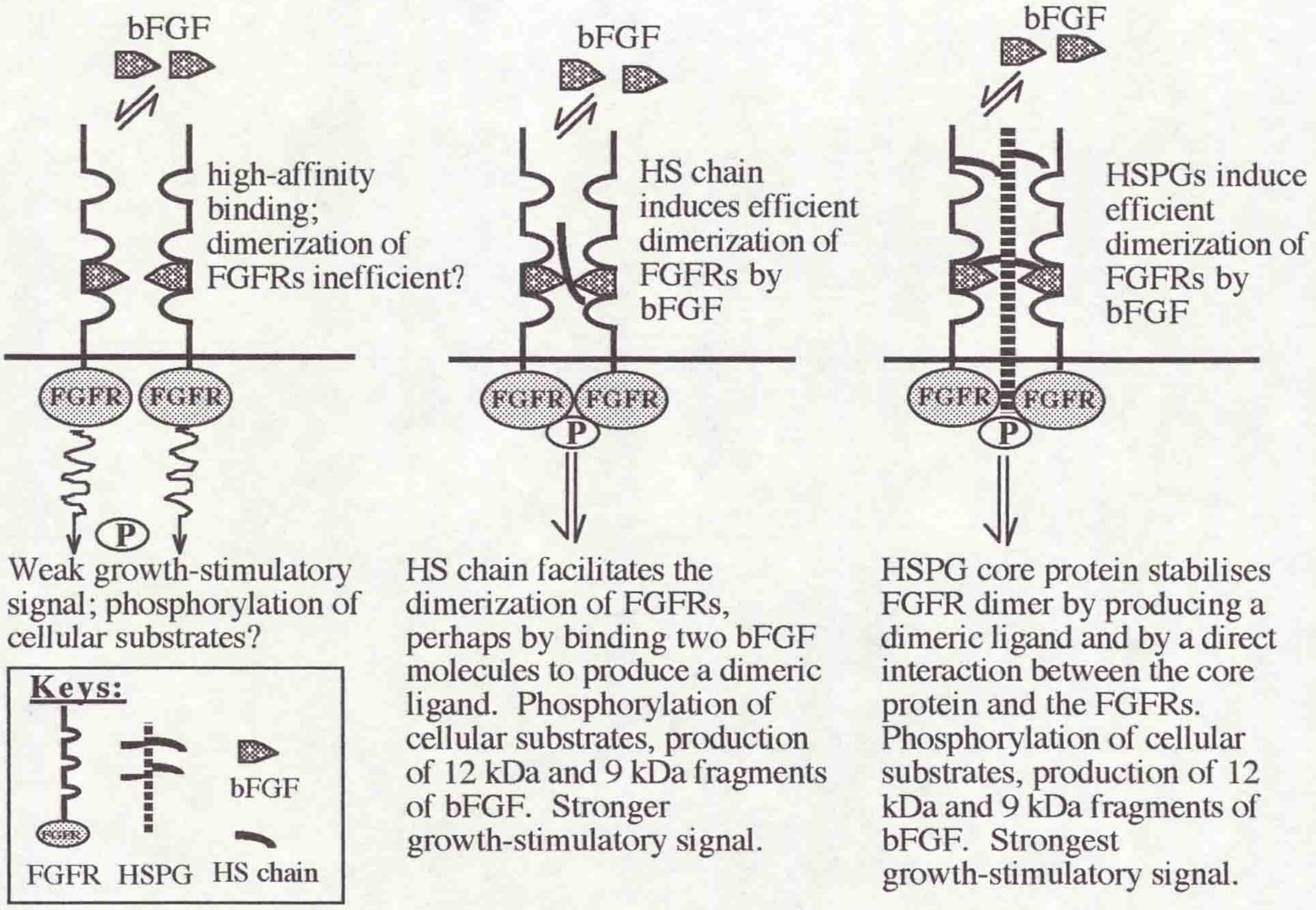
Taken together, the requirement for a dual receptor system and the constraints imposed on this receptor system as far as the generation of a growth-stimulatory response is concerned enable a preliminary answer to the question: "What signal(s) generated by the interaction of FGFs with their receptors on mammary cells lead to cell division?". Of the numerous events stimulated by the FGFs (Sections 1.2.2.2.3 and 1.2.3.3) two satisfy the requirements outlined above: the activation of the tyrosine kinase of the intracellular domain of the FGFRs by the FGF ligand (Section 1.2.2.2.3); the post-receptor processing of FGFs and the translocation to the nucleus of the FGFs and their fragments (Section 1 2.3.3).

The processing of bFGF to relatively large polypeptides occurs in a variety of mammary cells, all of which possess a functional dual receptor system. Although the time course of the processing of bFGF is consistent with the requirements described in Chapter 3, the signal-to-noise ratio is insufficient to make more than a qualitative judgement (Section 6.3). However, the subcellular fractionation studies show clearly that the processing of [^{125}I]-bFGF to 12,000 and 9,000 polypeptides only occurs in cells that possess a functional dual receptor system for FGFs and that are therefore capable of mounting a growth-stimulatory response to bFGF (Section 6.2.2). However, the translocation of these fragments to the nucleus does not correlate strictly with the growth-stimulatory response of the cells. Thus whilst the production of polypeptide fragments of bFGF in the cell cytoplasm may be important in itself, the translocation of such fragments in detectable quantities in the cell nucleus may be less important.

A simple model, which accounts for the above observations is as follows. A cell that possesses only FGFRs can bind bFGF with high affinity, but the bFGF-FGFR complex only produces growth stimulatory signals inefficiently (State 1 in Fig. 7.1). This may be the consequence of the inability of bFGF-FGFR complexes to dimerize rapidly or stably. A cell that possess a full dual receptor system binds bFGF with high affinity. The

receptor-ligand complex probably comprises, at the very least, two bFGFs bound to the HSPG and the FGFR, perhaps as depicted in Fig. 7.1 State 3. The presence of the HSPG may cause the dimerization of the bFGF, but this cannot be the only function of the HSPG receptor since soluble heparin cannot fully replace the cellular receptor. Thus the core protein of the HSPG may stabilise the formation of FGFR-FGFR dimers (Fig. 7.1). A stable HSPG-bFGF-FGFR complex may then be able to generate two types of long term signal: the continuous phosphorylation of cellular substrates by the FGFR kinase; the production of large polypeptide fragments of bFGF, which are translocated into the cytoplasm. A possible role of such fragments may be to relieve the inhibition of a latent signalling complex in the cell cytoplasm.

Fig. 7.1 Model of the dual receptor system for bFGF



Future Work

The issues raised in the preceding section point to three major questions that must be answered if the interaction of FGFs with mammary cells is to be understood:

(i) Is the tyrosine kinase activity of the FGFR part of the prolonged signal that is required if a cell is to divide?.

(ii) Which structures in the HS chains participate in the dual receptor system and are these the same as the ones that account of the low-affinity receptor function?

(iii) Which structures in FGFs, FGFRs and HSPGs actually interact at the molecular level in the dual receptor system?

The first question could be answered using antibodies to phosphotyrosine. These could be used to follow, first by immunoblotting, the activity of the FGFR tyrosine kinase between G_0- and S-phase DNA synthesis. Then a more precise measurement of the activity of this enzyme could be made by immunoprecipitating the FGFRs from cells between G_0- and S-phase DNA synthesis and measuring the activity of the enzyme directly in the immunoprecipitates.

Whilst it is relatively easy to define which FGF and which FGFR is present in an experimental system, no experimental systems use a chemically-defined HSPG receptor. One way to approach this problem is to use bFGF-binding assays, performed *in vitro*, and bFGF-activation assays performed with HS-deficient cells to isolate the minimum fragments in HS that are capable of replacing cellular HSPG receptors. A physiologically relevant source of HS would be the rat mammary cell lines representative of mammary development and cancer (Table 2.1). Once such structures are isolated, it is then possible to identify the structures in FGFs, FGFRs and HSPGs that participate at the molecular level in the dual receptor system. This could be approached with the assays used to isolate the defined fragments of HS, but by adding two new variables: mutant recombinant FGFs and transfection into HS-deficient cells of cDNAs encoding mutant FGFRs.

The definition of some of the above structures then allows structure-specific reagents,

e.g., monoclonal antibodies, to be used to examine the occurrence of such structures *in vivo*, and hence to predict whether the dual receptor system is in fact active during a particular phase of development of a tissue such as the mammary gland.

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