Fibroblast (Heparin-Binding) Growing Factors in Neuronal Development and Repair

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Contents

Abstract

Nearly thirty growth and trophic factors that have been purified from mammalian tissues in the last 15 yr have been found to share chemical identity. The results of their chemical purification and molecular cloning show that they are two distinct polypeptides (M_r 17,400 and 18,400), each of which gives rise to families of smaller size peptides. These peptides share a common affinity for heparin. In view of this property, a common nomenclature for the two principle peptide growth factors (heparin-binding growth factor classes 1 and 2; HBGF-1 and -2) has been proposed. However, the names acidic and basic Fibroblast Growth Factors (aFGF,bFGF), which were applied to them originally to describe their mitogenic activity, are more commonly in use and will therefore be adopted in this review. Brain tissue is one of the richest sources of FGFs. It has been used as a starting point for their chemical purification and to prepare genomic libraries for molecular cloning of the aFGF and bFGF genes. There is increasing evidence that these growth factors, expressed in neurons and glia throughout the mammalian nervous system, are implicated in neuronal cell proliferation, differentiation, and histiogenesis. FGFs have a strong affinity not only for heparin, but also for the related heparan sulphate proteoglycans that are abundant in neural tissues. This fact provides a clue to the importance of tissue-associated proteoglycans in mediating the release, sequestration, and activation of FGFs and the modulation of their receptor binding and bioactivity. The relevance of FGFs to neural development and their mechanisms of action in neurons will be considered in light of the existing literature describing their biological properties and activity in mesodermal cell types. Evidence is reviewed showing that FGFs have in vivo biological activity, ameliorating the degeneration of central and peripheral neurons after axotomy. The presence and implications of high levels of FGFs in adult mammalian brain provides a direction for future research into neural regeneration. The bioactivity of FGFs in neural tissue may not depend on the regulation of their expression *per se*, but on the subregional modification of their interaction with proteoglycans.

Index Entries: Heparin-binding growth factors; Fibroblast Growth Factors; neuronotrophic factors; glial cells; mitogens; messenger RNA.

Introduction

The isolation and complete chemical characterization of two ubiquitous, multifunctional mitogenic growth factors from bovine brain has been the culmination of five decades of biochemical research. The acidic and basic FGF polypeptides are chemically identical in most respects to growth factors that have been prepared from a variety of mammalian peripheral tissues and cell lines, all of which are now considered to be synonymous with them (Tables 1 and 2). Although first purified by conventional liquid chromatographic techniques, the FGFs were subsequently prepared by exploiting their high affinity for the proteoglycan, heparin. This characteristic is definitive for similar factors from all other tissue sources.

The FGFs not only show a chemical affinity for proteoglycans, but also depend on them for optimal biological activity. In this respect, they bear similarities with substratum-binding neural growth factors (Berg, 1984) that are also present in brain tissue and, like FGFs, stimulate neurite outgrowth and the expression of cell-specific markers in neurons from a variety of regions of the nervous system. These properties are in contrast to those of Nerve Growth Factor, which is believed to be trophic only toward nerve cells in the periphery and brain that are neural crest-derived. Substratum-binding growth factors with neuronotrophic activity have molecular sizes in the range 15,000–37,000 dalton (Da) under reducing conditions. Recent

Table 1
Tissues and Cells with Detectable Levels of bFGF[a]

Normal or Transformed Tissue	Cultured Normal Diploid Cells	Cultured Tumor Cells
Brain	Corneal endothelial cells	Y-1 adrenal cortical cells
Pituitary	Capillary endothelial cells	Osteosarcoma U 20 S
Kidney	Pituitary follicle cells	Ewing's Sarcoma
Placenta	Ovarian granulosa cells	Rhabdomyosarcoma
Corpus luteum	Adrenal cortex cells	Melanoma
Adrenal glands	Lens epithelial cells	Hepatoma (Sk HP-21)
Immune system	Uterine epithelial cells	Retinoblastoma
Macrophage–monocyte	Myoblasts	
Prostate	Retinal pigmented epithelial cells	
Retina		
Bone		
Cartilage		
Chondrosarcoma		
Melanoma		
Liver		

[a]aFGF has been detected in brain, retina, kidney, bone matrix (osteoblasts), and osteosarcoma. Adapted from Gospodarowicz et al. (1987b).

Table 2
Alternative Nomenclature for FGF Polypeptides[a]

aFGF	bFGF
Heparin-binding growth factor 1 (HBGF-1)	Heparin-binding growth factor 2 (HBGF-2)
Embryonic kidney-derived angiogenesis Factor 1	Embryonic kidney-derived angiogenesis Factor 2
Endothelial cell growth factor	Prostatic growth factor
Endothelial growth factor	Endothelial growth factor
Astroglial growth factor 1	Astroglial growth factor 2
Retina-derived growth factor	Macrophage growth factor
Prostatropin	Tumor angiogenesis factor
Eye-derived growth factor 1	Eye-derived growth factor 2
	Hepatoma growth factor
	Chondrosarcoma growth factor
	Myogenic growth factor
	Human placenta-purified factor
	Uterine-derived growth factor
	Embryonic carcinoma-derived growth factor
	Human pituitary growth factor
	Pituitary-derived chondrocyte growth factor
	Adipocyte growth factor
	Prostatic osteoblastic factor
	Mammary tumor-derived growth factor

[a]Adapted from Gospodarowicz et al. (1987b).

work in the characterization of neuro(glio)trophic activity from the brain shows that a considerable proportion of it can be attributed to polypeptides identical to the FGF family (Pettmann et al., 1985). However, other brain-derived growth factors, such as p30 (Rauvala and Pihlaskari, 1987) and chick retinal neurotrophic factor (Schubert et al., 1986), which also bind to heparin, exist and appear to differ from the FGFs on the basis of molecular size.

There is a large body of evidence that documents the biological profile of the FGFs, and readers are directed to several excellent, general reviews on the background to this article (Baird et al., 1986; Gospodarowicz et al., 1986b, 1987a,b; Thomas, 1987a). In the last 3 yr, the relevance of the FGFs to neural development has become apparent. Earlier work indicates that neuroglia are among the many cell types for which FGFs are mitogenic. More recent studies suggest that FGFs may play a physiological role in regulating aspects of neuronal development including induction, differentiation, and histiogenesis (Morrison, 1987). The work is reviewed in light of progress in the elucidation of the biosynthesis, structure, and cellular response to the FGFs in nervous tissue. The implications of the findings for regeneration in the nervous system will be discussed.

Isolation and Biochemical Characterization

The FGFs have been isolated and purified from a number of tissues that share, in common, a rich vascularization. The presence of bFGF in vascular endothelial cells may, in part, explain its abundance in these tissue sources (Schweigerer et al., 1987a). FGFs are not ideally suited to classical procedures of protein purification, being acid-, solvent-, and heat-labile (Westall et al., 1983). Earlier attempts to isolate the peptides from pituitary and brain used nondenaturing conditions with ion-exchange chromatography (Gospodarowicz et al., 1978), reversed-phase high pressure liquid chromatography, or immunoaffinity chromatography (Baird et al., 1986). Current methodology has made use of the affinity of both peptides for heparin to purify them from human, bovine, and chicken brain by a procedure based on one-pass heparin–sepharose affinity chromatography, either preceded by cation-exchange chromatography (Gospodarowicz, 1987; Huang et al., 1986; Klagsbrun et al., 1987) or without a preliminary purification step (Crabb et al., 1986; Mascarelli et al., 1987). Figure 1 illustrates the separation of the two FGFs from bovine brain by the latter approach. The biological activity of the mitogenic polypeptides, which are eluted from the heparin–sepharose column with a stepwise salt gradient, can be tested using a cell proliferation/DNA synthesis assay on a variety of cell types including bovine, endothelial, or BALB/c 3T3 cells. The heparin affinity chromatography procedure allows purification of both FGFs to apparent homogeneity in less than 2 d, giving a yield at this preparative stage of about 2.5 mg of each FGF polypeptide starting with 5 kg brain tissue (Gospodarowicz, 1987).

Subsequent purification and characterization of FGFs by high pressure liquid chromatography indicates that the two molecular species have different molecular weights (*see* Fig. 1) and separate p*I* values between 5 and 6 for bovine aFGF and 9.6 for bFGF. The acidic peptide does not resolve well by isoelectric focusing, a fact that reflects a conspicuous microheterogeneity of this peptide, which has been encountered in different laboratories (Gautschi, 1986 a,b; McKeehan and Crabb, 1987) working on both bovine and human aFGF. The degree of heterogeneity relates to the extraction conditions. At neutral or alkaline pH, aFGF isolates as three separate peaks, of which the largest and most predominant is a 154 amino-acid peptide acetylated at the *N*-terminus (M_r 17,400), and the remaining two of which are truncated forms lacking the *N*-terminal 14 and 20 amino acids,

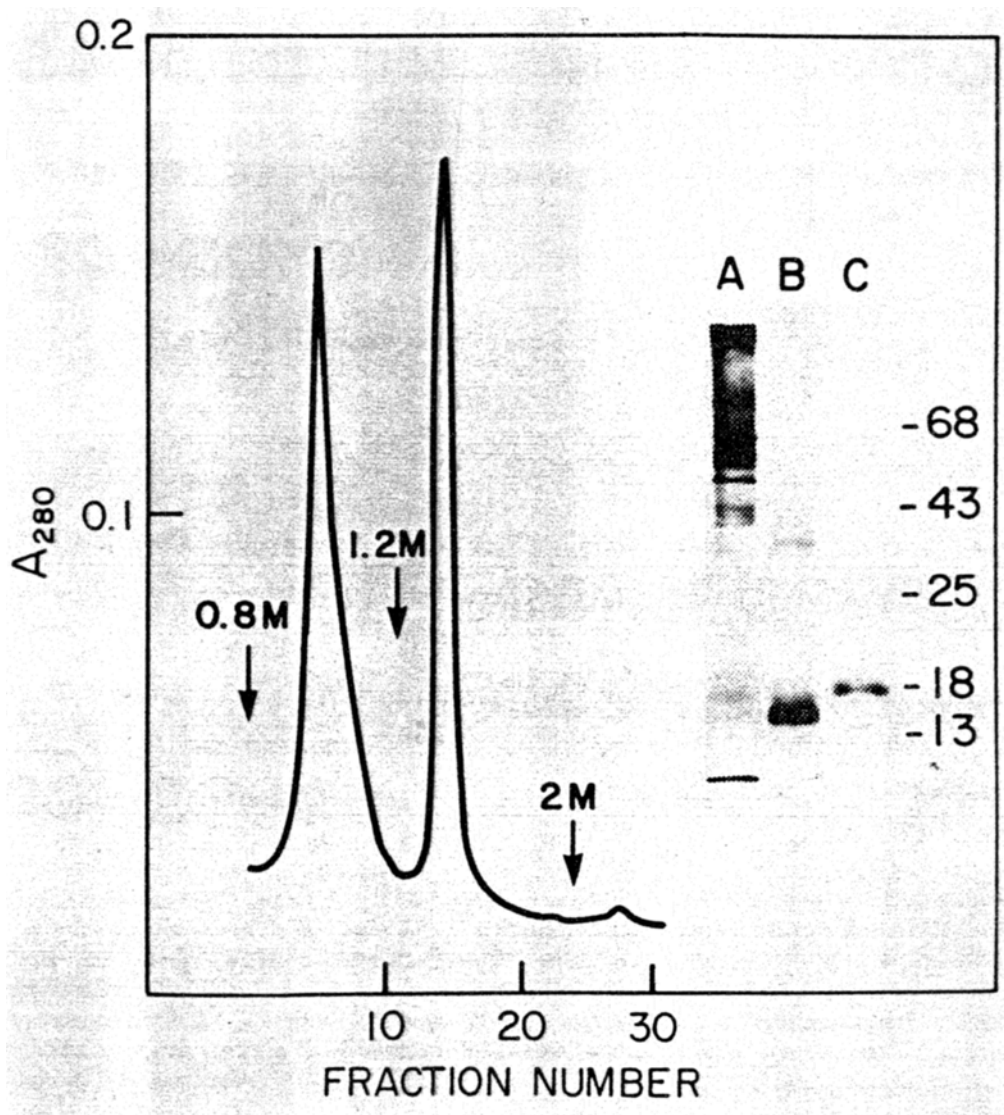

Fig. 1. Stepwise elution of brain FGFs from a heparin-sepharose column with, 0.8, 1.2, and 2.0*M* NaCl in 10 m*M* tris HCl (pH 7) as shown by arrows. Inset shows SDS-PAGE of fractions A, B, and C. Lanes B and C show the aFGF polypeptides with apparent mol wt of 16,000 and 18,000 Da, indicating the recovery of *N*-terminally-truncated aFGF peptides under these conditions. Molecular weights ($\times 10^{-3}$) are shown at right. From Lobb et al. (1986), with permission.

respectively. The fully-extended aFGF sequence from bovine brain is illustrated in Fig. 2, which indicates the extent of its homology with the basic peptide. bFGF shows comparable microheterogeneity (Story et al., 1987; Ueno et al., 1986) that also relates to differences in extraction procedures (Klagsbrun et al., 1987). The biological importance of the heterogeneity of either peptide is uncertain since both *N*-terminally-extended and shortened forms of aFGF and bFGF are equivalent in their mitogenic activity. It remains to be seen if the size and, hence, the tertiary configuration of different molecular forms of FGF affects their stability under natural conditions.

The FGF sequences show a 55% homology with one another. Between species there is considerable conservation of the structure of bFGF

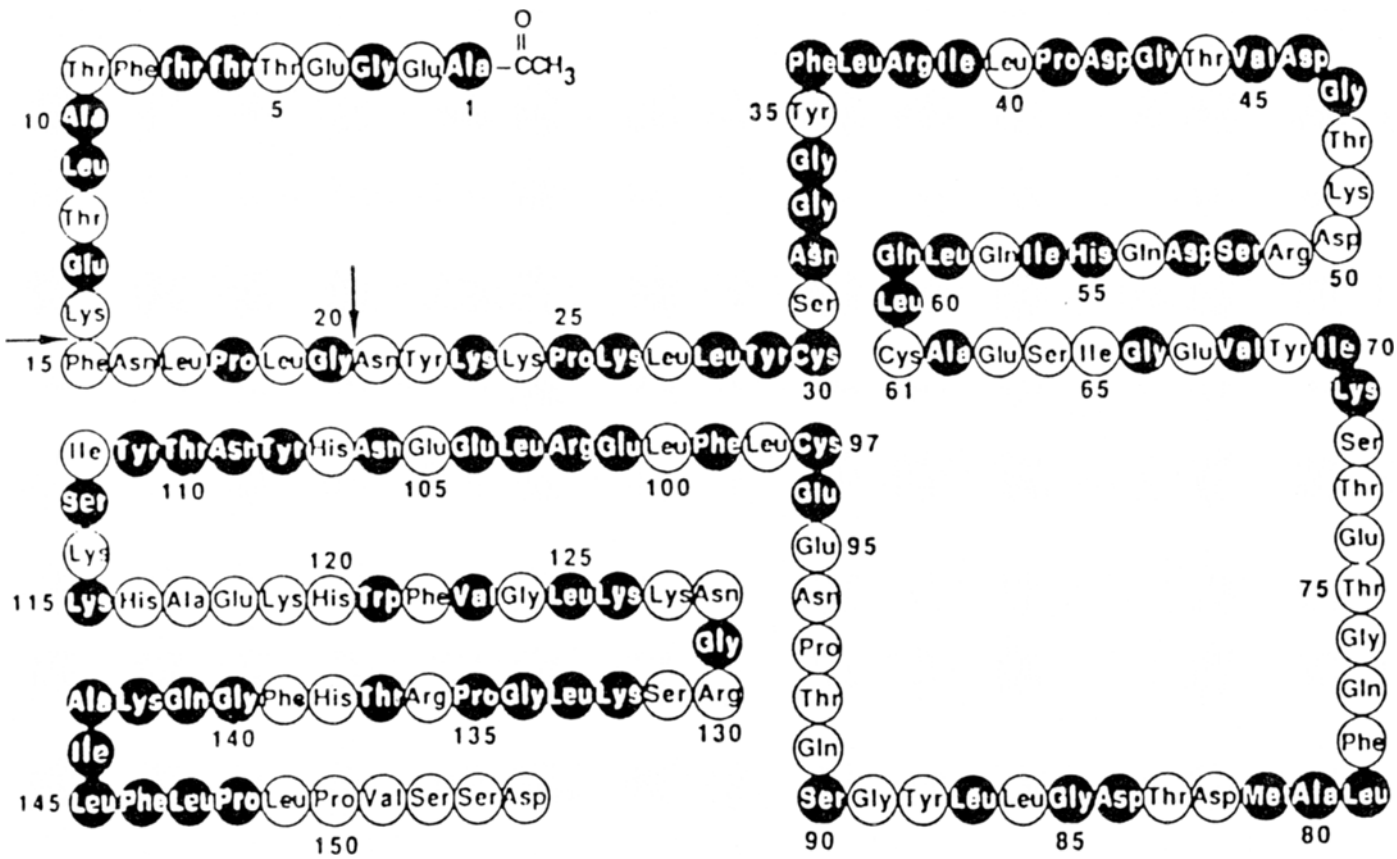

Fig. 2. Complete primary amino-acid sequence of *N*-acetylated bovine aFGF, isolated in the presence of protease inhibitors (phenylmethylsulphonyl fluoride, leupeptin, and pepstatin A). Bovine bFGF shares homology with aFGF in the shaded residues. It has an additional gly-gly-ser sequence between aFGF residues 14 and 15 and lacks aFGF residues lys^{119}, his^{120}, and asp^{154}. Arrows show sites of proteolytic cleavage commonly encountered during extraction of aFGF. bFGF is cleaved between residues 9 and 10 instead of 14 and 15. Note the highly-conserved sequence 140–148. Figure kindly provided by W. L. McKeehan.

(Abraham et al., 1986 a,b; Simpson et al., 1987). The sequence of human and bovine peptides differ in two amino acids. aFGF shows less interspecies homology, however, with the bovine and human peptides, differing in 11 amino acids (Gimenez-Gallego et al., 1986). There is 30% homology of sequence between FGFs and the interleukin-1 peptides (Thomas, 1987b). This may have functional relevance since interleukin-1 is also mitogenic toward fibroblasts. Both FGFs and interleukin peptides are unusual in their lack of a long leader *N*-terminal sequence of amino acids, characteristic of all polypeptides that are cellular secretory products. This property implies that FGFs are conveyed to their site of action by nonclassical mechanisms.

Molecular Cloning and Gene Expression

The sequence homology of the two FGF polypeptides suggests a corresponding genetic homology. The strategy for cloning the genes, therefore, was based on the use of synthetic oli-

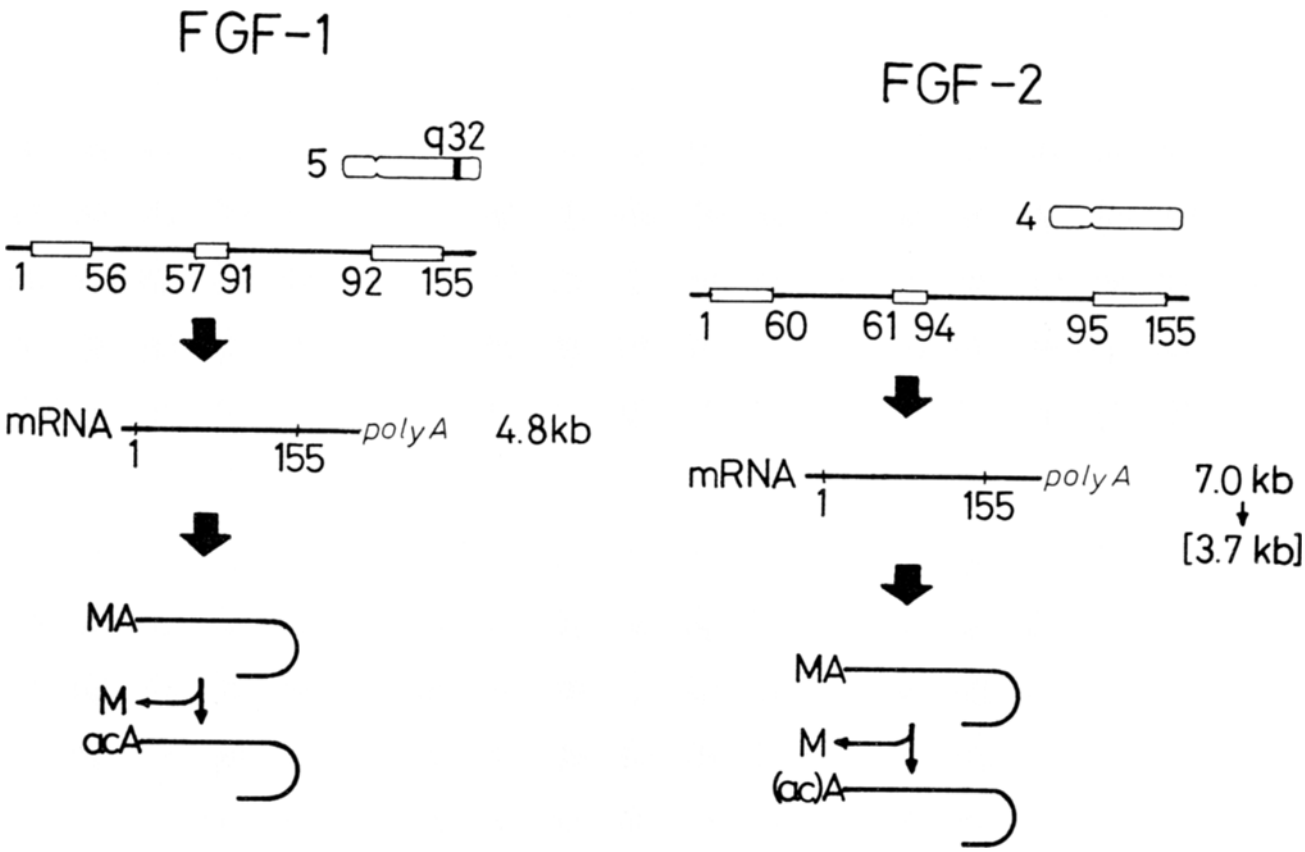

Fig. 3. Genomic organization of aFGF and bFGF loci in humans and processing of human messenger RNAs and translation products. (Top to bottom) chromosome (and locus for aFGF); encoding exons (open boxes); messenger RNA (mRNA) sizes (additional 3.7 k messenger RNA for bFGF was not originally reported in brain, but is present in other tissues); primary translation products and subsequent cleavage of N-terminal methionine (M).

gonucleotides containing putative codons for *N*-terminal amino-acid sequences well-conserved between the two peptides. The human aFGF (Jaye et al., 1986) and bFGF (Abraham et al., 1986a; Kurokawa et al., 1987) genes were cloned following the screening of brain genomic libraries. The genes for the bovine peptides were identified in the same way (Abraham, 1986 b,c). In all cases, a single locus is the source of each of the transcripts, indicating the various forms of aFGF and bFGF that have been isolated are biochemically interrelated. The genomic organization of human FGF loci is represented diagrammatically in Fig. 3. In both ox and man, aFGF and bFGF are translated as 155 amino acid peptides commencing with methionine, the putative translation initiation residue. In human (Lobb et al., 1986) and bovine brain, cleavage of methionine followed by *N*-acylation of the alanine[2] residue is a posttranslational step, although in other human tissues the *N*-terminus of bFGF may remain unblocked. The aFGF gene in humans is located on chromosome 5 (Jaye et al., 1987) and contains three encoding exons separated by long introns. The organization of the bFGF gene is homologous to that of aFGF (Abraham et al., 1986c). Its locus is on chromosome 4 (Mergia et al., 1986). In brain, the bFGF gene is transcribed to a single messenger RNA of 7.0 kilobases (kb). In cultured capillary endo-

thelial cells (Schweigerer et al., 1987a) and in most other tissues, a second 3.7 kb messenger RNA is detectable. The aFGF gene is transcribed to a single 4.8 kb messenger RNA. Although nervous tissue is well-vascularized, the source of FGFs in brain may not be primarily vascular endothelial cells (*see* below).

Genetic analysis confirms that the primary transcript of both aFGF (Jaye et al., 1986) and bFGF (Abraham et al., 1986b) genes does not encode a signal amino acid sequence or a hydrophobic leader sequence within the structure of either peptide. From aFGF and bFGF-encoding open reading-frame base sequences of isolated cDNAs, in each case methionine was predicted to be the initial amino acid. In both DNA sequences, the methionine codon coincides with a classic base consensus sequence for translation initiation in eukaryotic cells and is preceded in the aFGF-encoding sequence by a stop codon. The initial amino acid residues of aFGF are not sufficiently hydrophobic to constitute a leader sequence, and in neither aFGF–or bFGF is there evidence for a signal peptide cleavage site. In guinea pig brain, however, a 25,000 Da bFGF-like peptide has been isolated with full biological activity that can be cleaved to form bFGF in the presence of trypsin (Moscatelli et al., 1987). Although it seems unlikely, it may thus be argued that, in brain, a primary *N*-terminally-extended bFGF translation product exists with classic signal and leader sequences that may perhaps derive from another hitherto unidentified bFGF gene. Studies of promoter-directed expression of the bFGF gene in bacterial (Iwane et al., 1987) and eukaryotic (Abraham et al., 1986c) systems do not support this hypothesis, but indicate that gene products only the size of bFGF are translated but remain unsecreted.

"Forced" secretion of bFGF has been achieved by constructing an expression vector, including a signal peptide from immunoglobulin genes fused to the bFGF coding sequence. Cloned 3T3 cell lines translated the chimaeric protein, cleaved off the signal sequence and exported the biologically-active bFGF through the endoplasmic reticulum (Rogelj et al., 1988). Under normal conditions in mature brain cells that contain abundant FGFs, nonsecretion of peptides, therefore, may serve as a cytoplasmic control mechanism regulating their bioactivity.

Neural Cellular Localization

Bovine brain is one of the richest sources of FGFs. The bioassay of (presumptive) FGF bioactivity shows that it is evenly distributed throughout the bovine central nervous system (Logan and Logan, 1986) and, in rat brain, increases rapidly from embryonic gestational d 15, at which time it first becomes measurable by this method (Logan et al., 1985), during the period in which widespread neuronal differentiation is taking place. In *Xenopus* larvae, expression of a FGF messenger RNA been detected much earlier in development (Kimelman and Kirschner, 1987). In young adult rat brain, FGFs were localized in nearly all neurons (Fig. 4) by the immunocytochemical method, using antibodies that were cross-reactive to both aFGF and bFGF (Pettmann et al., 1986). The antiserum failed to localize FGFs to capillary endothelial cells or astrocytes. Ultrastructural studies demonstrated intraneuronal association of FGF immumoreactivity with many cytoplasmic structures, but no evidence of its presence at the cell surface (Janet et al., 1987). Significantly, FGFs appeared not to be associated with membranes of the endoplasmic reticulum and other organelles, but to be abundant in the cytoplasmic matrix. These observations are not consistent with the likelihood that FGFs are actively being exported from nerve cells in the rat brain, in contrast to the status of FGF regulation in neuroblastoma cells (Gospodarowicz et al., 1986a). aFGF immunoreactivity is also present in cultures of human neuronal tumor cells (Huang et al., 1987), and immunocytochemical studies suggest that FGFs are of exclusively

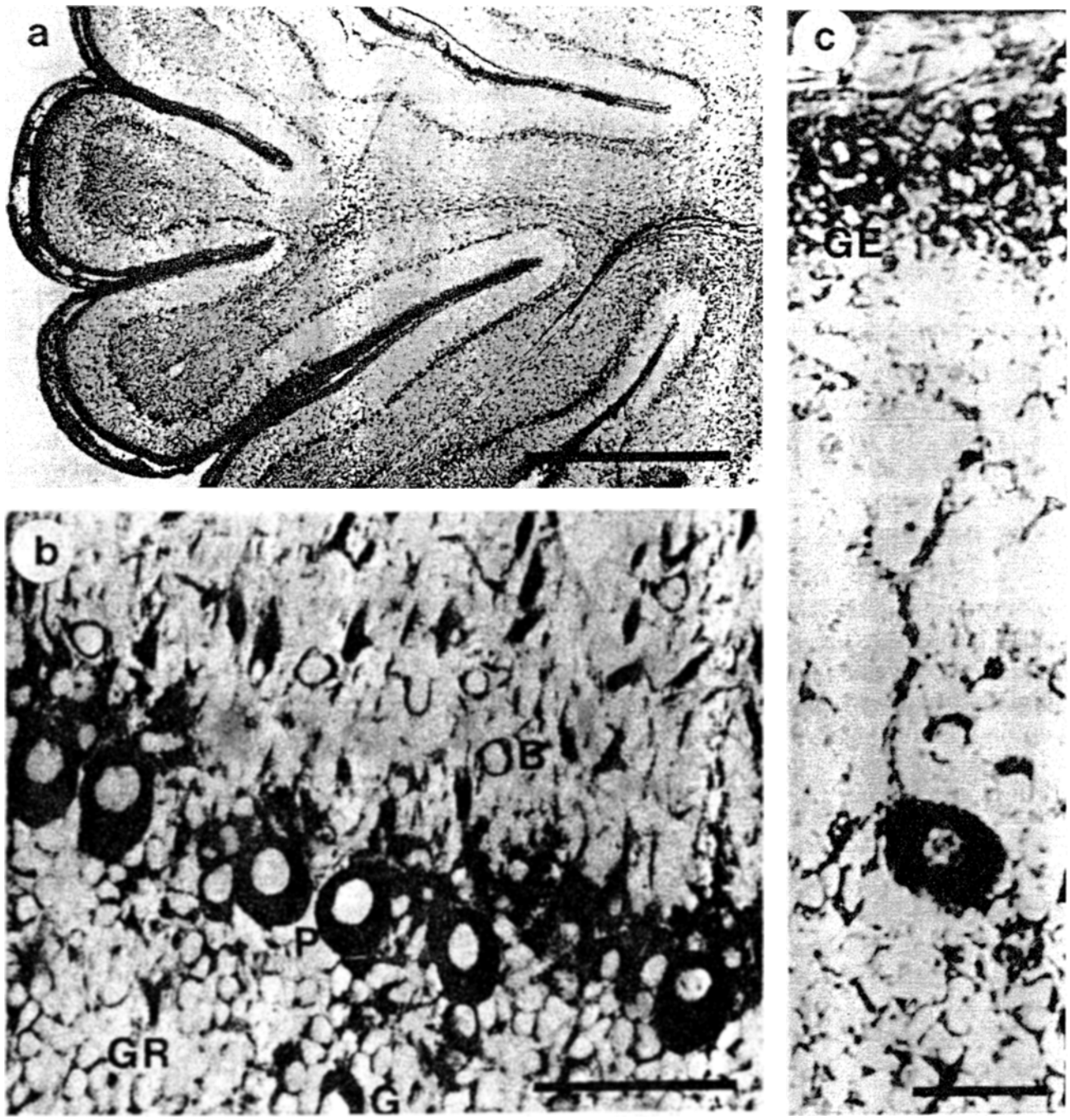

Fig. 4. Immunocytochemical localization of FGFs in 18-d-old rat cerebllum using primary mouse monoclonal antibody against bFGF. P, Purkinje cells; G. Golgi cells; B, basket cells; B, basket cells. Calibration bars: a, 1 mm; b, 100 mm; c, 50 mm. Reproduced with permission from Pettmann et al. (1986).

neuronal localization in cultures of embryonic rat and chick central nervous systems and sensory and autonomic ganglion cultures from both species (Janet et al., 1988). However, bFGF has been immunoprecipitated from lysates of pure mouse brain astrocyte cultures (Hatten et al., 1988) and is expressed by malignant glial cells (Libermann et al., 1987). There is also evidence for the biosynthesis of bFGF in pigment epithelium (neuroepithelial-derived) cells (Schweigerer et al., 1987b) and in cells of retinoblastoma, a tumor of probable neural origin (Schweigerer et al., 1987c).

Release, Bioactivity, and the Role of Proteoglycans

The primary sequence of the two FGF peptides reveals the existence of two stretches of basic residues (in aFGF between residues 23–26 and 114– 116) that are the likely sites at which the peptides interact with heparin. The corresponding site of interaction on the heparin molecule is likely to be the sulphate-rich pentasaccharide moiety that is the binding domain for antithrombin (Lindahl et al., 1983)(Fig. 5). The binding of heparin and other proteoglycans to the FGFs is a concept central to hypotheses of the mode of transport and biological activation of these growth factors.

Transport

The absence of a signal peptide in the sequence of the FGF messenger RNA primary translation products indicates that the peptides are not transported to the lumen of the endoplasmic reticulum for export in a manner similar to hormones and growth factors that are secreted in the classical manner. It follows that they must either be coexpressed with their receptor proteins to activate them intracellularly, or that alternative export mechanisms of the peptides to the cell surface are made available. In endothelial and smooth muscle cells, in which autocrine regulation of proliferation by FGFs takes place (Schweigerer et al., 1987a; Winkles et al., 1987), FGF binding sites can be localized on the cell surface, and the peptides can be released from the cell surface by exposing the cells to heparinase (Baird and Ling, 1987). Since much of the FGF in nervous tissue is present in neurons, it is likely that the neuronotrophic actions of FGFs are also autocrine although it is possible that aspects of neuronal responses are of a paracrine nature (*see* sections on Second Messengers and Neuronal Differentiation). FGFs share their affinity for proteoglycans of the heparan sulphate subgroup with laminin, which also has neurotrophic activity (Edgar et al., 1985).

Although as yet unproven, it appears likely that FGFs in neurons, as in endothelial cells, are transported to the cell surface without being released and remain complexed with extracellular heparan sulphate proteoglycans (Vlodavsky et al., 1987a,b). Their activation may thus depend on enzymatic remodeling of the extracellular matrix, bringing them into contact with their receptor sites.

Biological Activation

The biological activity of FGFs in vitro is enhanced in the presence of both heparin and proteoglycan-rich subcellular fractions. Although both FGFs bind with equal affinity to heparin in a cell-free environment, heparin differently influences the cellular responses to the two peptides. Heparin and heparan sulphate proteoglycans substantially augment mitogenic responses to aFGF (Thomas, 1987b), while having a lesser effect on bFGF. This may reflect a greater biological amplification of the response to aFGF at the receptor site, or the greater stabilization of its tertiary conformation in the extracellular milieu. The adsorption of heparin in vitro can not only protect both FGFs from inactivation by denaturing acid and heat treatment

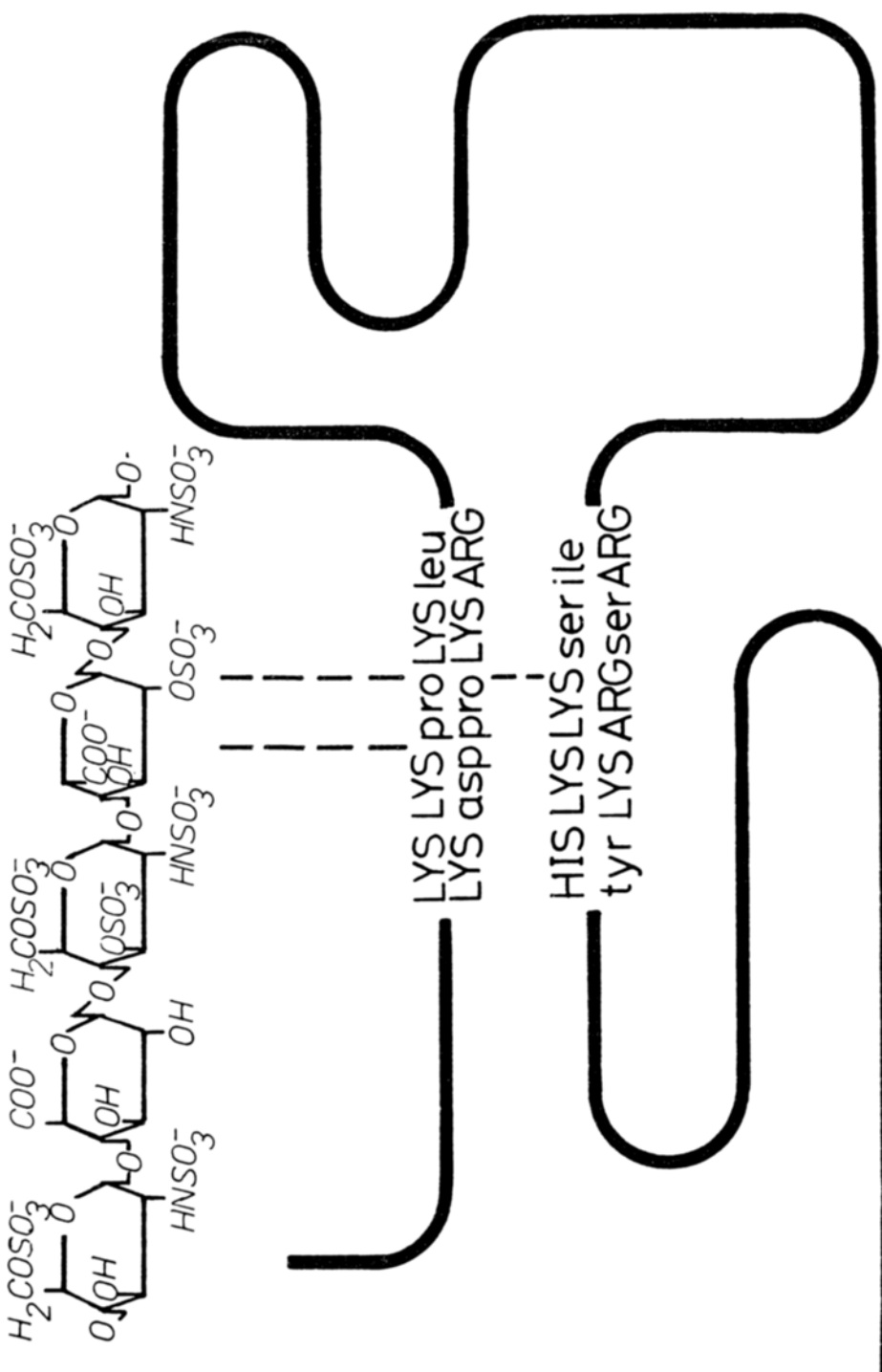

Fig. 5 . Binding domains for interaction of aFGF peptide with polysaccharide glycosaminoglycan sidechains of heparin and heparan sulphate.

Table 3
Properties of the HBGF Receptor in PC12 Cells
and Characteristics of Responses with Relevance to the Nervous System[a]

Mol wt	145,000 Da
Enzyme activity	Tyrosine kinase
Ligand	aFGF and bFGF
Binding dissociation constants (K_d)	400 and 20 pM
Site density/cell	1600–3600
Responsive cells in the nervous system	Endothelial cells, neurons, astrocytes, and oligodendrocytes
Time to binding equilibrium (endothelial cells)	15 min

[a]Data from Neufeld et al. (1987) and other sources.

(Gospodarowicz and Cheng, 1986), but can also restore a large component of their biological activity lost on storage in glycerol at -20°C (Schreiber et al., 1985). The biological implication of these findings is that heparan sulphate proteoglycans may play an important part in stabilizing the conformation of FGFs at their receptor site and, indeed, may be essential to receptor activation. Under "physiological" conditions, FGFs have an in vitro half-life of only a few hours (Westall et al., 1983). The formation of a complex between FGF molecules and the extracellular matrix may thus be necessary for the retention of full biological potency during the entire period between biosynthesis and receptor binding.

Nerve cells capable of differentiation in culture exhibit a neuronotrophic response to FGFs (*see* section on Neuronal Differentiation). In the neural PC12 pheochromocytoma cell line, stimulation of neurite outgrowth by both aFGF and bFGF is modified by heparin, the effect being particularly evident for aFGF, whose biological activity is increased nearly threefold in the presence of 10mg/mL heparin (Neufeld et al., 1987). The cooperativity of heparin with bFGF in the neurite outgrowth response of PC 12 cells is less apparent, as for the mitogenic response to this peptide in endothelial cells (Ulrich et al., 1986a). Cooperativity in the mitogenic response has been demonstrated between aFGF and the pentasaccharide antithrombin binding sequence of heparin alone (Ulrich, 1986b). This finding supports the hypothesis that FGFs and antithrombin share a common heparin-binding domain that, in isolation from the proteoglycan, is sufficient to stabilize FGF peptide tertiary structure.

In the nervous system, the state of processing of heparan sulfate proteoglycans may itself have a bearing on the biological activity and sequestration of FGFs. The maturation of neurons, both naturally and in vitro in response to neuronal growth factors, results in an incease in the sulphation of proteoglycans (Margolis et al., 1987). It is possible that this directly influences the binding affinity of FGFs to the extracellular matrix through an increase in the number of sulphate-rich glycosaminoglycan binding moieties.

Receptors and Second Messengers

Binding Sites

By comparison to other growth factors (epidermal and platelet-derived growth factors), the affinity of receptors for FGFs is higher, and the site number per cell lower, by an order of magnitude (Huang and Huang, 1987), as dem-

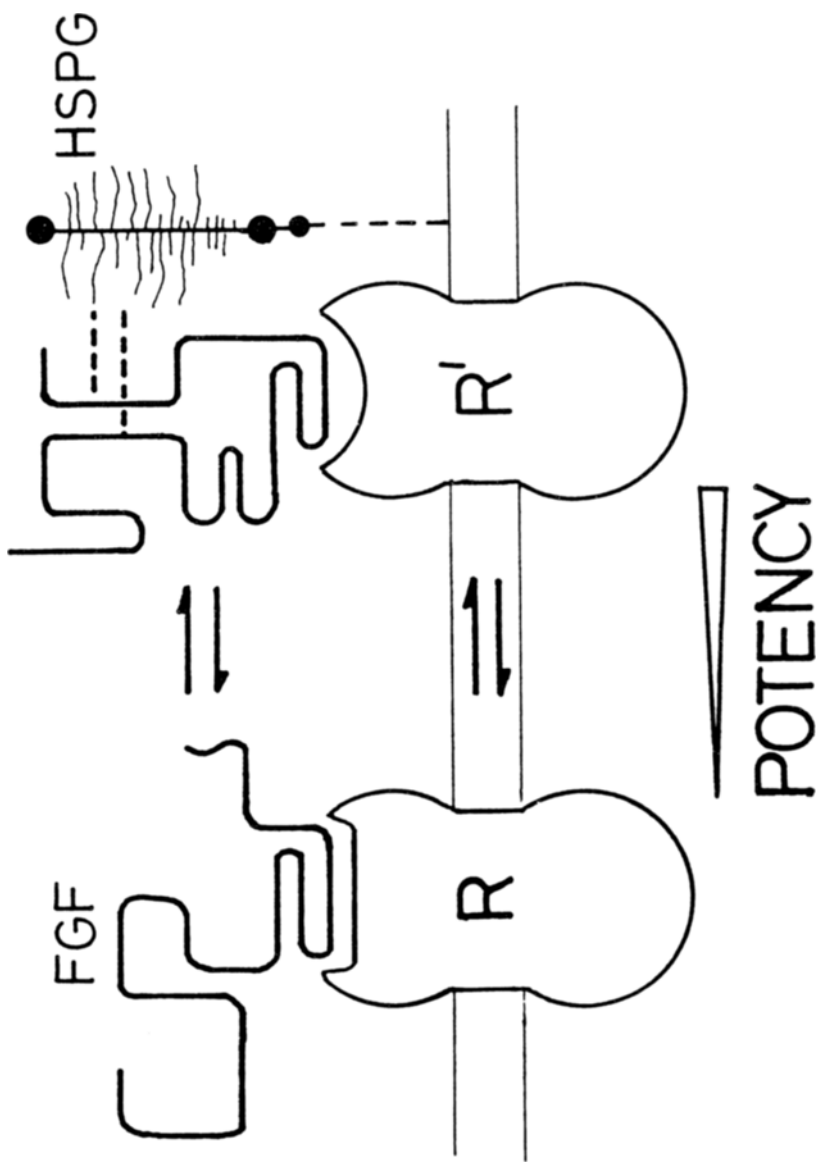

Fig. 6. Hypothesis for the interaction of FGFs with heparan sulphate proteoglycans (HSPG) and the FGF receptor site. HSPG (anchored to the cell surface via hyaluronate receptors and other binding sites) binds FGF, altering its conformation and rendering it more stable. This results in an inceased affinity of FGF for its receptor and greater potency, possibly involving changes in the equilibrium between the two ligand-bound states (R and R'). Differences may exist in the nature of the interaction and degree of peptide–receptor stabilization for aFGF and bFGF.

onstrated by affinity labeling with ^{125}I-bFGF (Table 3). Although slightly different kinetic data are obtained using aFGF as the primary ligand, both FGFs can be cross-linked in PC12 cells to a single 145,000 Da membrane-associated receptor protein, and both FGFs displace either primary ligand with equal potency (Neufeld et al., 1987). These results are in agreement with earlier findings with BHK-21 cells (Neufeld and Gospodarowicz, 1986) indicating that, despite differences in their primary structure, both FGFs probably activate the same receptor. Low-affinity sites of 10-fold high density are also present in both cell lines (Moscatelli, et al., 1987; Neufeld et al., 1987). The binding of bFGF to the low-affinity sites is competed strongly by heparin, and much of it is abolished following heparinase treatment. This suggests that the low-affinity binding of bFGF to cells represents its association, either directly or indirectly, with extracellular heparan sulphate proteoglycans rather than low-affinity membrane receptor sites. The FGF receptor-ligand complex reaches equilibrium more quickly, and is internalized and degraded more slowly than other growth factors (Huang et al., 1986). This may be a direct result of proteoglycan-dependent stabilization of a (cell-surface) FGF ligand-receptor complex and could explain both the requirement of FGFs for less binding sites and high receptor binding affinity in comparison to other growth factors. Heparin increases the affinity of bFGF for its receptor (Neufeld and Gospodarowicz, 1986) and does so presumably through its stabilization of the growth factor, but also possibly by stabilizing the bound state of the receptor–ligand complex. (Fig. 6).

Peptide Receptor Binding Sequence of FGFs

The functional domain of the bFGF molecule has partly been elucidated by systematic structure-activity studies of mitogenic activity within a panel of synthetic peptide fragments (Baird et al., 1986). These show that a sequence of 34 amino acids in the center of the molecule, containing inverted repeat sequences and resembling the cellular fibronectin receptor-binding site, may be responsible for receptor activation. The same sequence (peptide F) is also able to potentiate the neurite outgrowth response of PC12 cells to bFGF and to inhibit the binding of the growth factor to its receptor. The presence of two amphiphilic sequences (residues 41–46: leu-arg-ile-his-pro-asp and 57–67: asp-pro-his-ile-lys-leu-gln-leu-gln-ala-glu, in bovine bFGF) give this peptide a b-pleated structure that accords with the physical chemistry of receptor-binding sequences in polypeptide hormones.

Second Messengers

The primary response to FGF-receptor activation is the phosphorylation of a small group of medium to high mol wt proteins. The FGF receptor, like those for epidermal and platelet-derived growth factors, has an intracellular domain with tyrosine kinase activity (Huang and Huang, 1987). However, unlike these related growth factors, FGFs probably do not stimulate autophosphorylation of their receptor, but the phosphorylation of a discrete 80,000–90,000 Da protein (Blackshear et al., 1985; Coughlin et al., 1988) as well as smaller proteins that are substrates for protein kinase C. The activation of protein kinase C and the mobilization of intracellular calcium through the products of polyphosphoinositide metabolism, are generally believed to be early events following FGF receptor activation (Gospodarowicz et al., 1986a). There is evidence that protein kinase C activation is linked to the sequential induction of c-*fos* (Takeyama et al., 1986) and c-*myc* (Kaibuchi et al., 1986) oncogenes in cells undergoing mitotic stimulation. In some cell types, however, polyphosphoinositide hydrolysis and protein kinase C activation, in the presence of FGFs, has not been detected (Magnaldo et al., 1986; Zeytin and Delellis, 1987)

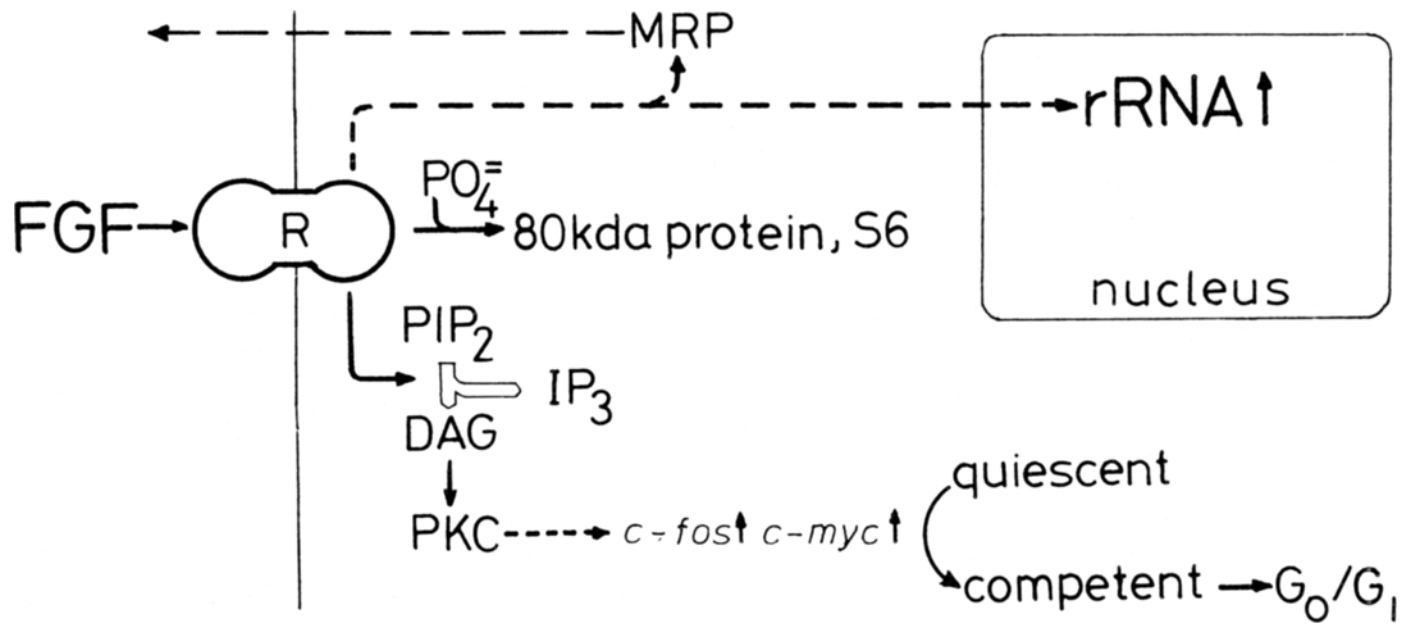

Fig. 7. Major intracellular regulatory events following interaction of the FGF receptor and implications for mitogenesis. These include transcription-independent mechanisms: cytoplasmic protein phosphorylation (S6, ribosomal protein), activation of inositol phospholipid pathway ($PIP_{2)}$ phosphatidylinositol 4,5-bisphosphate; IP_{3}, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C), release of mitogen-related (glyco) protein (MRP), transcription-dependent mechanisms following translocation of the ligand-receptor complex to the nucleus, and increased synthesis of ribosomal RNAs. These events result in the movement of cells from the quiescent state to the G_0/G_1 phase of the mitotic cycle. Similar events may underly the survival/differentiation-promoting actions of FGFs in neurons.

despite the presence of a mitogenic response. These conflicting results have not adequately been resolved. They could be explained on the basis of large variations in the numbers of FGF receptors on diferent cells, or the contamination of FGF preparations with other growth factors.

Cell Proliferation

General Hypothesis

Recently, it has been shown that FGFs accumulate in the nuclei of quiescent fibroblasts minutes after activation (Bouche et al., 1987), and their presence is accompanied by *de novo* DNA synthesis and the transcription of ribosomal RNA. This work suggests that, having activated specific cytoplasmic reactions, the FGF-receptor complex migrates to the nucleus and binds to ribosomal RNA genes. Cellular oncogene expression, the phosphorylation of ribosomal S6 protein and an increase in the level of ribosomal RNA, signals the induction of quiescent cells and their transition to the G_0phase of the mitotic cycle. The progression of cells to the S phase is uniquely dependent upon the increase of intracellular pH following activation of the plasma membrane Na^+/H^+ antiport system (Pouysségur et al., 1985). In cells induced to competence by FGFs, changes in extracellular pH can initiate cell division in vitro (Bravo et al., 1986). These changes can be brought about by serum factors, or in the absence of serum, by hormones such as insulin (Pouysségur et al., 1985), or neurotransmitters (Isom et al., 1987). Most FGF-responsive cells, including neuronal stem cells (Gensburger et al., 1987), proliferate in response to FGFs in serum-free culture providing hormones are present, indicating the role of FGFs in competence-induction. A tentative summary of putative intracellular events leading to the mitotic response based on studies in non neural cells is depicted in Fig. 7. FGFs induce the secondary secretion and release of a number of cellular products that may explain the apparent paracrine nature of some of their actions. These include a 39,000 Da mol, the major excreted protein, a thiol-dependent cathepsin (Gospodarowicz et al., 1987b), and the mitogen-related 34,000 Da glycoprotein (Nilsen-Hamilton et al., 1986). The latter has a core protein sequence highly homologous to prolactin and growth hormone, and may function as a secondary growth factor. The ability of other hormones and growth factors to modulate FGF responses may be related to the control of release of this glycoprotein.

Neural Cell Proliferation

Studies on the actions of bFGF on neural cell types have revealed a mitogenic response in vitro that is relatively nonselective. However, the validity of some of the observations is questionable in light of the very high growth factor concentrations that have often been employed (e.g., nanomolar range for induction of DNA synthesis in oligodendrocytes, Eccleston and Silberberg, 1985). Astrocytes show a proliferative and morphological response to polypeptide growth factors that have been known to exist in brain for several years (Pettmann et al., 1981), and were later shown to be identical to the FGFs. bFGF can replace serum in inducing competence in immature astrocytes (Kniss and Burry, 1988), thus, acting through a mechanism common to other cell types. However, the astrocyte proliferation in culture is seen only at relatively high FGF concentrations (Morrison and DeVellis, 1981). Moreover, the response of astrocytes to FGFs is not unique and other growth factors both endogenous (Ito et al., 1986) and exogenous to astrocytes (Besnard et al., 1987; Lemke and Brockes, 1984; Perraud et al., 1987; Pruss et al., 1982; Wu et al., 1985; Yong et al., 1988) may be of equal or greater effectiveness in stimulating cell division and stabilizing the phenotype of immature astrocytes in the developing brain.

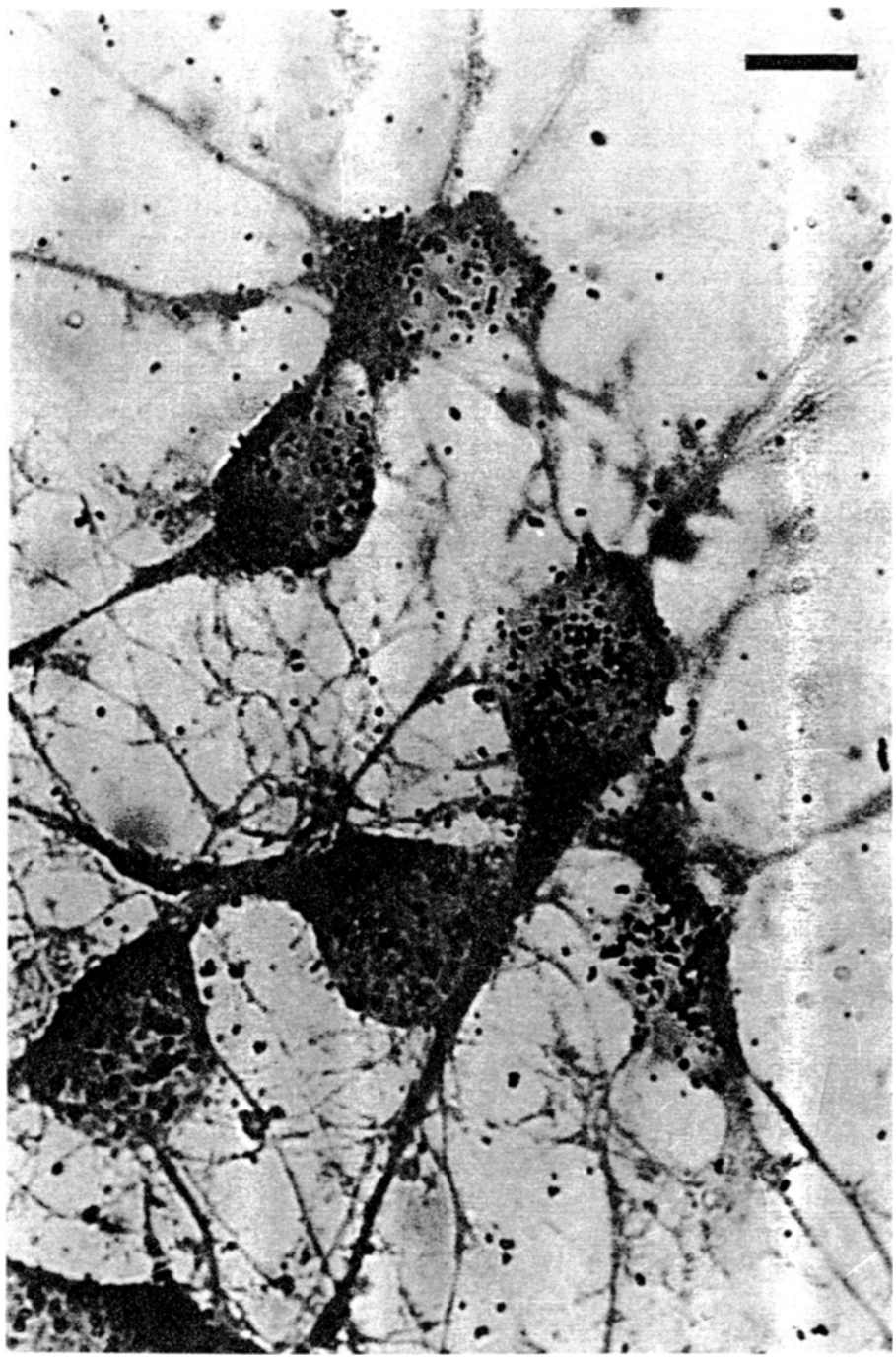

Fig. 8. Immunocytochemical localization of neurofilament protein with ^{3}(H)-thymidine autoradiography confirms that bFGF stimulates DNA synthesis in proliferating neuronal stem cells in cultures of embryonic rat brain. Reproduced with permission from Gensburger et al. (1987).

In striking contrast, enriched cultures of immature neurons from early embryonic rat brain show a 100% increase in DNA synthesis in the presence of picomolar concentrations of bFGF (Gensburger et al., 1987). This response could not be reproduced with epidermal growth factor, platelet-derived growth factor, or insulin-like growth factor-1. Thrombin was also ineffective in stimulating mitosis in these cells. These results indicate that the primary response of mammalian neuroblasts to bFGF was one of competence-induction among a quiescent cell population. Autoradiography with immunocytochemical labeling confirmed that the responsive cells expressed neurofilament protein (Fig. 8). These studies, together with those reviewed in the next section, provide good evidence that the autocrine and putative paracrine responses of neurons to FGFs span a number of stages in their development and include the proliferation of stem cells, as well as the stabilization and survival of the differentiated state.

Neuronal Differentiation

Role of Glial vs Neuronal FGF

Although neural crest-derived neurons show an absolute requirement for Nerve Growth Factor for their survival and differentiation (Greene and Shooter, 1980), in vitro experiments have revealed that many neurons of the embryonic central nervous system are refractory to Nerve Growth Factor, but can survive and differentiate in the presence of astrocytes or astrocyte-conditioned culture medium (Banker and Cowan, 1979; Barbin et al., 1984). Embryonic cortical and hippocampal neurons, in culture in the presence of bFGF, survive for longer durations than controls and form an extensive neurite outgrowth (Morrison et al., 1986; Walicke et al., 1986). FGFs, as well as other growth factors, can replace serum-free conditioned medium in maintaining brain cell growth and differentiation (Eccleston et al., 1985). Astrocytes in vitro are a source of bFGF, but not aFGF (Hatten et al., 1988), and it now appears that much of the astrocyte-derived neurogliotrophic activity is attributable to bFGF (Pettmann et al., 1985). The neurite growth response of mixed cultures of cerebellar granule cells and astrocytes could be reduced by affinity-purified antibodies against bFGF and restored with pure bFGF added exogenously (Hatten et al., 1988). Pure cultures of neurons could be grown only in the presence of added bFGF, suggesting that the level of biosynthesis/export of endogenous bFGF in neurons cultured alone is not sufficient to support their growth (Fig. 9). bFGF is neuronotrophic at concentrations that are mitogenic toward fibroblasts and endothelial cells, but below those that are required to stimulate glial proliferation. It is one of the few growth factors with both mitogenic and neurite stimulating activity in neurons. In both central and peripheral neurons of chick and rat embryos, FGFs promote both morphological growth and chemical differentiation (Rydel and Greene, 1987); Unsicker et al., 1987). The stabilization of the differentiated form by FGFs has been reported for endothelial cells, chondrocytes, adipocytes, and nonneuronal as well as neuronal cell lines (Gospodarowicz et al., 1986b). This general observation suggests that morphogenic changes allied to alterations in the pattern of ganglioside and glycopeptide distribution on the cell surface, together with accompanying changes in developmentally-regulated gene expression, are among the responses that FGFs elicit.

Cellular Mechanisms

The mechanisms by which FGFs exert their cell survival and differentiation-promoting neuronotrophic influences have been elucidated in studies of their actions on embryonic peripheral autonomic neurons and PC12 pheochromocytoma cells, both of which provide a

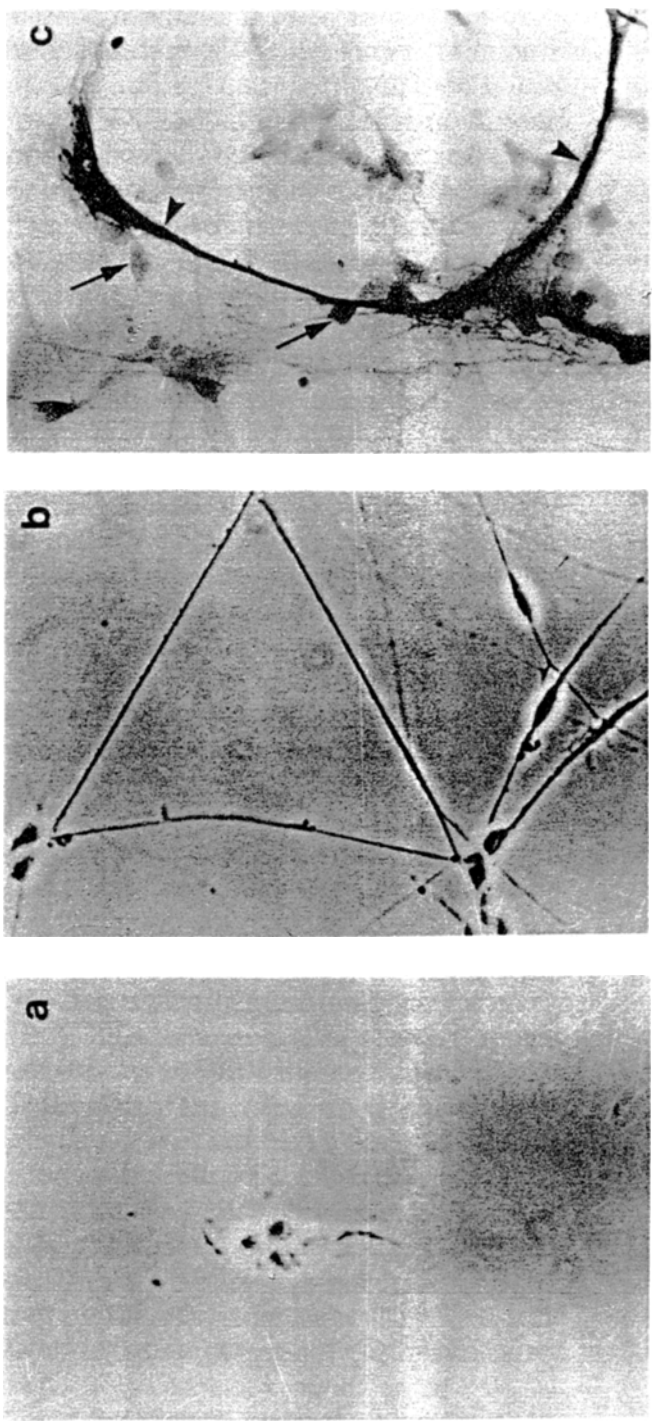

Fig. 9. Effect of bFGF on neurite extension in pure cultures of newborn mouse cerebellar granule cells, (a) control; (b) bFGF (1–4 nm). When mixed with cultures enriched in astrocytes, bFGF did not increase or decrease the degree of morphological interaction with glia, identified by immunocytochemical labeling with antiserum to glial fibrillary acidic protein (c), ×600. Reproduced with permission from Hatten et al. (1988).

homogeneous population of cells capable of differentiation into the neuronal phenotype. In ciliary ganglion cells, bFGF is 100 times more potent than aFGF in supporting neuronal survival, but, as in the mitogenesis assay *(see* previous section on Release, Bioactivity, and the Role of Proteoglycans), heparin acting synergistically with aFGF increases its potency to a level equivalent to bFGF (Unsicker et al., 1987). The importance of optimizing the relative amounts of heparin and FGF added to cultures for neurite outgrowth has been demonstrated both in primary cultures of embryonic neurons and PC12 cells (Neufeld et al., 1987). The presence of 10–100-fold excess heparin may enable all low-affinity, heparinase-sensitive binding sites on the cell surface to be occupied, giving maximum cooperativity with FGF. A further excess of heparin is inhibitory to FGF action, indicating that heparin may interfere with FGF binding in a negative manner at sufficiently high concentrations.

The time-course of neurite formation in PC12 cells, in the presence of FGF, indicates a delayed onset of the response, distinct from that seen in the presence of cholera toxin (Wagner and D'Amore, 1986). This observation confirms that FGFs, like Nerve Growth Factor, act through an adenylate cyclase-independent mechanism. In serum-free medium, the short-term response to FGFs bears many similarities to Nerve Growth Factor. The polypeptides are approximately equipotent. Both cause an increase in cellular ornithine decarboxylase and the phosphorylation of microtubule-associated protein (MAP 1.2), tyrosine hydroxylase and a nuclear protein (Rydel and Greene, 1987; Togari, 1985). However, in serum-supplemented medium, the long-term effects of the two factors differ. The rate of neurite initiation (increase in the percent of neurite-bearing cells with time) in response to Nerve Growth Factor is nearly twice that of bFGF, the neurite outgrowth response being more prolific with Nerve Growth Factor, developing into a dense network of fibers. After a few days, the morphological response to bFGF ceases, despite the continued presence of the peptide. However, these differences in vitro may be an artifact of the culture conditions, since they are not noticeable in serum-free conditions. In the presence of serum, despite the absence of neurite outgrowth, bFGF continues to act through transcription-dependent mechanisms to induce cell-surface glycoproteins (Nerve Growth Factor-inducible large external (NILE) glycoprotein and thy-1) characteristic of the differentiated phenotype (Rydel and Greene, 1987).

It is clear that FGFs act as neuronotrophic factors through separate receptor mechanisms, but through common intracellular pathways with Nerve Growth Factor and perhaps of other neuronal survival/growth factors. The differences that have yet to be explained may partly be reconciled on the basis of the complex interdependence of FGFs and extracellular proteoglycans, a property not shared by Nerve Growth Factor.

The specificity of basal lamina components in promoting the FGF neuronotrophic response is broader than previously suggested for the mitogenic action of the peptides. This has elegantly been demonstrated in PC12 cells, which show patterns of cell association mimicking histiogenesis in response to bFGF, that differ in complexity depending on the type of proteoglycan (heparan or chondrotin sulphate) used to coat the substratum (Schubert et al., 1987). These results are consistent with the report that classes of sulphated proteoglycan other than heparin and heparan sulphate potentiate the response of FGFs, but with reduced potency (Damon et al., 1988). Even association with an artificial substratum of polyornithine dramatically increases the neuronotrophic potency of FGFs (Unsicker et al., 1987). These important studies provide a preliminary indication that the responses of neurons to FGFs may extend to intercellular organization under the appropriate conditions. Studies of the progress of tissue organization in

primary reaggregating cultures of embryonic brain, in the presence of FGFs, are required to strengthen this hypothesis.

Neural Regeneration

The presence of FGF peptides in adult nervous tissue suggests their potential involvement in neural regenerative processes. Peripheral tissue regeneration has been studied extensively in urodele amphibians. A neural component is important in the early (blastema cell proliferation), but not later (differentiation) phases of limb regeneration (Singer, 1974). It has been suggested that a neurally-derived mitogen may stimulate the mesodermal blastema cells to divide at the early stage of regeneration. bFGF has been shown to replace neural extracts in stimulating blastema cell proliferation in vitro, and blastema formation after nerve transection and limb amputation in vivo, at physiological concentrations (Gospodarowicz et al., 1987b). In the adult, as opposed to the developing mammalian nervous system, the neuronotrophic rather than the mitogenic activity of FGFs may be expected to assume the greatest physiological relevance. The effectiveness of FGFs in promoting the survival of neurons has been demonstrated after axotomy in the central nervous system. After transection of the optic nerve in rats, gel foam matrices, impregnated with aFGF or bFGF, were implanted near the proximal stump. Both aFGF and bFGF reduced the number of retinal ganglion cells that survived within 30 d after nerve transection. There was a slight increase in the numer of retinal glial cells in FGF-treated animals that may have contributed in part to the neurotrophic response (Sievers et al., 1987). The ability of bFGF to exert longer-range neuronotrophic effects at deep brain sites in vivo has also been demonstrated (Anderson et al., 1988). Following unilateral fimbria-fornix transection in both young and aged rats, death of half of the cholinergic neurons of the medial septal nucleus and diagonal band of Broca takes place within 2 wk. Infusion of less than 6 ng/d bFGF during this period, with autologous serum as carrier by osmotic mini-pump into the lateral ventricle, rescued between 66 and 74% of the cholinergic cells in these nuclei that would otherwise have died in young rats and between 45 and 47% of these cells in aged animals. If infusion was commenced 2 d after the lesion, cholinergic neurons of the diagonal band, but not of the medial septal nucleus, were still amenable to recovery by bFGF.

The influence of bFGF in promoting neuronal survival has also been demonstrated in peripheral ganglia (Otto et al., 1987). Unilateral sciatic nerve transection results in the loss of one-third of the sensory ganglion cells on the same side of the animal with little contralateral cell loss. bFGF, applied in a silicone chamber attached to the proximal stump of the transected nerve, protected about half the neurons from death, whereas Nerve Growth Factor administration completely prevented nerve cell death. Despite the reduced effectiveness of bFGF, as compared to Nerve Growth Factor in this experiment, the results suggest that bFGF or its ligand-receptor complex can be retrogradely transported by mature sensory nerve axons to the cell nucleus. An alternative view is that the endothelial cell response to FGFs and stimulation of revascularization of the injured sciatic nerve are at least of equal importance to the neurotrophic response in nerve regeneration (Cuevas et al., 1986). The response does not appear to be selective for certain cell types, but neurons of a variety of sizes are among those that survive.

Summary

Two heparin-binding growth factors, the FGFs, of mol wt 17,400 and 18,400 Da, have been isolated from brain tissue, purified, and biochemically characterized. They are synonymous with Astroglial Growth Factors 1 and 2, Eye-Derived Growth Factors 1 and 2, Endothelial Cell Growth Factors, and Prostatropin,

among other previously characterized factors of common identity. Genetic cloning has revealed the presence of two separate loci and of discrete messenger RNAs from which the two FGFs are translated. The peptides each occur as shortened forms, some of which may be the artifacts of biochemical extraction and purification. Both peptides are produced throughout the mammalian and human brain. Their biosynthesis is developmentally regulated and continues into adult life. Both neurons and astrocytes contain FGFs, but the evidence suggests that astrocytes contain only bFGF. FGFs lack a signal peptide and leader sequence and are not secreted in the classical sense. They are probably transported to the cell surface in association with heparan sulfate proteoglycans, although there is no immunocytochemical evidence for their cell-surface localization in adult mammalian brain in this context. The working hypothesis is that regulation of FGF bioactivity takes place at the level of intracellular translocation so that the peptides are expressed, but not functional, under certain conditions. The association of FGFs with heparan sulfate proteoglycans in the natural environment stabilizes the peptide tertiary structure, preventing enzyme degradation and influencing the interaction of FGFs with their receptor. Both FGFs contain heparin-binding domains, but heparin influences the biological potency of aFGF more than bFGF in vitro, suggesting that the acidic peptide or its ligand-receptor complex exhibits a lower stability than the basic peptide in the absence of heparin.

Both FGFs bind with very high affinity to a single membrane receptor site that posesses tyrosine kinase activity in common with several other growth factor receptors. The mitogenic response to FGFs is elicited following the phosphorylation of a 80,000–90,000 Da cytoplasmic protein and probably the activation of protein kinase C. Evidence also suggests that the receptor is internalized and binds to DNA. FGFs cause quiescent cells to move to the G_0/G_1 stage of the mitotic cycle. Although glial cells are responsive to high concentrations of FGFs, it is likely that the FGFs are naturally targeted to neurons during the period of neuronal proliferation early in the embryogenesis of the central nervous system. There is evidence to support both the hypothesis that neuronal cell division is regulated by FGFs in an autocrine manner and that it is somehow regulated in a paracrine manner by astrocytic bFGF. The latter hypothesis must be extended to include the possibility of autocrine action within the astrocyte and the release of a secondary astrocyte-derived growth factor.

The response of neurons to FGFs lasts into the postmitotic phase during which the FGFs may promote the survival and stabilize the differentiated form of maturing neurons in different parts of the brain and peripheral nervous system. Early and late phases of the neuronal FGF response can be identified. These are exemplified by various transcription-dependent and independent processes that closely resemble those elicited by Nerve Growth Factor. In vivo, FGFs promote the survival of neurons undergoing postlesion degeneration. Thus, there is evidence that they can replace both serum in vitro and neuronotrophic influences in vivo to maintain the viability of developing and regenerating nerve cells.

The implication of these findings is that FGFs may contribute to the process of neural repair in the adult central nervous system. The mechanism of the in vivo neural response to FGFs is not yet understood, but will be clearer when the nature of bioregulation and precise sites of action of the FGFs in the nervous system is determined. The regulation of biosynthesis and processing of extracellular matrix components may be of greater importance than FGF gene expression in this respect. Since the FGFs are almost ubiquitous in the brain, as in many other tissues, their biological status during development, injury, and repair of neural cells may be regionally controlled by the nature and degree of their association with sulphated proteoglycans.

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