Mini-Review

Recent Developments in the Cell Biology of Basic Fibroblast Growth Factor

Daniel B. Rifkin and David Moscatelli

Department of Cell Biology and the Kaplan Cancer Center, New York University Medical School and the Raymond and Beverly Sackler Foundation, New York 10016

In the past four years there has been an enormous increase in research involving basic fibroblast growth factor (FGF)[1] as a result of the development of effective methods for the isolation of the protein and the availability of characterized nucleic acid probes, specific antibodies, and recombinant growth factor. Several reviews have been published describing much of the basic biology of acidic FGF (aFGF) and basic FGF (bFGF) (5, 25, 33). Therefore, in this mini-review we have focused on specific areas in bFGF biology in which uncertainties exist. For simplicity, we have confined this review to bFGF. However, some of the questions concerning bFGF also apply to aFGF and are discussed.

History

FGF was originally identified as an activity in extracts of pituitary and brain that stimulated the growth of 3T3 cells (3, 23). The activity was shown to be due to two proteins. One of them, aFGF, had an acidic pI (5.6) and eluted from heparin-Sepharose with 1 M NaCl (34, 65). The second, bFGF, had a basic pI (>9.0), eluted from heparin-Sepharose at 1.5 M NaCl, and had 55% sequence homology to aFGF (14).

The introduction of heparin-affinity chromatography facilitated the isolation of sufficient quantities of bFGF for structural studies. Bovine pituitary bFGF was found to be a 146-amino acid protein with a molecular weight of 16,400 (14), while human placental bFGF had 157 amino acids with a molecular weight of 17,500 (63). However, smaller forms of both bovine and human bFGF have been identified which apparently arise by truncation at the amino terminus (43). The shortest molecule that retained biological activity lacked the first 15 amino acids present in bovine pituitary bFGF (14). In some instances these truncated forms of bFGF seem to be generated by proteases released during the isolation procedure (31). The 146-amino acid bovine bFGF may, itself, derive from a larger molecule since extraction of pituitaries in the presence of protease inhibitors yielded a larger form (66). This idea is supported by data on the potential translation initiation sites identified from the cDNA for bFGF (see below).

The amino acid sequence of bFGF has some unusual attributes. bFGF contains four cysteines. Two are conserved among all members of the FGF family (71) and may be important in determining the tertiary structure of bFGF through intramolecular disulfide bonds. The two remaining cysteines are not essential for biological activity (19). In recombinant bFGF produced in *Escherichia coli*, these two cysteines appear to remain reduced (19). However, the two nonessential cysteines in natural bFGF may be involved in disulfide bonds with free cysteines since the initial amino acid analysis of bovine pituitary bFGF yielded six cysteines (14).

bFGF has two sequences (residues 18–22 and 107–110 of bovine pituitary bFGF) that have the characteristics of heparin binding domains (14), and synthetic peptides that include these amino acid sequences bind heparin (6). However, peptides that include neighboring sequences also bind heparin (sometimes with a higher affinity) suggesting that the heparin-binding activity is not restricted to simple domains (6). The protective effect of heparin against denaturation as well as proteolytic degradation of bFGF (24, 55, 62) indicates that significant changes in the tertiary structure of the protein must occur when it is complexed to this polysaccharide. The precise nature of the heparin-bFGF interactions remain to be characterized.

Analysis of a genomic clone of human bFGF revealed that the bFGF gene is interrupted by at least two introns and spans over 40 kb (2). The human genome contains one copy of the bFGF gene located on chromosome 4 (35). From two to five mRNA species that cross react with probes for bFGF have been described (1, 32) and differ in the length of their 3′ untranslated regions (32).

Synthesis

The biosynthesis of bFGF appears to involve a relatively unique mechanism. The bFGF first isolated from the bovine pituitary began with the amino acid sequence Pro-Ala-Leu-Pro (14). bFGFs from several other tissues and cell lines were also isolated and characterized shortly thereafter. Amino acid sequence studies indicated that degradation could take

1. *Abbreviations used in this paper*: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; FGF, fibroblast growth factor; GAG, glycosaminoglycans; HSPG, heparan sulfate proteoglycans.

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place during isolation as both shorter and longer forms of bFGF were found (see above). When the bovine pituitary bFGF amino acid sequence was compared with the amino acid sequence predicted from the cloned bFGF cDNA, a possible ATG (Met) initiation codon was found at position −9 from the CCC codon corresponding to the amino terminal Pro (1, 32). Therefore, this ATG codon was proposed as the initiation site for bFGF translation. However, amino acid sequencing of the placental form of human bFGF yielded a protein with an amino terminal sequence Gly-Thr-Met-Ala-Ala-Gly-Ser-Ile-Thr-Thr-Leu-Pro-Ala-Leu-Pro which agreed with the sequence predicted from the cDNA and indicated that additional higher molecular mass forms of bFGF must exist (63). Indeed, Moscatelli et al. (42) isolated a 25-kD form of bFGF from guinea pig brain. This protein was as active as 18 kD human bFGF, was cross reactive with antibodies to 18-kD human bFGF (42), and was extended at the amino terminal end (63a). Western blots and immunoprecipitations from numerous cell lines always reveal bFGF forms with molecular weights of 24, 22.5, and 22 kD in addition to the 18-kD form (unpublished observations). These higher molecular mass forms are as abundant as the 18-kD form, and pulse-chase data shows no conversion of the higher molecular mass forms into the 18-kD form. The mechanism of generation of these higher molecular mass forms of bFGF is not obvious since no additional ATG codons occur in the cDNA 5′ to the originally proposed ATG initiation codon.

In an elegant series of experiments, Florkiewicz and Sommer (16) have demonstrated translational initiation at CUG (Leu) codons 5′ to the AUG (Met) codon. In COS cells transfected with cDNA in which the ATG codon was changed to GCT, only bFGF species with molecular masses of 22, 22.5, and 24 kD were formed. The insertion of a frame-shift mutation 5′ to the ATG codon eliminated the synthesis of the 22-, 22.5-, and 24-kD forms, while the specific mutation of the CTG codon −167 to −165 to CTT (Leu) resulted in the loss of the 24-kD form. Similar results were obtained by Prats et al. (48). These results must be confirmed by additional protein sequencing of the higher molecular mass forms. The biological consequences of translation initiation at CUG and the synthesis of multiple forms of bFGF are not obvious but can be explored in cells stably transfected with unique cDNAs. Arg levels in the amino terminal extensions may be as high as 29%. This may confer unique properties to these proteins. Unpublished observations (Renko, M., and D. B. Rifkin) on the subcellular distribution of the 18-, 22-, 22.5-, and 24-kD forms of bFGF indicate that the forms are not equally distributed within subcellular compartments. The 18-kD form is primarily cytoplasmic, whereas the other forms are not. Thus, the forms of bFGF may differ functionally as well as structurally. Biological experiments demonstrating functional differences have not been described.

Distribution

bFGF has been found in all organs, solid tissues, tumors, and cultured cells examined (5, 25, 33). Although bFGF was reported to be present in serum (38), the validity of the assay used has been questioned (21). It is unclear whether the ubiquitous distribution of bFGF in cultured cells represents its in vivo distribution. In cultures of bovine endothelial cells, bFGF has autocrine activities which may be required for their maintenance (57). Thus, the expression of bFGF may be an adaptive response that allows cell survival in culture. Clearly, the limited number of immunolocalization studies that have been published show a restricted distribution of the antigen in vivo (18, 26, 28), consistent with the conjecture that the wide-spread occurrence of bFGF in cultured cells may be artifactual.

Biological Activities

At the present time, the normal function(s) of bFGF is unknown. In vitro, the molecule displays a broad spectrum of activities on many types of cells, including increased growth, induction of plasminogen activator, interstitial and type IV collagenase, and increased cell migration (25, 37, 41, 50). In vivo, the administration of exogenous bFGF induces a variety of responses. bFGF causes a rapid neovascularization in the cornea, kidney capsule, or skin as well as fibroplasia in the dermis (9, 17, 27, 59), supporting the hypothesis that it is involved in neovascularization and wound repair.

Perhaps, the most intriguing role proposed for bFGF is that of an inducer during embryonic development. In 1979, Mescher and Gospodarowicz (36) proposed that FGF enhanced limb regeneration in amphibians. More recently, Slack et al. (61) observed that purified bFGF mimicked the mesenchymal inducer in *Xenopus* embryos. In these experiments, the application of purified bFGF to ectodermal explants of early *Xenopus* embryos induced the differentiation of mesenchyme. In addition, when the animal and vegetal pole explants were separated by a nylon gauze, the inclusion of heparin in the assay blocked the normally observed induction of mesoderm formation in the animal pole explant. The authors concluded that under these conditions binding to heparin blocked endogenous bFGF from interacting with its receptor. The reports that bFGF mRNA and protein are present in *Xenopus* oocytes and early embryos (30, 60) support the hypothesis that endogenous bFGF may act as an embryonic inducer.

The major question which persists in studies with bFGF is whether results obtained after the addition of exogenous bFGF represent the normal role of this protein. Several members of the FGF family may share a common receptor (see below), and the addition of bFGF to a system which uses any of these proteins may induce a response. Since bFGF seems to be inefficiently released by cells, its role in extracellular inductions during embryonic development or tissue proliferation is not obvious. One of the members of the FGF family that is secreted, such as hst/K-fgf or FGF-5 (see below), may be more suited to such a role. One approach to this problem is through the use of reagents, such as antibodies, to neutralize the activities of specific members of the FGF family. The ability of neutralizing antibodies to bFGF to block an endogenous in vivo process would be a powerful demonstration of a natural role for this growth factor. This is a problem when considering the normal role of all growth factors and has only been answered successfully for nerve growth factor.

Related Proteins

bFGF is a member of a family of related proteins or gene products. In addition to aFGF (34), two other homologous proteins have been identified. The hst/K-fgf gene product is

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a 206–amino acid protein which has a 43% homology to bFGF. The hst/K-fgf gene was identified by transfection assays as an oncogene from a Kaposi's sarcoma (10) and independently from a human stomach tumor (64). The protein is a potent mitogen for fibroblasts and endothelial cells, increases the production of plasminogen activator by these cells and, like aFGF, is potentiated by heparin (11, 12). FGF-5, a 267–amino acid protein that is 43% homologous to bFGF, was identified after transfection of NIH-3T3 cells with tumor cell DNA followed by selection in growth factor deficient medium (72). FGF-5 is mitogenic for both 3T3 and endothelial cells and binds to heparin. The fifth member of the family is int-2 (13). The synthesis of int-2 mRNA is enhanced in MMTV-induced tumors, presumably as a result of the integration of the virus adjacent to the int-2 gene and increased transcription driven by the viral promoter. The protein encoded by int-2 mRNA has not been isolated, but the predicted sequence has a 44% homology to bFGF. When compared with bFGF, both the int-2 and FGF-5 proteins contain extensions at their carboxy-terminal ends, while hst/K-fgf has an extension at its amino terminal end. Both hst/K-fgf and FGF-5 are initially translated with hydrophobic signal sequences and are secreted into the culture medium of transfected cells. A classic signal sequence does not appear to be present in the predicted int-2 translation product. In this respect int-2 resembles bFGF and aFGF. While the expression of these bFGF-related proteins has been studied primarily in tumor cells, it is reasonable to expect that synthesis in normal cells will be found. The regulation of hst/K-fgf in differentiating teratocarcinoma cells (67) and int-2 in the mouse embryo (70) indicates that the expression of these proteins may be required for specific developmental events, but this remains to be definitively proven.

Transforming Potential

Within the past year, several papers have appeared demonstrating that high expression of 18-kD bFGF in NIH-3T3 (8, 53), BALB/c 3T3 (56), or BHK-21 (46) cells yields a transformed phenotype. Rogelj et al. (53) were the first to demonstrate that transfection of NIH-3T3 cells with bFGF expression vectors yielded transformed cells. They found, however, that transformation occurred only with a bFGF construct that contained the immunoglobulin signal sequence fused 5′ to the bFGF coding sequence. Transfection with constructs coding only for bFGF yielded no transformants. Similar results were observed by Blam et al. (8) using a bFGF cDNA fused to the growth hormone signal sequence cDNA. Other groups observed growth in low serum, increased saturation density, and growth in soft agar with cells transfected with bFGF expression constructs lacking a signal sequence (46, 51, 56). Interestingly, in no instance was >1% of the 18-kD bFGF found in the culture medium, even in the transfectants studied by Rogelj et al. (53). In these experiments, the inclusion in the medium of neutralizing antibodies to bFGF had no effect on the phenotype with the exception of the study of Sasada et al. (56) in which a small alteration in morphology and a decrease in growth in soft agar was observed. Thus, transformation by bFGF may result from the protein acting intracellularly as described for the sis oncogene (7) or from extracellular protein which cannot be neutralized by antibody.

bFGF does not appear to be a potent transforming factor, however. The amount of bFGF produced in transformants is quite high and may approach 0.1% of the total cellular protein. The amount of bFGF synthesized by transfectants also appears to correlate directly with the phenotype of the cell, with low producers having a nontransformed morphology and high producers having a transformed morphology (51). A comparison of the amounts of bFGF and hst/K-fgf required to transform NIH-3T3 cells revealed that 10–100× more bFGF than hst/K-fgf was necessary (51). This may relate to the fact that hst/K-fgf is a secreted protein and is efficiently released, while bFGF is not. Therefore, the differences observed by various groups in the transforming potential of bFGF may reflect differences in expression levels. Constructs without a signal sequence will transform only when expressed at high levels, while constructs with a signal sequence can transform at lower levels of expression. Thus, it is unlikely that enhanced bFGF expression is a primary event in the etiology of natural tumors since sufficient levels of expression would be difficult to achieve.

Receptors

Receptors for bFGF have been identified on a variety of cultured cells (39, 44, 47). Binding affinity for bFGF has been estimated to be 2×10^{-11} M to 2×10^{-10} M. The number of receptors per cell ranges from ~3,000 per cell for bovine aortic endothelial cells (unpublished observations) to 80,000 per cell for BHK cells (44). There seems to be an approximate inverse relationship between the number of receptors per cell and the content of endogenous bFGF (39), suggesting that receptors are down-regulated by endogenous growth factor. Chemical cross-linking of labeled bFGF to cell surface proteins has identified two putative receptors with molecular weights of 125,000 and 145,000. The relationship between the two species of receptor is presently not understood.

Receptors for aFGF also have been identified on a variety of cells (20, 29). bFGF will compete for the binding of aFGF to its receptors, and aFGF will compete for the binding of bFGF to its receptors (45, 47). These results suggest that the two molecules share receptors, although there is evidence that the 145-kD receptor has a stronger affinity of bFGF, while the 125-kD receptor has a stronger affinity for aFGF (45). Recent work indicates that a third member of the FGF family, the hst/K-fgf protein, also shares receptors with bFGF (Newman, K. M., and D. Moscatelli, unpublished observations). These results suggest that the members of the FGF family may have overlapping affinities for the FGF receptors.

Release

Considerations of the action of bFGF must be tempered by the fact that the mechanism for its release is not understood. This is also true for aFGF as well as interleukin-1. All of the translational products of bFGF mRNA appear to lack a signal sequence which would direct their release via the normal secretory pathway. The first nine amino acids in the 18-kD form of bFGF, which were missing from the original isolates of bovine bFGF and are slightly hydrophobic, have been suggested to function as a signal (1). However, this sequence is probably too short to act as a classical signal in vectorial translation. While a few reports have described the occur-

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rence of soluble extracellular bFGF (46, 58), the concentrations have always been so low that release by cell death cannot be excluded.

Soluble extracellular bFGF may always be present at low concentrations, however, since neutralizing antibodies to bFGF alter several in vitro cellular properties. These include cell morphology, plasminogen activator synthesis, cell migration, and growth in soft agar (56, 57). These results indicate that bFGF may act as an autocrine or paracrine factor. While the possibility that the neutralizing antibodies react with a second uncharacterized antigen that shares an epitope with bFGF and controls the phenomena studied has not been rigorously eliminated, the simplest explanation of these observations is via released bFGF. However, no defined mechanism for release has been described other than cell death. Whether this is the only mechanism for release is one of the more important questions in FGF biology.

Matrix Interactions

The strong interaction of bFGF with heparin probably reflects an in vivo affinity of bFGF for extracellular matrix (ECM) heparan sulfate proteoglycans (HSPG) and glycosaminoglycans (GAG) (55, 68). Several groups have shown that bFGF is found in the ECM (4, 18, 69). In this state, the growth factor appears to be bound to HSPG since it is solubilized by heparinase treatment (69). While the overall binding of bFGF to HSPG is not as strong as to heparin, subspecies of HSPG exist with binding affinities for bFGF equal to that of heparin (55). Immunohistochemical staining of basement membranes such as Descemet's membrane has revealed the presence of bFGF in these structures (18) indicating that bFGF may be concentrated in the ECM in vivo as well as in vitro.

The biological significance of this interaction is not clear, but several roles for the bFGF–HSPG interaction have been suggested. Folkman et al. (18) and Vlodovsky et al. (69) have proposed that bFGF may be stored in these extracellular structures and mobilized when needed by remodeling of the basement membrane or ECM through the production of hydrolases. Moscatelli (39, 40) has shown that exogenously added bFGF equilibrates between matrix binding sites and plasma membrane receptors and that removal of bFGF from its high affinity receptor with low pH results in a reequilibration of bFGF from the matrix with the receptor. Thus, matrix binding of bFGF may provide a reservoir of growth factor.

In a recent series of experiments, Presta et al. (49) and Flaumenhaft et al. (15) demonstrated that the extended action of bFGF on the production of plasminogen activator by endothelial cells after a brief exposure to growth factor requires the presence of matrix-bound bFGF. Both of these groups observed that transient (10–60 min) exposure of cells to growth factor resulted in long term stimulation of plasminogen activator production if the bFGF was simply removed from the cultures by washing with PBS. However, if the growth factor was removed by conditions that stripped bFGF from the ECM, no stimulation of protease activity was observed. These observations were interpreted as indicating that the matrix-bound bFGF provides a continuous source of ligand for the bFGF receptor and that continuous occupancy of the receptor is required for long term (24-h) stimulation. Similar results were obtained when DNA synthesis was measured after a brief exposure of cells to bFGF (15).

Gospodarowicz and Cheng (24) demonstrated that the interaction of bFGF with heparin stabilizes the protein to thermal denaturation and extremes of pH. When bFGF is bound to either heparin or heparan sulfate, it is also protected against degradation by trypsin or plasmin (55, 62). Heparin has also been shown to protect aFGF from plasmin degradation (54). Heparin interaction may be important in stabilizing bFGF to proteases generated during wound healing and tumor growth: processes in which bFGF may play a role.

Vlodavsky et al. (69) have shown that bFGF can be released from the ECM by the action of heparinase, while Saksela and Rifkin (unpublished observations) have found that plasmin releases bFGF complexed to HSPG or GAG. In the case of plasmin, the bFGF is released as a noncovalent complex of bFGF and HSPG–GAG. bFGF complexed to heparin or HSPG appears to interact with its receptor in an identical manner to free bFGF (39, 55). Since both heparinase and plasmin may be present at sites of tissue remodeling or growth, they may afford a mechanism for enhanced bFGF release. A question that remains is what are the relative contributions of heparinase and plasmin to bFGF release.

These studies have suggested the following model for bFGF interaction with cells and matrix. Upon release from the cell, the growth factor binds to HSPG. The bFGF bound to the matrix may be mobilized either by heparinase or by plasmin and is released as a complex of either bFGF–HSPG or bFGF–GAG. This complex is capable of diffusing through the stroma to the target cells and binding to the high affinity plasma membrane receptor. This model has several attractive features. It provides for a constant extracellular source of bFGF under conditions where the initial release of the growth factor may be as a bolus after cell death. The solubilization from the ECM of bFGF as a complex with HSPG or GAG yields a form of bFGF that is stable to denaturation and proteolytic degradation. The bFGF–HSPG or bFGF–GAG should diffuse away from the matrix more readily than free bFGF since bFGF–heparin or bFGF–HSPG complexes do not bind to the ECM (39, 55). While the biological significance of bFGF–matrix interactions remains to be proven, a functional requirement for interaction with matrix may exist for other growth factors. For example, two recent reports describe a role of HSPG in localizing colony-stimulating factors to the stromal cell matrix where interaction with stem cells occurs (22, 52).

Conclusions

In this review we have described a number of the features of bFGF. We have focused on specific aspects of bFGF biology that are of particular interest. bFGF has several unusual properties not associated with other growth factors that indicate that it must act in a unique fashion. Foremost among these is its lack of a signal sequence and intracellular localization. The intracellular localization of this growth factor raises questions concerning how and when bFGF is released. Moreover, the existence of hst/K-fgf and FGF-5 suggests that processes requiring a secreted molecule with FGF-like activity may use one of these factors and that bFGF has a different function. Thus, the lack of a signal sequence may be crucial to the specific biological role of bFGF. The generation of multiple forms of bFGF through translation initiation at both CUG and AUG codons is highly unusual and implies that the

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various bFGF species may have different functional roles. Finally, the strong interaction of bFGF with matrix HSPG may be indicative of a novel type of extracellular functional control which has not been described for other growth factors.

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