Differential Effects of Heparin, Fibronectin, and Laminin on The Phosphorylation of Basic Fibroblast Growth Factor by Protein Kinase C and the Catalytic Subunit of Protein Kinase A

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Abstract. Basic fibroblast growth factor (FGF) is synthesized as a phosphoprotein by both bovine capillary endothelial and human hepatoma cells in culture. Because basic FGF is characterized by its high affinity for heparin and its association in vivo with the extracellular matrix, we examined the possibility that the phosphorylation of this growth factor by purified protein kinase C (PK-C) and the catalytic subunit of cAMP-dependent protein kinase-A (PK-A) can be modulated by components of the extracellular matrix.

Heparin and other glycosaminoglycans (GAGs) inhibit the ability of PK-C to phosphorylate basic FGF. In contrast, heparin can directly increase the phosphorylation of basic FGF by PK-A. While fibronectin, laminin, and collagen IV have no effect on the ability of PK-C to phosphorylate basic FGF, they all can inhibit the effects of PK-A. Thus, there is a differential effect of extracellular matrix-derived proteins and GAGs on the phosphorylation of basic FGF. The enhanced phosphorylation of basic FGF that is mediated by heparin is associated with a change in the kinetics of the reaction and the identity of the amino acid targeted by this enzyme. The amino acids that are targeted by PK-C and PK-A have been identified by phosphopeptide analyses as Ser^{64} and Thr^{112}, respectively. In the presence of heparin, basic FGF is no longer

phosphorylated by PK-A at the usual PK-A consensus site (Thr^{112}), but instead is phosphorylated at the canonical PK-C site (Ser^{64}). Accordingly, heparin inhibits the phosphorylation of basic FGF by PK-C presumably by masking the PK-C dependent consensus sequence surrounding Ser^{64}. Thus, when basic FGF is no longer phosphorylated by PK-A in the receptor binding domain (Thr^{112}), it loses the increased receptor binding ability that characterizes PK-A phosphorylated basic FGF.

The results presented here demonstrate three novel features of basic FGF. First, they identify a functional effect of the binding of heparin to basic FGF. Second, they establish that the binding of heparin to basic FGF can induce structural changes that alter the substrate specificity of protein kinases. Third, and perhaps most important, the results demonstrate the existence of a novel interaction between basic FGF, fibronectin, and laminin. Although the physiological significance of this phosphorylation is not known, these results clearly suggest that the biological activities of basic FGF are regulated by a complex array of biochemical interactions with the proteins, proteoglycans, and glycosaminoglycans present in the extracellular milieu and the cytoplasm.

H EPARIN and related glycosaminoglycans (GAGs)[1] appear to be important regulators of the activity and binding of basic FGF. It is the discovery that basic FGF has a high affinity for heparin that first established the link between GAGs and this growth factor (20, 31). Since that

time, heparin has been demonstrated to protect the growth factor from enzymatic degradation and modulate its biological activities (19, 32). Even its binding to target cells has been linked to the presence of GAGs (28). As an example, two distinct binding components have been identified on the surface of basic FGF-responsive cells. The first is a high-affinity glycoprotein membrane receptor that appears to interact with both acidic and basic FGF (12, 29, 30). The second component is a cell surface GAG that is heparinase-sensitive (28), and thus considered to be heparin related. Recently, several groups have established that basic FGF is associated with the extracellular matrix and associated with heparin-like GAGs

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; FGF, fibroblast growth factor; GAG, glycosaminoglycan; PK-A and PK-B, protein kinase A and protein kinase B.

(2, 33). Although the functional significance of this discovery is currently the topic of extensive research, it has been suggested that the association of basic FGF with the basement membrane regulates the bioavailability of the growth factor (1, 16). For this reason, we have been examining the possibility that mechanisms exist to regulate the interaction between basic FGF and GAGs. In the course of these studies, we have discovered that basic FGF is in fact a phosphoprotein.

In a recent study we established (13) that basic FGF is a substrate for phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase-A (PK-A) and the phospholipid and calcium dependent protein kinase-C (PK-C) and suggested that these processes might be involved in the regulation of basic FGF. Although the physiological significance of phosphorylation remains unknown, basic FGF is phosphorylated by endothelial and hepatoma cells in culture (13), establishing that it exists in vivo as a phosphoprotein. Because the phosphorylation of basic FGF did not alter the growth factor's affinity for immobilized heparin, we examined the possibility that interactions with the extracellular matrix (ECM) might modify the phosphorylation of basic FGF. We report here that ECM-derived proteins and GAGs have differential effects on the capacity of PK-A and PK-C to phosphorylate the growth factor. The identification of a novel interaction between basic FGF, fibronectin, collagen, and laminin establishes that the growth factor interacts with the protein as well as with GAG-related components of the extracellular matrix.

Materials and Methods

Materials

Recombinant human basic FGF (4) was generously provided by Chiron Corporation. PK-C purified from bovine brain was a generous gift from Dr. J.-M. Pelosin (Unité INSERM 244, Grenoble, France), and PK-A from porcine skeletal muscle was a generous gift from Dr. S. Taylor (University of California, San Diego, CA). Fibronectin was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Laminin was obtained from E. Y. Laboratories, Inc. (San Mateo, CA), collagen IV, GAGs, and other reagents were purchased from Sigma Chemical Co. Trypsin-TPCK was purchased from Worthington (Freehold, NJ). $[\gamma^{32}P]ATP$ (3,000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA) and ^{125}I-Na from Amersham Corp. (Arlington Heights, IL).

Phosphorylation Assay

Aliquots of recombinant human basic FGF were incubated with purified PK-C or the catalytic subunit of PK-A for 10 min at 30°C in a 20-μl reaction volume containing 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM $[\gamma^{32}P]ATP$ (5,000 cpm/pmol) and the indicated amounts of GAGs and adhesion proteins. The buffers also contained 0.6 mM $CaCl_2$, 40 μg/ml phosphatidyl serine and 0.8 μg/ml dioctanoylglycerol in PK-C assays. The reactions were stopped by boiling for 2 min after the addition of 5 μl of a 5× concentrated Laemmli sample buffer (25). Phosphorylated proteins were separated on 0.8-mm-thick, 15% polyacrylamide SDS gels and visualized by autoradiography.

Specificity Studies

The possibility that the GAGs and ECM proteins were interacting directly with PK-C or PK-A to modify the phosphorylation of basic FGF was examined using histone H1 and histone IIA as substrates. The phosphorylations were performed as described above except that the reaction solutions contained 25 μg of histone H1 (PK-C) or 100 μg of histone IIA (PK-A). The incubations were stopped by precipitation with 30 μl of BSA (50 mg/ml) and 20% TCA. The pellet obtained by centrifugation was dissolved in 200 μl of 1 N NaOH and reprecipitated with 20% TCA. The solution was then centrifuged and the pellet resuspended in 100 μl of 1 N NaOH, neutralized with 1 N HCl, and counted.

Phosphoamino Acid Analysis and Tryptic Mapping

Radiolabeled peptides were extracted from polyacrylamide gels with 0.05 M ammonium bicarbonate pH 7.3–7.6 supplemented with 0.1% SDS and 1% 2-mercaptoethanol. After precipitation with 50% TCA, the pellet was dissolved in 6 N HCl and the protein was hydrolyzed for 60 min at 110°C. Phosphoamino acids were separated by two-dimensional high voltage electrophoresis as described by Cooper et al. (7). The plates were run for 20 min at 1.5 kV (pH 1.9) and then for 16 min at 1.3 kV (pH 3.5). Standard phosphoamino acids were mixed with the samples before electrophoresis and were visualized by ninhydrin staining. Radioactive phosphoamino acids were detected by autoradiography using films and intensifying screens (XAR5; Eastman Kodak Co., Rochester, NY). For tryptic mapping, the phosphoproteins were extracted from the gel, alkylated, and subjected to proteolytic cleavage as described by Hunter and Sefton (22). The phosphopeptides generated were resolved on 100-μm cellulose thin-layer plates by electrophoresis at pH 1.9 for 25 min at 1 kV in the first dimension followed by ascending chromatography in the second dimension. Approximately 500 Cerenkov cpm were routinely loaded per plate and the phosphopeptides were localized by autoradiography overnight using films and intensifying screens from Eastman Kodak Co.

Identification of the Sites of Phosphorylation

Peptide mapping of the fragments generated by the *Staphylococcus aureus* V8 protease digestion of recombinant human basic FGF was used to identify the sites of phosphorylation. Phosphorylated recombinant human basic FGF was prepared with PK-C, PK-A, of PK-A + heparin as described earlier. The reactions were stopped by heating at 80°C for 3 min. The solution was cooled, and basic FGF was immunoprecipitated with antibodies to basic FGF(1–24) conjugated to Affigel-10 beads (Bio-Rad Laboratories, Cambridge, MA). After mixing for 30 min, the solution was centrifuged and the pellet washed with 1.0 M NaCl in 10 mM Tris-Cl (pH 7.4). After a second wash, the pellet was resuspended in water, centrifuged and basic FGF was eluted from the pellet with 1 ml of 1 N acetic acid. A total of 150,000 cpm was recovered in the PK-C phosphorylated basic FGF and 500,000 cpm in the PK-A phosphorylated basic FGF and 160,000 cpm in the PK-A/heparin phosphorylated basic FGF.

Before enzymatic degradation, carrier recombinant human basic FGF (500 μg) was added to the samples of radiolabeled FGF and dissolved in 1 ml 0.2 M N-ethyl morpholine acetate, pH 8.6, 6 M guanidine-HCl, 3 mM EDTA, 1 mM DTT. After purging with argon, and incubating at 37°C for 1 h, the cysteine residues were pyridylethylated by the addition of 1 μl of 4-vinyl pyridine (Aldrich Chemical Co., Milwaukee, WI) to achieve a final concentration of ~40 mM. The samples were then purged with argon, and incubated at room temperature for over 2 h. The reaction was stopped by either the addition of 50 μl of 50 mM DTT, or by immediate desalting by reverse-phase chromatography on a 25.0 × 0.46 cm C-4 column.

The desalted samples, dried in vacuo, were dissolved in 100 μl 0.1 M ammonium acetate, pH 4.0, and digested by the addition of *Staphylococcus* V8 protease (1:20 mol, enzyme: basic FGF). The samples were incubated overnight at room temperature. An equal amount of protease in the absence of human basic FGF was incubated in parallel as a control. The peptides generated during the digestion were separated on a 25 × 0.46 cm C-18 reverse-phase column (Vydac). Samples were loaded onto the column in 100% Buffer A (0.1% TFA) and eluted with a 50-min linear gradient from 10 to 40% Buffer B (0.1% TFA in acetonitrile). Absorbance at 230 nm was monitored, and the peaks collected and identified by amino acid analysis. The identities of the peaks were deduced by comparison with the expected amino acid content of the predicted peptide products. In an effort to identify the fragments containing phosphorylated amino acids, aliquots of each column fraction were collected, counted, and matched to their corresponding peaks. Recovery of the radiolabel was 30–50% after reverse-phase HPLC.

Biological Assays of FGF Activity

Basic FGF was phosphorylated with the catalytic subunit of PK-A in the presence or absence of heparin under the conditions described above but with unlabeled ATP. Under these conditions, 0.6–0.8 phosphates are incorporated into basic FGF. Controls consisted of basic FGF treated under iden-
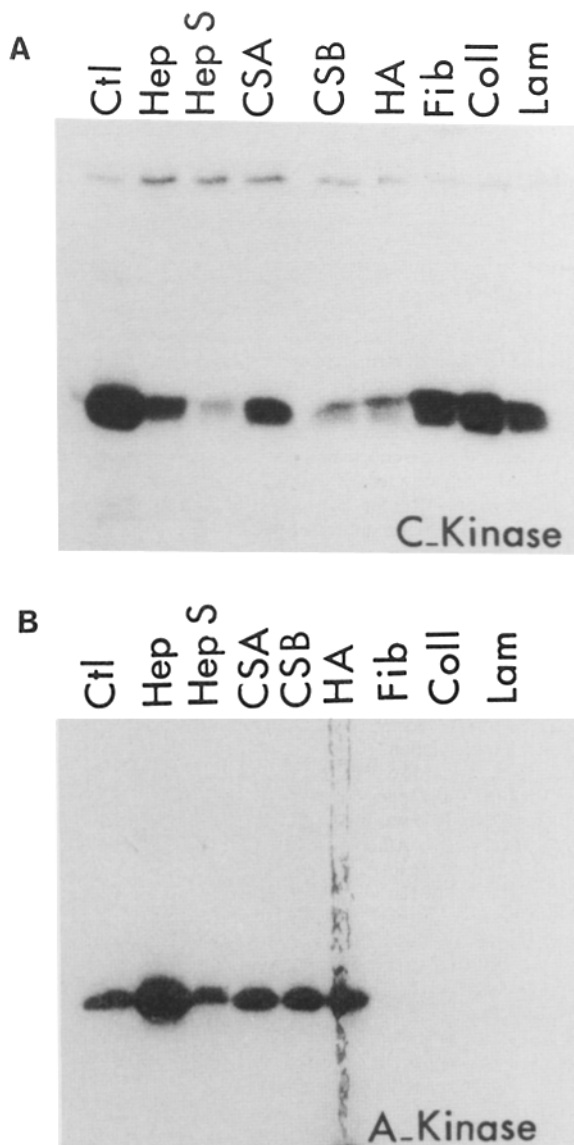
Figure 1. Effect of GAGs and adhesion proteins on the phosphorylation of human basic FGF by PK-C and PK-A. Recombinant human basic FGF (1 μg) was phosphorylated in the absence (*Ctl*) or presence of 5 μg heparin (*Hep*), chondroitin sulfate A (*CSA*), chondroitin sulfate B (*CSB*), hyaluronic acid (*HA*), collagen IV (*Coll*), laminin (*lam*), or of 3 μg fibronectin (Fib). *A,* Purified PK-C (40 ng), or *B,* purified PK-A (80 ng) were present in the reaction. After electrophoresis on a 15% polyacrylamide, 0.1% SDS gel, the radiolabeled proteins were visualized by autoradiography (XAR5 film, Eastman Kodak Co.).

tical conditions, but in the absence of kinase. The recovery of basic FGF from the reaction tubes was determined by the addition of trace amounts of radiolabeled growth factor. In these experiments, the phosphorylation of basic FGF with cold ATP was monitored by the addition of trace amounts of [γ^{32}P]ATP to the reaction mixture and autoradiography after SDS-PAGE confirmed the phosphorylation reaction. The phosphorylated mitogens were tested for their capacity to compete with the binding of ^{125}I-basic FGF to its high and low affinity receptor using the baby hamster kidney (BHK) cell assay described by Moscatelli (28). Serial dilutions of the phosphorylated FGFs and their respective unphosphorylated controls were added to BHK cells. After a 2-h incubation with 500 pg radioiodinated recombinant human basic FGF, the cells were washed twice with 1 ml PBS, twice with 0.5 ml of 2 M NaCl in 10 mM Tris-Cl, and then the membranes were solubilized with 0.1% Triton in PBS (pH 8.4) and counted on a γ-counter.

The effect of the PK-A-phosphorylated basic FGF was compared to a preparation of unphosphorylated basic FGF that was treated in an identical fashion as the phosphorylated protein except that no kinase was present in the reaction. Similarly, the effects of the PK-A/heparin-phosphorylated basic FGF was compared to an unphosphorylated basic FGF that was treated in an identical fashion as the phosphorylated protein including incubation buffer in the presence of heparin. Thus, the only difference between the phosphorylated and unphosphorylated forms tested in the receptor assay is the presence (or absence) of kinase in the binding medium. In a separate series of experiments, the various phosphorylated FGFs were prepared as described, and the "mock" phosphorylated basic FGFs were prepared in the absence of ATP. This approach gave identical results.

Results

GAGs and Basement Membrane Proteins Affect the Phosphorylation of Basic FGF by PK-C and PK-A

Recombinant human basic FGF was phosphorylated with purified PK-C or with the catalytic subunit of PK-A and a fivefold excess (wt/wt) of various GAGs or adhesion-stimulating proteins. As shown in Fig. 1 *A,* heparin, heparan sulfate, chondroitin sulfate A, chondroitin sulfate B, and hyaluronic acid reduced the amount of phosphate incorporated into basic FGF by PK-C. At the concentrations used, Hep-S, CSB, and HA were clearly the most effective, however, no dose curve analyses were performed. In contrast, fibronectin, collagen IV, and laminin had only small effects. When basic FGF was phosphorylated by the catalytic subunit of PK-A (Fig. 1 *B*), a very different profile was obtained. Heparin markedly increased the phosphorylation of basic FGF while the other GAGs had no apparent effect. Fibronectin, collagen IV, and laminin completely prevented the phosphorylation of basic FGF by PK-A.

A series of control experiments were performed to establish the specificity of these observations. First, we examined the possibility that the GAGs and proteins might be competing with basic FGF for phosphorylation. None of these molecules was found to be a substrate for either kinase (data not shown). Accordingly, an examination of the autoradiogram shown in Fig. 1 fails to show the presence of phosphorylated products other than the autophosphorylated kinase and basic FGF. Because GAGs are known to modulate the activity of casein kinases (14), we also tested the possibility that the GAGs or proteins were directly inhibiting PK-C/PK-A activity. The addition of any of these molecules to the reaction did not affect the ability of PK-C to phosphorylate histone H1 or that of PK-A to phosphorylate histone HIIA (Fig. 2). Therefore, we concluded that the effects outlined in Fig. 1 were because of direct interactions with basic FGF and not interactions with the kinases. Thus, the suitability of basic FGF as a substrate for PK-C decreases as a function of its interaction with GAGs. The interaction between basic FGF and proteins of the ECM results in a decrease in its suitability as a substrate for PK-A. Because one of the most remarkable effects of the GAGs and ECM proteins was the ability of heparin to increase the phosphorylation of basic FGF, it was further investigated.

Heparin Modifies the Kinetics of the Phosphorylation Reaction

The kinetics of the phosphorylation of basic FGF by the catalytic subunit of PK-A were examined to identify the mechanism through which the addition of heparin to the reaction

Effect of GAGs and ECM Proteins on the Phosphorylation of Histones

Histone I with PK-C
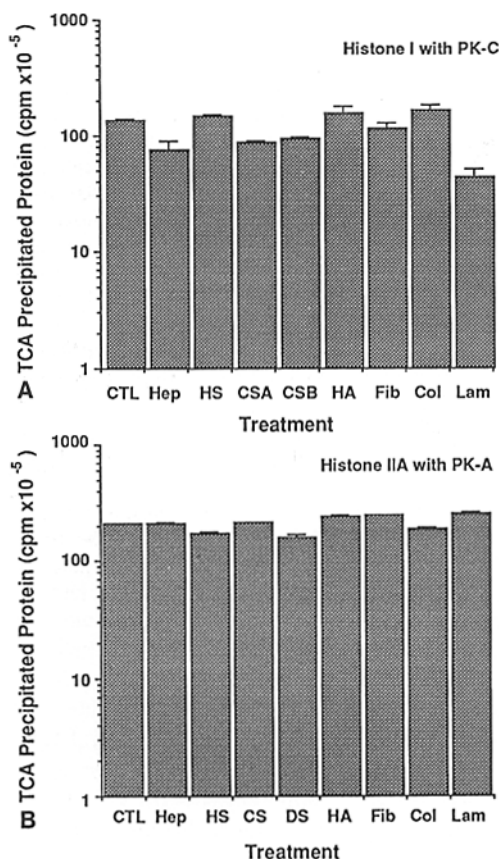
A

Histone IIA with PK-A

B

Figure 2. Effects of GAGs and ECM proteins on the phosphorylation of histones by PK-C and PK-A. *A*, Histone 1 (1.25 mg/ml) was incubated as described in the text with PK-C in the presence of heparin (*Hep*), heparan sulfate (*HS*), chondroitin sulfate A (*CSA*), chondroitin sulfate B (*CSB*), hyaluronic acid (*HA*), fibronectin (*Fib*), collagen IV (*Col*), laminin (*Lam*), or no additive (*Ctl*), and the amounts of radioactivity incorporated into TCA precipitable protein was measured by liquid scintillation counting. The K_m for the reaction is 150 μg/ml. *B*, Histone IIA was incubated as described in the text with the catalytic subunit of PK-A and the same various additives. Results are the mean of three determinations. The K_m of this reaction is 200 μg/ml.

mixture increases the phosphorylation reaction. A Lineweaver-Burke analysis of the reaction was carried out in the absence or presence of 50 μg/ml heparin (Fig. 3). Although the previous specificity studies established that the effects of heparin were because of interactions with basic FGF itself and not on the kinases, the K_m value for this reaction, while decreased, was only reduced from 17 μM in the absence of heparin to 13 μM in its presence. Thus, the binding of basic FGF to heparin had very little effect on the affinity of PK-A for basic FGF. In contrast, these concentrations of heparin dramatically increased the V_{max} of the reaction, suggesting that either the enzyme was in fact directly affected by the presence of heparin or that when bound to heparin, basic FGF was phosphorylated at a novel, distinct site. Because of these significant changes in the kinetics of phosphorylation, it was necessary to establish whether the site of PK-A dependent phosphorylation was changed by the presence of hepa-

rin. For this reason, the PK-C, PK-A and PK-A/heparin sites of phosphorylation were all identified.

Heparin Changes the Amino Acid Targeted by PK-A

In a previous study (13), we showed that human basic FGF is phosphorylated on a serine by PK-C and is phosphorylated by PK-A on a threonine. As expected from the consensus sequences that are required for phosphorylation, the sites are thus distinct. It was thus unexpected that when human basic FGF is phosphorylated by PK-A in the presence of heparin the site of phosphorylation is changed to a serine residue (Fig. 4 *B*). There is in fact no evidence for threonine phosphorylation and the partial hydrolysis products are remarkably different. In the case of PK-A/heparin (Fig. 4 *B*), the partially hydrolyzed products are very hydrophobic and dramatically different from those observed after phosphorylation by PK-A alone. Thus, the presence of heparin in the reaction buffer changes the site targeted by PK-A presumably by conferring a tertiary structure to the growth factor that reveals a cryptic site for PK-A dependent phosphorylation. It is presumably this change in the site of phosphorylation that accounts for the changes in V_{max} rather than K_m of the reaction observed earlier (Fig. 3).

Several methods were used to identify the sites of phosphorylation. Tryptic peptide mapping of the phosphorylated basic FGFs confirmed that the sites of PK-A-dependent phosphorylation in the presence or absence of heparin are different (Fig. 5). When basic FGF is phosphorylated by PK-A alone, only one major phosphopeptide is detected (Fig. 5 *A*). This peptide has considerable chromatographic mobility and has the features of a charged molecule. In contrast, when ba-
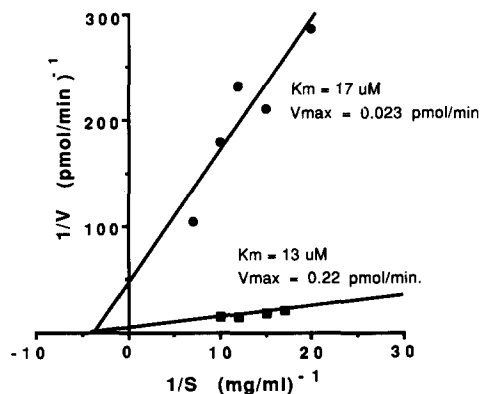
Km = 17 uM
Vmax = 0.023 pmol/min

Km = 13 uM
Vmax = 0.22 pmol/min.

Figure 3. Effect of heparin on the kinetic parameters of the phosphorylation of basic FGF by PK-A. Various amounts of recombinant human basic FGF (0.05–0.14 mg/ml; 1 mM = 18 mg/ml) were phosphorylated under the standard conditions described in Materials and Methods. The assay was performed in the presence of 4 μg/ml purified PK-A and in the absence (●) or presence (■) of 0.05 mg/ml heparin. After a 15-min incubation at 30°C, the reaction was stopped by 50% TCA precipitation. After addition of BSA as a carrier, the solutions were centrifuged. The pellets were dissolved in 0.1 ml in NaOH N and reprecipitated with TCA. The final pellets were dissolved in 0.1 ml in NaOH, neutralized with HCl, and the radioactivity content were determined by liquid scintillation counting. The results were plotted according to Lineweaver and Burke, and the kinetic parameters were calculated from each linear regression.
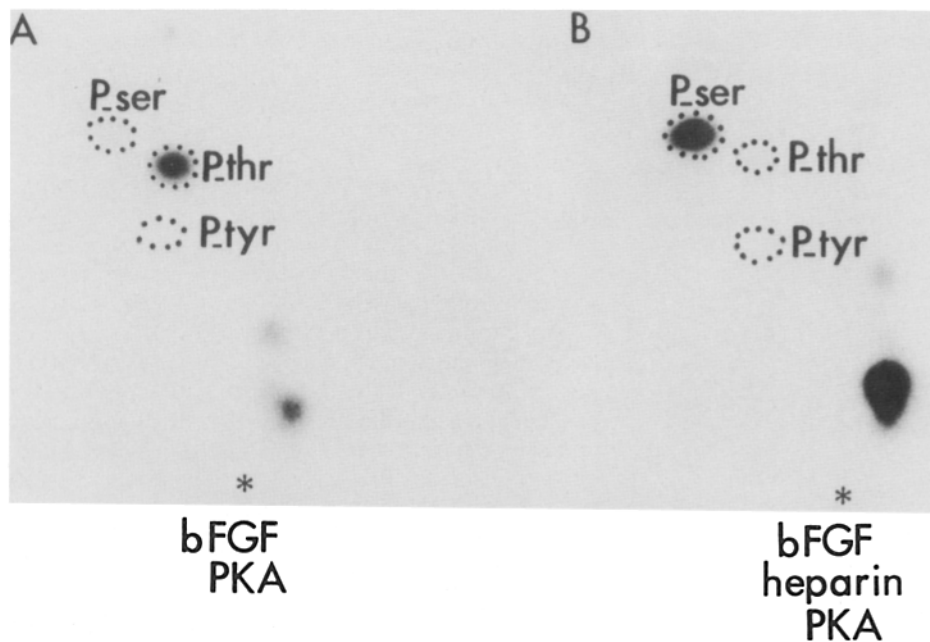
Figure 4. Phosphoamino acid analysis of human basic FGF phosphorylated by PK-A in the absence or presence of heparin. Recombinant human basic FGF was phosphorylated by PK-A in the absence (A) or presence (B) of heparin under the same conditions described in the legend to Fig. 1. The ³²P-labeled basic FGF was localized by autoradiography of the 15% SDS-PAGE, cut and extracted from the gel, hydrolyzed with HCl and analyzed by two-dimensional high voltage electrophoresis on cellulose thin-layer plates. Radiolabeled phosphoamino acids were identified after overnight autoradiography with intensifying screens. Asterisks represent the origin of migration of the samples, the circles show the position of the phosphoserine (P-ser), phosphothreonine (P-thr), and phosphotyrosine (P-tyr) standards. The results were obtained with 200 (A) and 500 (B) Cerenkov cpm.

sic FGF is phosphorylated PK-A/heparin, at least four phosphopeptides are detected (Fig. 5 B), all of which are considerably less hydrophobic than the one found with PK-A alone. The profiles are, in fact, clearly distinct as illustrated

when phosphopeptides are analyzed in combination (Fig. 5 C) suggesting that the sites of phosphorylation are not in physical proximity.

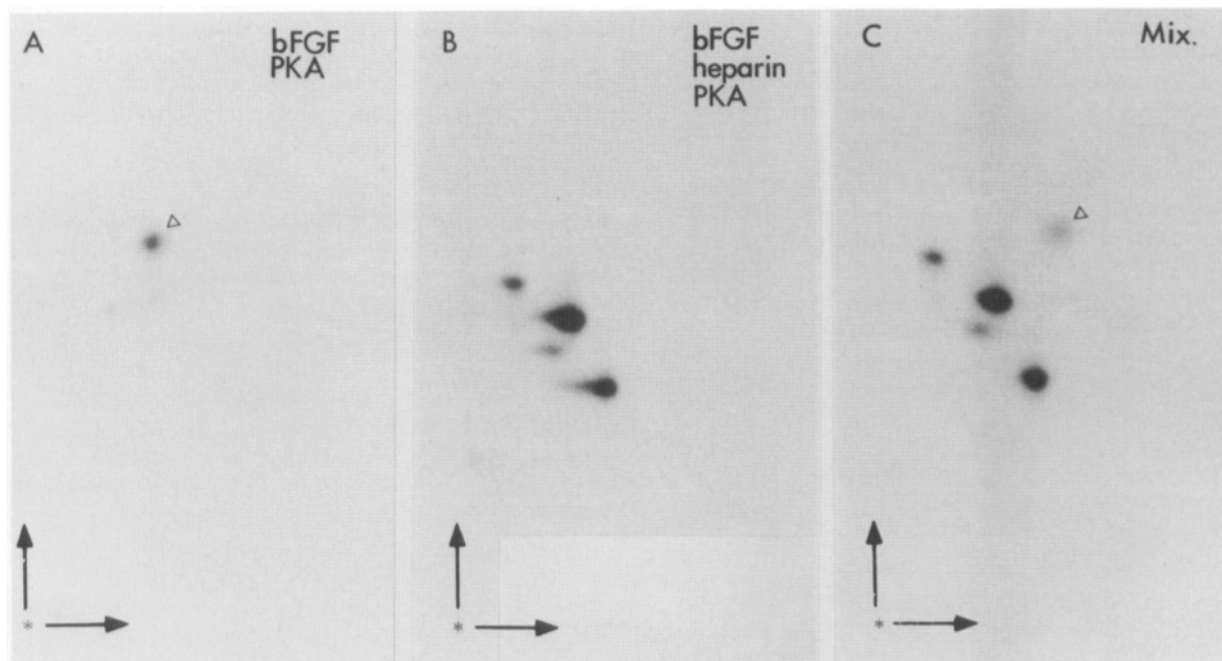An analysis of the tryptic peptide maps generated after the

Figure 5. Phosphotryptic mapping analysis of human basic FGF phosphorylated by PK-A in the absence or presence of heparin. Recombinant human basic FGF was phosphorylated by PK-A in the absence (A) or presence (B) of heparin under the same conditions described in Fig. 1. The ³²P-labeled basic FGF was localized by autoradiography after 15% SDS-PAGE, cut and extracted from the gel, digested extensively with TPCK-trypsin and the phosphopeptides were separated as described in the text. Asterisks indicate the points of sample application with ascending chromatography in the vertical direction. Electrophoresis of tryptic phosphopeptides generated from basic FGF phosphorylated in the absence of heparin (100 Cerenkov cpm, A), of basic FGF phosphorylated in the presence of heparin (500 Cerenkov cpm, B), and of a mixture of the phosphopeptides (C) was performed in the horizontal dimension with the anode on the left.

Figure 6. Comparative phosphotryptic mapping analysis of basic FGF phosphorylated by PK-C and PK-A/heparin. Recombinant human basic FGF was phosphorylated by PK-C (*A*) or by PK-A/heparin (*B*) as described in the text. The ^{32}P-labeled basic FGF was localized by autoradiography after 15% SDS-PAGE, cut and extracted from the gel and digested extensively with TPCK-trypsin as described in the text. The phosphopeptides were separated in two-dimensions after the application of 400 (*A*) and 600 (*B*) Cerenkov cpm. Asterisks indicate the points of sample application.

phosphorylation of basic FGF by PK-C shows a similar pattern to the PK-A/heparin phosphorylation and reveals the presence of highly hydrophobic radiolabeled peptides (Fig. 6 *A*). These peptides are characterized by the fact that they are phosphorylated on serine residues (Fig. 4) (13) and that they are hydrophobic and lack significant charge. The first interpretation of this finding was the surprising possibility that, in the presence of heparin, PK-A phosphorylates basic FGF at a PK-C consensus sequence. In an effort to determine if indeed the PK-C site and the PK-A/heparin site are one and the same, the peptides were analyzed by peptide mapping of human basic FGF on HPLC.

Identification of the PK-C, PK-A, and PK-A Heparin Sites of Phosphorylation

Staphylococcus aureus V8 protease digestion of recombinant human basic FGF generates five peptides (P[−8]–[+5]; P6–45; P46–58; P60–78; and P70–91) each of which contains a single serine residue (Ser[−5]; Ser9; Ser47; Ser64; and

V8 PROTEASE MAPPING OF RECOMBINANT HUMAN BASIC FGF

Figure 7. *Staphylococcus* V8 digestion of human basic FGF. Recombinant human basic FGF was resolved to nine major peptides after digestion with V8 protease by reverse phase HPLC. Peptides 5 and 9 contain classical PK-C sites of phosphorylation characterized by the S-X-K and S-X-R sequence shown. Peptide 9 contains two PK-A sites characterized by RXXT and KXXS. The numbering system corresponds to the 146 amino acid sequence reported by Esch et al. (10).
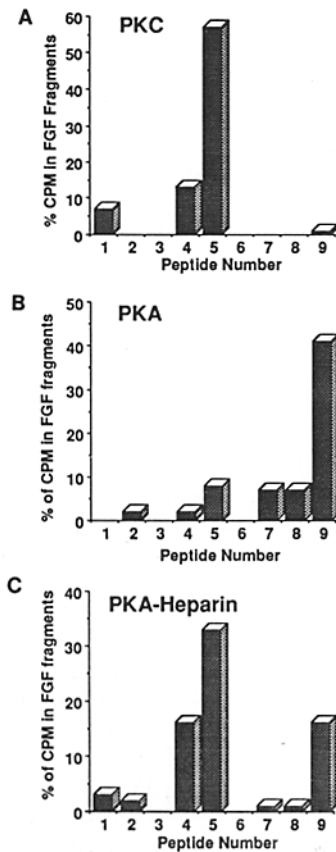
V8 Protease Mapping of Human Basic FGF

Figure 8. Distribution of radioactivity in the peptides generated by V8 protease digestion. The peptides generated by the enzymatic degradation of human basic FGF were resolved and identified by reverse phase HPLC and fractions were assayed for the presence of ^{32}P. Relative incorporation of label is expressed as the percentage of the total counts recovered.

Ser85, respectively) and a sixth C-terminal peptide (P100-146) containing 5 serine residues (Fig. 7). Phosphorylation was accomplished in vitro by incubation with PK-C, PK-A, or PK-A in the presence of heparin. For FGF phosphorylated by PK-C, the radioactivity eluted with a single peptide which by sequencing was identified as basic FGF(60-78) (Fig. 8 A). Because this peptide contains only one serine (at position 64), it was concluded that Ser[64] is the site of PK-C-dependent phosphorylation of basic FGF. A significant portion (15%) of the radioactivity also comigrated with a minor protein peak that was designated as the partial cleavage product P59-78 (see Fig. 7). Although this peptide is an unusual product that is not normally detected in the digests, it is possible that the phosphorylation of the Ser[64] makes the neighboring cleavage site less susceptible to digestion by the S. aureus V8 protease.

Unlike PK-C, PK-A phosphorylates basic FGF at a site in the C-terminus (P100-146, Fig. 8 B). The purified radiolabeled phosphopeptide contains four threonine residues of which only one (Thr[112]) meets the criteria of a PK-A-dependent consensus sequence (Fig. 7). We used the fact that bovine basic FGF has a substituted serine at this site (Ser[112]) to help identify Thr[112] as the residue phosphorylated by PK-A in human basic FGF. As such, when bovine basic FGF is phosphorylated by PK-A, a phospho serine is identified by phosphoamino acid analyses (Fig. 9 B).

Mapping of the basic FGF phosphorylated by PK-A/heparin established that heparin confers a new site of PK-A-dependent phosphorylation to basic FGF. Peptide maps (Fig. 8 C) gave results similar to those seen with PK-C and in agreement with the 2D-tryptic maps. The fact that the labeled peptide contains only 1 serine (Fig. 7) thus establishes the identity of Ser[64] as the site targeted by PK-A/heparin. Heparin thus conceals the site Thr[112] and induces a structural conformation to a PK-C site to make it compatible for PK-A phosphorylation.
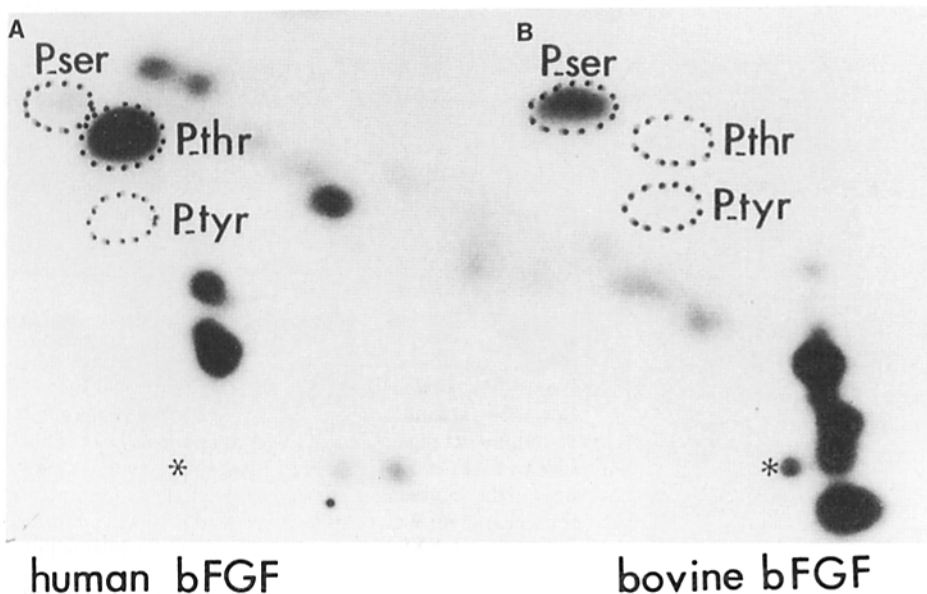

Figure 9. Phosphorylation of human and bovine basic FGF by the catalytic subunit of PK-A. The growth factors, by the substitution of Thr[112] for Ser[112] were phosphorylated according to Materials and Methods and the phosphoamino acids identified by two-dimensional, high voltage electrophoresis in cellulose thin layer plates.
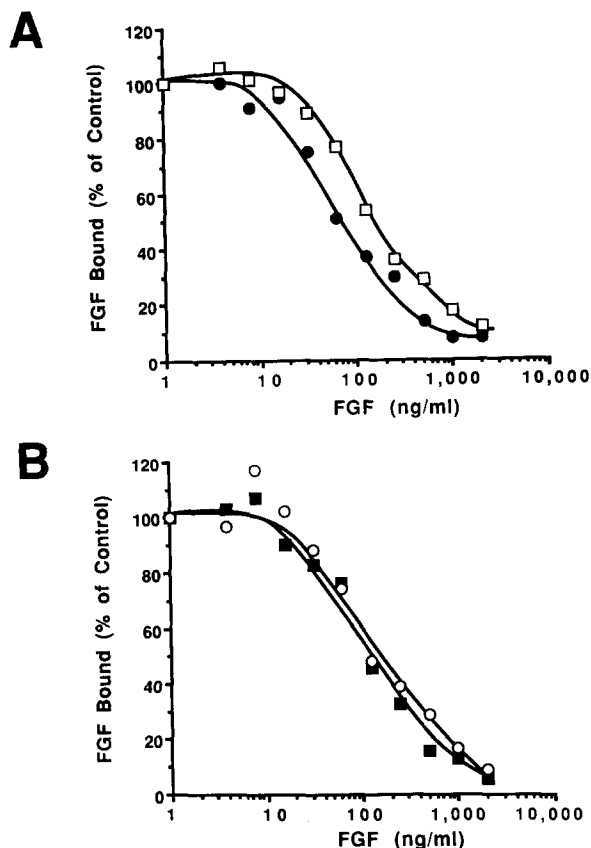
A

B

Figure 10. Effect of phosphorylation by PK-A and PK-A/heparin on the binding of basic FGF to its cell surface receptor. Recombinant human basic FGF (2.5 μg) was phosphorylated for 25 min at 30°C in the presence (●) or absence (□) of PK-A (*A*) and in the presence (○) or absence (■) of heparin (*B*), and each of the FGFs were tested for their capacity to displace [^{125}I]-basic FGF in a radioreceptor assay. The binding to low affinity sites was assessed in a 2 M NaCl wash of the cells and the binding to high affinity sites was measured in a Triton X-100 extract of the cells. No differences were seen in the low affinity sites and the results presented are those obtained for the high affinity receptor.

Heparin Modifies the Functional Effects of PK-A-dependent Phosphorylation

Previous studies in our laboratory had demonstrated that Thr^{112} is in the receptor binding domain of human basic FGF. Furthermore, its phosphorylation results in the generation of a form of basic FGF that has an increased capacity to displace [^{125}I]basic FGF from its receptor on BHK cells (13). We reasoned that if basic FGF is no longer phosphorylated in the receptor binding domain when it is phosphorylated by PK-A/heparin, it should be indistinguishable from recombinant FGF. We had previously shown just such an effect when basic FGF is phosphorylated by PK-C (13). As expected then, when basic FGF is phosphorylated by PK-A in the receptor domain, it is more potent than unphosphorylated recombinant basic FGF at displacing the radiolabeled ligand (Fig. 10 *A*). In contrast, this increased activity is not detected when basic FGF is phosphorylated by PK-A/heparin. Like PK-C–phosphorylated FGF, it is equipotent to the recombinant growth factor (Fig. 10 *B*). Thus, the failure of PK-A/heparin to phosphorylate basic FGF in its receptor

binding domain (Thr^{112}) prevents the generation of a functionally activated basic FGF.

Discussion

The results presented in Fig. 11 summarize the effects of GAGs and proteins derived from the ECM on the phosphorylation of basic FGF. Basic FGF is a substrate for both PK-A and PK-C. PK-A phosphorylates basic FGF in the receptor binding domain and this phosphorylated basic FGF has a greater affinity for its receptor on BHK cells. When basic FGF is associated with ECM proteins like fibronectin, laminin, and collagen, the site of phosphorylation is masked and the mitogen is no longer a substrate for PK-A. In the presence of heparin, basic FGF is phosphorylated by PK-A at a cryptic site that is not a PK-A consensus sequence. Phosphorylation at this site is dependent on the interaction between heparin and basic FGF. It is thus consistent that the changes conferred by heparin that make Ser^{64} recognizable by PK-A mask this sequence to the phosphorylation by PK-C.

In view of the fact that phosphorylation is generally regarded as an intracellular event (21), it would appear difficult to reconcile the findings reported here with a physiological role for heparin, phosphorylation, and the regulation of basic FGF activity. Recently however, there have been reports suggesting the existence of ecto kinases capable of phosphorylating proteins outside of the cell (8, 9, 24) and which might phosphorylate the basic FGF that is localized in the ECM. Furthermore, a series of intracellular heparinlike molecules have been detected (11, 17, 23) that could potentially be capable of modulating the phosphorylation of basic FGF inside the cell. Basic FGF is potentially available at multiple sites for phosphorylation. Clearly, it will be of paramount importance to establish the functional significance of this posttranslational change.

Among the substances tested, only heparin enhanced the phosphorylation of basic FGF. The increase in phosphorylation was because of an increase in the velocity of the reaction
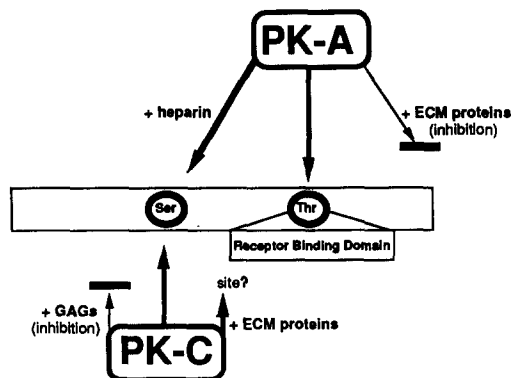
The Phosphorylation of Basic FGF

Figure 11. Summary of the effects of ECM components on the phosphorylation of basic FGF by PK-A or PK-C. The two sites of phosphorylation in the sequence of human basic FGF are shown in circles. For comparative purposes, the amino acid numbers are based on the sequence of basic FGF originally reported for bovine pituitary basic FGF.

and a change in the site of phosphorylation from Thr[112] to Ser[64]. Heparin thus induces conformational changes in basic FGF that result in the masking of the normal phosphorylation site and the unmasking of a new one. The fact that Ser[64] is phosphorylated by PK-C or PK-A/heparin agrees with the PK-C consensus sequence (ser/thr-x-arg/lys) proposed by Woodgett et al. (34), but does not correspond to the consensus sequence required for a PK-A site (6). The sequence (Fig. 7) surrounding the targeted serine (-gly-val-val-SER-ile-lys-) does not have any basic amino acid on the N-terminal side of the phosphorylated serine residue. Thus, heparin binding presumably alters the tertiary structure to basic FGF to turn a classical PK-C site (Ser[64]) into a PK-A site. Accordingly, in the presence of heparin these same residues lack the features of a PK-C consensus sequence, and basic FGF becomes a weak substrate for PK-C.

A close examination of the differential effects of the GAGs and ECM-derived proteins on the phosphorylation of basic FGF can suggest that sequences of basic FGF are interacting with each of these molecules. The ability of fibronectin, laminin, and collagen to inhibit the effects of PK-A but not PK-C suggests that they are associating themselves with the receptor binding domain of basic FGF and preventing the accessibility of PK-A to the consensus substrate sequence located around Thr[112]. This possibility is supported by the observation that the proteins have no effect on PK-C-catalyzed phosphorylation that targets sequences outside of this domain (Ser[64]). The effects of GAGs appear more complex, presumably because their interactions with basic FGF confer changes in its tertiary structure. These changes are suggested by the results here but also by the fact that heparin protects basic FGF from proteolysis and denaturation (19, 32). Both results are consistent with the observation that heparin has multiple binding sites on basic FGF (2).

The identification of a functional effect of heparin on the phosphorylation of basic FGF coupled with the demonstration of a novel interaction between basic FGF, fibronectin, and laminin emphasizes the potential role of components of the ECM in regulating the activity, stability, and storage of basic FGF (1, 2, 16, 33). While it remains premature to propose a specific physiological function for the phosphorylation of basic FGF, the results presented here would be consistent with a regulatory function. Certainly, the effects reported here establish the fact that basic FGF is interacting with a complex array of GAGs, proteoglycans, and basement membrane proteins in the ECM. It will thus be important to consider the contribution of each of these molecules in the regulation of this growth factor's activity in vivo and the possibility that they modulate phosphorylation dependent activities of the FGFs.

References

1. Baird, A., and P. A. Walicke. 1989. Fibroblast growth factors. Brit. Med. J. 45:458–452.
2. Baird, A., and N. Ling. 1987. Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implication for a role of heparinase-like enzymes in the neovascular response. Biochem. Biophys. Res. Commun. 142:428–435.
3. Baird, A., D. Schubert, N. Ling, and R. Guillemin. 1988. Receptor and heparin-binding domains of basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 85:2324–2328.
4. Barr, P. J., L. S. Cousens, C. T. Lee-Ng, A. Medina-Selby, F. R. Masiarz, R. A. Hallewell, S. Chamberlain, J. Bradley, D. Lee, K. S. Steimer, L. Poulter, A. L. Burlingame, F. Esch, and A. Baird. 1988. Expression and processing of biologically active fibroblast growth factors in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 263:16471–16478.
5. Bouché, G., N. Gas, H. Prats, V. Balsin, J. P. Tauber, J. Teissié, and F. Amalric. 1987. bFGF enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing G0-G1 transition. Proc. Natl. Acad. Sci. USA. 84:6770–6774.
6. Carlson, G. M., P. J. Bechtel, and D. J. Graves. 1979. Chemical and regulatory properties of phosphorylase kinase and cyclic AMP-dependent protein kinase. In Advances in Enzymology and Related Areas of Molecular Biology. A. Meister, editor. John Wiley & Sons Inc., New York. 41–115.
7. Cooper, J. A., B. M. Sefton, and T. Hunter. 1983. Detection and quantitation of phosphotyrosine in proteins. Methods Enzymol. 99:387–402.
8. Dusenberg, K. E., J. R. Mendiola, and K. M. Skubitz. 1988. Evidence for ecto-protein kinase activity on the surface of human neutrophils. Biochem. Biophys. Res. Commun. 153:7–13.
9. Ehrlich, Y. H., T. B. Davis, E. Bock, E. Kornecki, and R. H. Lenox. 1986. Ecto-protein kinase activity on the external surface of neural cells. Nature (Lond.). 320:67–70.
10. Esch, F., A. Baird, N. Ling, N. Ueno, F. Hill, L. Denoroy, R. Klepper, D. Gospodarowicz, P. Böhlen, and R. Guillemin. 1985. Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine acidic FGF. Proc. Natl. Acad. Sci. USA. 82:6507–6511.
11. Fedarko, N. S., and H. D. Conrad. 1986. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. J. Cell Biol. 102:587–599.
12. Feige, J. J., and A. Baird. 1988. Glycosylation of the basic fibroblast growth factor receptor. The contribution of carbohydrate to receptor function. J. Biol. Chem. 263:14023–14029.
13. Feige, J. J., and A. Baird. 1989. Basic fibroblast growth factor is a substrate for protein phosphorylation and is phosphorylated by capillary endothelial cells in culture. Proc. Natl. Acad. Sci. USA. 86:3174–3178.
14. Feige, J. J., F. Pirollet, C. Cochet, and E. M. Chambaz. 1980. Selective inhibition of a cyclic nucleotide-independent protein kinase (G-type casein kinase) by naturally occurring glycosaminoglycans. FEBS (Fed. Eur. Biochem. Soc.) Lett. 121:139–142.
15. Florkiewicz, R. Z., and A. Sommer. 1989. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate from non-Aug codons. Proc. Natl. Acad. Sci. USA. 86:3978–3981.
16. Folkman, J., M. Klagsbrun, J. Sasse, M. G. Wadzinski, D. Ingber, and I. Vlodavsky. 1988. A heparin-binding angiogenic protein, basic fibroblast growth factor, is stored within basement membrane. Am. J. Pathol. 130:393–400.
17. Fransson, L. A. 1987. Structure and function of cell associated proteoglycans. Trends Biochem. Sci. 12:406–411.
18. Gonzalez, A. M., M. Buscaglia, M. Ong, and A. Baird. Distribution of basic FGF in the 18-d rat fetus: localization in the basement membranes of diverse tissues. J. Cell Biol. In press.
19. Gospodarowicz, D., and J. Cheng. 1986. Heparin protects basic and acidic FGF from inactivation. J. Cell. Physiol. 128:475–484.
20. Gospodarowicz, D., J. Cheng, G. M. Lui, A. Baird, and P. Böhlen. 1984. Isolation of brain fibroblast growth factor by heparin-Sepharose affinity chromatography: identity with pituitary fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 81:6963–6967.
21. Hunter, T. 1987. A thousand and one protein kinases. Cell. 50:823–829.
22. Hunter, T., and B. M. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA. 77:1311–1315.
23. Ishihara, M., N. S. Fedarko, and H. E. Conrad. 1986. Transport of heparan sulfate into the nuclei of hepatocytes. J. Biol. Chem. 261:13575–13580.
24. Kübler, D., W. Pyerin, E. Burow, and V. Kinzel. 1983. Substrate-effected release of surface-located kinase from intact cells. Proc. Natl. Acad. Sci. USA. 80:4021–4025.
25. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.
26. Lobb, R. R., and J. W. Fett. 1984. Purification of two distinct growth factors from bovine neural tissue by heparin affinity chromatography. Biochemistry. 23:6295–6299.
27. Mascarelli, F., D. Raulais, and Y. Courtois. 1989. Fibroblast growth factor phosphorylation and receptors in rod center segments. EMBO (Eur. Mol. Biol. Organ.) J. 8:2265–2273.
28. Moscatelli, D. 1987. High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding

in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J. Cell. Physiol.* 131:123–130.

29. Neufeld, G., and D. Gospodarowicz. 1985. The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. *J. Biol. Chem.* 260:13860–13868.

30. Olwin, B. B., and S. D. Hauschka. 1986. Identification of the fibroblast growth factor receptor of Swiss 3T3 cells and mouse skeletal muscle myoblasts. *Biochemistry.* 25:3487–3492.

31. Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science (Wash. DC).* 223:1296–1299.

32. Sommer, A., and D. B. Rifkin. 1989. Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J. Cell. Physiol.* 138:215–220.

33. Vlodavsky, I., J. Folkman, R. Sullivan, R. Fridman, R. Ishai-Michaeli, J. Sasse, and M. Klagsbrun. 1987. Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 84:2292–2296.

34. Woodgett, J. R., K. L. Gould, and T. Hunter. 1986. Substrate specificity of protein kinase C. *Eur. J. Biochem.* 161:177–184.