Endothelial Cell-derived Heparan Sulfate Binds Basic Fibroblast Growth Factor and Protects It from Proteolytic Degradation

Olli Saksela, David Moscatelli, Andreas Sommer,* and Daniel B. Rifkin

Department of Cell Biology and the Kaplan Cancer Center, New York University School of Medicine, New York 10016; and *Synergen Inc., Boulder, Colorado 80301

Abstract. Cultured bovine capillary endothelial (BCE) cells were found to synthesize and secrete high molecular mass heparan sulfate proteoglycans and glycosaminoglycans, which bound basic fibroblast growth factor (bFGF). The secreted heparan sulfate molecules were purified by DEAE cellulose chromatography, followed by Sepharose 4B chromatography and affinity chromatography on immobilized bFGF. Most of the heparinase-sensitive sulfated molecules secreted into the medium by BCE cells bound to immobilized bFGF at low salt concentrations. However, elution from bFGF with increasing salt concentrations demonstrated varying affinities for bFGF among the secreted heparan sulfate molecules, with part of the heparan sulfate requiring NaCl concentrations between 1.0 and 1.5 M for elution. Cell extracts prepared from BCE cells also contained a bFGF-binding heparan sulfate proteoglycan, which could be released from the intact cells by a short proteinase treatment. The purified bFGF-binding heparan sulfate competed with ^{125}I-bFGF for binding to low-affinity binding sites but not to high-affinity sites on the cells. Heparan sulfate did not interfere with bFGF stimulation of plasminogen activator activity in BCE cells in agreement with its lack of effect on binding of ^{125}I-bFGF to high-affinity sites. Soluble bFGF was readily degraded by plasmin, whereas bFGF bound to heparan sulfate was protected from proteolytic degradation. Treatment of the heparan sulfate with heparinase before addition of plasmin abolished the protection and resulted in degradation of bFGF by the added proteinase. The results suggest that heparan sulfate released either directly by cells or through proteolytic degradation of their extracellular milieu may act as carrier for bFGF and facilitate the diffusion of locally produced growth factor by competing with its binding to surrounding matrix structures. Simultaneously, the secreted heparan sulfate glycosaminoglycans protect the growth factor from proteolytic degradation by extracellular proteinases, which are abundant at sites of neovascularization or cell invasion.

A variety of sulfated glycosaminoglycans are found in mammalian tissues. The major forms are usually covalently linked to a protein core to form large proteoglycan macromolecules with several glycosaminoglycan chains branching from a single protein core (12). Proteoglycans are classified either according to their glycosaminoglycan composition or according to the properties and antigenicity of the core protein (13). Proteoglycans are widely distributed in tissues as basement membrane components and are found as components of cartilage and connective tissues, intercalated membrane proteoglycans, and intracellular vesicle proteoglycans (13). Their physiological function is not well-understood, although heparan sulfate has been suggested to play a role in regulation of cell growth (5, 8, 32, 37) and in cell attachment and spreading (6, 22).

Fibroblast growth factors are a family of structurally related polypeptides characterized by a high affinity for heparin (9). The members of this growth factor family are divided into two groups, partly on the basis of their different elution patterns from immobilized heparin. The acidic fibroblast growth factors elute with lower salt concentrations (at ~1 M NaCl) than the basic fibroblast growth factors (bFGF)[1], which require 1.5 M NaCl or higher for elution. The heparin-binding growth factors are among the most potent known protein inducers of neovascularization in experimental models. They are mitogenic and chemotactic for endothelial cells and are capable of inducing plasminogen activator (PA) and collagenase production by the cells (27, 31). Increased proteolytic activity is thought to be essential for the migration of capillary endothelial cells through limiting structures such as basement membranes and intercellular matrices (11). Many cell types, including endothelial cells, possess specific high-affinity receptors for bFGF on their surface (25, 29, 30), which mediate the cellular effects of

1. *Abbreviations used in this paper*: BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; PA, plasminogen activator; TX-100, Triton X-100.

bFGF. Abundant low-affinity binding sites have also been described but these appear to have no direct effect on signal transduction (25). bFGF has been isolated from numerous tissues, including placenta, pituitary gland, brain, corpus luteum, retina, adrenal gland, kidney, bone, and prostate (see reference 9), and is synthesized by several cultured normal and malignant cell lines (9, 19, 26, 31). bFGF has also been reported to be associated with the extracellular matrix (2, 36).

Among other sites, heparan sulfate proteoglycans are found in the micro- and macrovasculature (3). Cloned aortic endothelial cells synthesize heparan sulfate proteoglycans, and a small proportion of these molecules have the disaccharide structure of heparin (28) and exhibit anticoagulant activity indistinguishable from that of heparin (24). In the present article, we have characterized an interaction between the endothelial cell–secreted heparan sulfate and bFGF. The binding of bFGF has no detectable effect on the interaction of bFGF with its high-affinity receptor but abolishes its interaction with the low-affinity binding sites. Moreover, bFGF bound to heparan sulfate is protected from proteolytic digestion by plasmin. These interactions may help to explain some of the physiologically important features in the action of the heparin-binding growth factors.

Materials and Methods

Cell Cultures

Bovine capillary endothelial cells were isolated from adrenal cortex by published methods (7, 11). The cloned cells were confirmed to be of endothelial origin by immunohistochemical staining for factor VIII-related antigen (7). The cells were grown in alpha-modified minimal essential medium containing 10% calf serum. When the cultures reached confluency, the serum concentration was reduced to 5%. The cells were used between passages 7–11.

Reagents

bFGF was purified from term placentas (27, 31). The purified molecule has been sequenced and is highly homologous with bFGF purified from the bovine pituitary (34). Recombinant bFGF was prepared and kindly provided by Synergen Inc., Boulder, CO.

Trypsin, plasmin, soybean trypsin inhibitor, chondroitinase ABC, and the purified Flavobacterium heparinum heparinase (EC 4.2.2.7) were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available. The heparinase preparations used were tested for possible contaminating proteolytic activity. They showed no activity when tested at concentrations of 50 IU/ml for 3 h in the ^{125}I-fibrin degradation assay.

Reactigel used for immobilizing bFGF was obtained from Pierce Chemical Co. (Rockford, IL), and was used according to manufacturer's procedure. DE 52 was purchased from Whatman Ltd. (Maidstone, Kent, England), and Sepharose 4B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Rabbit antiserum against placental bFGF was prepared as described (31) and further affinity purified on a Reactigel-recombinant bFGF column.

Metabolic Labeling of BCE

Confluent cultures of BCE cells grown on 100-mm petri dishes were washed with serum-free medium and fresh serum-free alpha-modified minimal essential medium containing L-[^{35}S]methionine or [^{35}S]sodium sulfate (50 μCi/ml; New England Nuclear, Boston, MA) and 0.1 mM aprotinin (Sigma Chemical Co.) was added to the cells. The cultures were incubated for 48 h or as indicated, and the conditioned medium was collected, centrifuged at 2,000 *g* for 20 min, and used for isolation of the bFGF-binding molecules.

Purification of the bFGF-binding Heparan Sulfates Secreted by BCE Cells

Conditioned medium from metabolically labeled BCE cells containing 0.5% Triton X-100 (TX-100) was chromatographed on a column of DE 52 equilibrated with 0.01 M PO_4, pH 7.4, 0.15 M NaCl, and 0.5% TX-100. The column was washed with the equilibration buffer followed by 0.25 M NaCl in the same buffer. The remaining bound material was eluted with 0.5 M NaCl, resulting in a relatively selective release of the glycosaminoglycans (12). The eluate was adjusted to contain 0.25 M NaCl, and the molecules were rebound to a small volume of DE 52 (0.5 ml) for concentration. The column was washed with 0.01 M Tris-HCl, pH 7.4, containing 0.25 M NaCl, and 0.5% TX-100, and the bound material eluted with a small volume of 2.0 M NaCl in the above buffer. The eluate was loaded on a Sepharose 4B column (1 × 50 cm) equilibrated with 0.01 M Tris–HCl buffer, pH 7.4, containing 2 M NaCl, and 0.5% TX-100, and the column eluted at a flow rate of 4 ml/h. 1-ml fractions were collected, and the radioactivity of the fractions was determined.

As a final step after Sepharose 4B chromatography, the fractions containing the radioactively labeled material were pooled, adjusted to contain 0.15 M NaCl, and applied to a Reactigel column (vol = 1 ml) containing 1 mg recombinant bFGF. A control column without bFGF was used to monitor nonspecific binding of the labeled material. The column was washed with 5 ml of 0.15 M NaCl in 0.01 M Tris–HCl, pH 7.4, containing 0.5% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and with 10 ml of the same buffer lacking the detergent. The bound material was eluted with a gradient from 0.15 to 2.0 M NaCl in 0.01 M Tris–HCl, pH 7.4. The radioactivity of the collected fractions was measured, and the salt concentration of the fractions was determined by measuring their conductance values. The peak fractions were pooled, diluted to 0.25 M NaCl, and concentrated by binding the material to 0.5 ml DE 52, from which it was eluted with a small volume of 0.5 M NaCl in 0.01 M PO_4, pH 7.4, and diluted to a final concentration of 0.15 M NaCl.

Isolation of the Cell Layer–associated bFGF-binding Heparan Sulfate

Trypsinization of metabolically labeled cultures was carried out by washing the dishes briefly with 0.05% (wt/vol) trypsin solution containing 1 mM EDTA followed by scraping the slightly rounded cells after 2–3 min into 2 ml of 0.01 M PO_4, pH 7.4, containing 0.15 M NaCl and 0.5% soybean trypsin inhibitor. The cells were removed by centrifugation at 2,000 *g* for 10 min. The trypsinate was used for isolation of the bFGF-binding heparan sulfate as described for the conditioned medium.

Immunoprecipitation of bFGF-bound Heparan Sulfate

For some experiments, heparan sulfate from conditioned medium, cell extracts, or matrix preparations was directly bound to bFGF immobilized by affinity-purified antibodies on protein A–Sepharose beads (Pharmacia Fine Chemicals). The washed beads (25 μl containing 5 μg bFGF antibodies and 200 ng bFGF) were incubated with the samples for 20 min on an end-over rotator, and the beads were subsequently washed with 0.25 M NaCl in 0.01 M PO_4 buffer, pH 7.4, containing 0.5% TX-100. The bound molecules were eluted with reducing Laemmli sample buffer and analyzed by electrophoresis in a 3–16% continuous gradient polyacrylamide gel (21) followed by autoradiography.

Plasminogen Activator Assay

BCE cells used for the PA assays were grown in 24-well Falcon dishes. The cells were extracted with 0.5 ml of 0.1 M Tris–HCl buffer, pH 8.1, containing 0.5% TX-100. The PA activity of the extracts was determined by the ^{125}I-fibrin assay as described earlier (11). 25 μl of the cell extract was used in the assay, and the PA activity was presented in Ploug units using a urokinase preparation as standard (Calbiochem-Behring Corp., La Jolla, CA).

Receptor Assays

Confluent cultures of BCE cells grown on either 24-well plates or on 60-mm dishes were washed and incubated at 4°C for 4 h in serum-free medium containing indicated concentrations of the purified proteoglycans and ^{125}I-bFGF. The binding of ^{125}I-bFGF to high- and low-affinity sites was assayed as earlier described for baby hamster kidney (BHK) cells (25).

Results

BCE Cell-conditioned Medium and Cell Extract Contain a bFGF-binding Molecule

Conditioned medium from metabolically labeled BCE cells was absorbed with bFGF bound to affinity-purified bFGF antibodies immobilized on protein A–Sepharose. When the material bound to immobilized bFGF was analyzed by PAGE and the gel was exposed for autoradiography, a high molecular mass protein heavily labeled with [^{35}S]methionine and barely entering the gel was detected (*arrow* in Fig. 1 *a*). The interaction between bFGF and the macromolecule was specific, since the molecule did not bind to affinity-purified bFGF antibodies in the absence of bFGF (Fig. 1 *b*). Moderate concentrations of heparin (5 μg/ml) were sufficient to inhibit this binding (Fig. 1 *c*). A similar bFGF binding protein was associated with the cells. Cell extracts prepared by solubilizing the cells with a detergent mixture (1% TX-100, 1% sodium deoxycholate, and 0.5% SDS in 0.1 M Tris–HCl buffer, pH 7.4) contained a major high molecular mass protein seen at the top of the polyacrylamide gel (Fig. 1 *d*). The molecule did not bind to affinity-purified bFGF antibodies alone (Fig. 1 *e*) and the binding was inhibited by heparin (Fig. 1 *f*). A small amount of background binding to the Protein A–Sepharose particles was seen in the control samples of Fig. 1, *b*, *c*, *e*, and *f*.

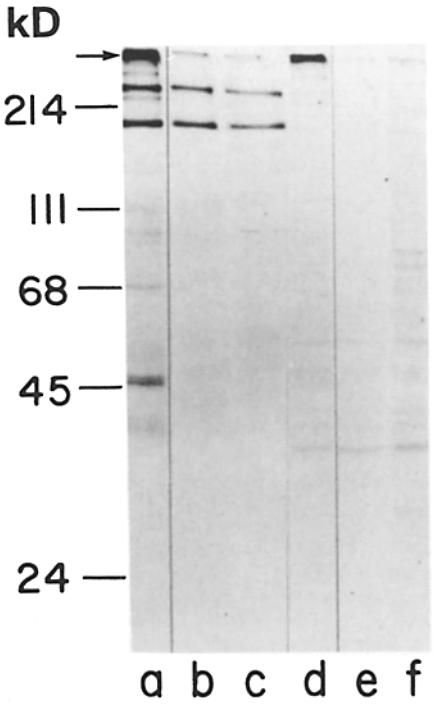

Figure 1. The binding of [^{35}S]methionine-labeled polypeptides from BCE cell cultures to immobilized bFGF. Confluent cultures of BCE cells were labeled with [^{35}S]methionine in serum-free medium. After 24 h, the medium was collected and the cultures washed with PBS and extracted in a detergent solution. Both the collected medium and the cell extract were absorbed with bFGF immobilized by affinity-purified bFGF antibodies to protein A–Sepharose particles. The bound material was analyzed on a 9% polyacrylamide gel with a 3% stacking gel under reducing conditions. A high molecular mass molecule on top of the 9% gel (*arrow*) that bound to bFGF was observed both in the conditioned medium (*a*) and in the cell extract (*d*). The molecule did not bind to the immobilized bFGF antibodies alone (*b* and *e*). 5 μg/ml heparin prevented the binding to immobilized bFGF of the high molecular mass molecule from both the medium (*c*) and the cell extract (*f*).

When the cultures were labeled with $^{35}SO_4$, and the conditioned medium was assayed for bFGF-binding molecules, a high molecular mass–sulfated molecule was detected (data not shown). In addition, a diffuse 250-kD band was seen in the autoradiograms of polyacrylamide gels of the $^{35}SO_4$-labeled material. No related 250-kD [^{35}S]methionine-labeled molecules were seen to bind bFGF (Fig. 1 *a*). The results suggested that the major binding molecules were heparan sulfates.

The conditioned medium contained minor amounts of other proteins that also bound selectively to bFGF (Fig. 1 *a*) and were not detected after precipitation with purified antibodies alone (Fig. 1 *b*). These included proteins with molecular masses of 47, 68, 230, and ~280 kD. These proteins were not further characterized. The binding of the other metabolically labeled proteins seen in Fig. 1, *b* and *e* was nonspecific, since it occurred when nonimmune IgG was used instead of the bFGF antibodies (data not shown).

Isolation of bFGF-binding Molecules Secreted by BCE

To isolate the bFGF-binding molecules, the purification methods for heparan sulfate glycosaminoglycans were adopted (12). Secreted glycosaminoglycans in the conditioned medium of $^{35}SO_4$-labeled or [^{35}S]methionine-labeled BCE cultures were bound to DE 52 as described in Materials and Methods. The column was washed extensively with 0.25 M NaCl in 0.01 M PO_4 buffer, pH 7.3, containing 0.5% TX-100. This salt concentration is sufficient to elute most of the secreted glycoproteins and leaves the highly negatively charged glycosaminoglycans and proteoglycans bound to the column. The column was then eluted with 0.5 M NaCl. After concen-

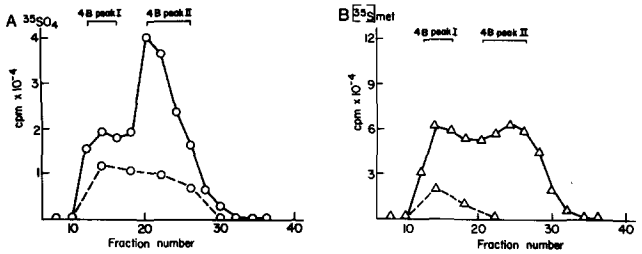

Figure 2. Sepharose 4B chromatography of DE 52 binding material from the conditioned medium of $^{35}SO_4$-labeled (*A*) or [^{35}S]methionine-labeled (*B*) BCE cells. Confluent cultures of BCE cells were metabolically labeled for 48 h in serum-free medium. The medium was applied to a DE 52 column, and the material eluted between 0.25–0.5 M NaCl. After concentration, the eluate was applied to a 1 × 50-cm Sepharose 4B column equilibrated with 2.0 M NaCl in 0.01 M Tris–HCl buffer, pH 7.4, containing 0.5% TX-100. The column was eluted at a flow rate of 4 ml/h, 1 ml fractions were collected, and the radioactivity of the fractions was counted. The peak fractions were pooled as indicated in the figure. The dashed lines indicate the amount of radioactivity bound to antibody-immobilized bFGF in 0.15 M NaCl from aliquots of the marked fractions.

tration, the eluate was applied to a Sepharose 4B column (Fig. 2). Two radiolabeled peaks were detected in both $^{35}SO_4$-labeled (Fig. 2 *A*) and [^{35}S]methionine-labeled (Fig. 2 *B*) preparations. A high-salt concentration (2 M NaCl) was used during the chromatography to avoid possible ionic interactions between the glycosaminoglycans and other secreted molecules. The presence of bFGF-binding molecules in the Sepharose 4B fractions was demonstrated by absorbing aliquots of the fractions on antibody-immobilized bFGF after first diluting them to 0.15 M NaCl (Fig. 2, *dashed line*).

For purification of the bFGF-binding molecules, fractions from the two peaks observed after Sepharose 4B chromatography of both $^{35}SO_4$-labeled and [^{35}S]methionine-labeled material were pooled and diluted to contain 0.15 M NaCl and were bound to Reactigel-immobilized bFGF. Approximately 50% of the $^{35}SO_4$-labeled molecules from the first peak and ~25% of the labeled molecules from the second peak remained bound to the column after extensive washing. The nonbinding fraction probably represented molecules other than those that were heparan sulfate related (see below). The material bound to bFGF was eluted with a 0.15–2.0 M NaCl gradient (Fig. 3 *A*). The majority of the radiolabeled molecules from the two $^{35}SO_4$-labeled Sepharose 4B peaks eluted with 0.75 M NaCl, although some of the molecules in the second Sepharose 4B peak required higher NaCl concentrations for elution. When material from the second Sepharose 4B peak bound to immobilized bFGF was eluted with sequential extensive washes with increasing NaCl concentrations, 22% of the total counts recovered eluted with 0.5 M, 63% with 1.0 M, 13% with 1.5 M, and 2% with 2.0 M NaCl. When the $^{35}SO_4$-labeled material that eluted with concentrations >1.0 M NaCl was rebound to bFGF at 0.5 M NaCl, washed with 1.0 M NaCl, and re-eluted with 2.0 M NaCl, >60% of the material was recovered in the 2 M eluate, confirming that this fraction includes molecules that have a high affinity for bFGF. The [^{35}S]methionine-labeled material from the first Sepharose 4B peak eluted from immobilized bFGF with the same salt concentration as the corresponding $^{35}SO_4$-labeled molecules (Fig. 3 *B*). All of the [^{35}S]methionine-labeled material from the second Sepharose 4B peak eluted from the immobilized bFGF at low-salt concentrations. Most likely the second peak represented proteins that initially bound to DE 52 via the proteoglycans but had dissociated during the Sepharose 4B chromatography at 2 M NaCl, or proteins that bound directly to DE 52 with relatively high affinity.

Characterization of the bFGF-binding Molecules

Binding of the $^{35}SO_4$-labeled molecules to immobilized bFGF was abolished by pretreatment with purified Flavobacterium heparinase (5 IU/ml; incubated for 6 h at 37°C) but not by chondroitinase ABC (5 IU/ml; incubated for 6 h at 37°C; Fig. 4). After separation of the DE 52 eluted material in the Sepharose 4B column, the first peak from the column contained a SO_4-labeled high molecular mass bFGF-binding molecule as visualized after SDS-PAGE and autoradiography (Fig. 5 *a*). A similar molecule was seen in preparations of the conditioned medium from [^{35}S]methionine-labeled BCE cell cultures (Fig. 5 *c*). These molecules appeared to be identical to the molecule initially observed to bind to bFGF (Fig. 1 *a*). When the $^{35}SO_4$-labeled and the [^{35}S]methionine-labeled high molecular mass molecules were pretreated with 0.05% (wt/vol) trypsin and the enzyme was inhibited after 6 h incubation at 37°C by adding 0.5% soybean trypsin inhibitor, a conversion of the $^{35}SO_4$-labeled molecule to a smaller form still binding to bFGF was seen (Fig. 5 *e*). No [^{35}S]methionine-labeled molecules bound to bFGF after trypsin treatment (data not shown).

A smaller $^{35}SO_4$-labeled heparinase-sensitive molecule from the second peak of the Sepharose 4B column bound to immobilized bFGF (Fig. 5 *b*). Neither the size nor binding of this molecule was affected by trypsin (Fig. 5 *f*). After [^{35}S]methionine labeling, no corresponding molecules were found bound to immobilized bFGF (Fig. 5 *d*). The results suggest that the high molecular mass bFGF-binding molecule is a heparan sulfate glycosaminoglycan, which has a protein core sensitive to degradation by trypsin. A core protein for the smaller secreted heparan sulfate species could not be identified by metabolic labeling with methionine (or cysteine) or by sensitivity to trypsin treatment.

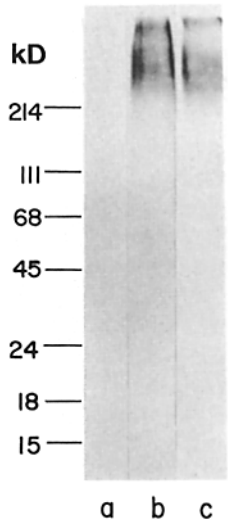

Figure 4. Effect of heparinase and chondroitinase ABC treatment on the binding of $^{35}SO_4$-labeled molecules to bFGF. Concentrated metabolically labeled samples from DE 52 were incubated for 6 h at 37°C with heparinase (*a*), chondroitinase ABC (*b*), or without additions (*c*). The treated material was bound to immobilized bFGF and analyzed by SDS-PAGE. The bound molecules were visualized by autoradiography.

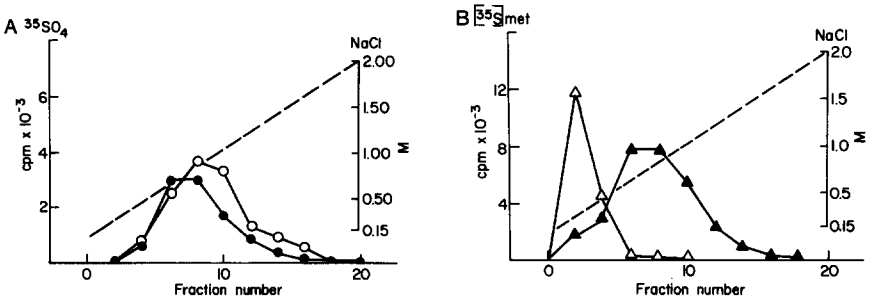

Figure 3. Gradient elution of $^{35}SO_4$-labeled (*A*) or [^{35}S]methionine-labeled (*B*) molecules from immobilized bFGF. Labeled material from the Sepharose 4B peaks I and II (Fig. 2) was applied to a Reactigel–bFGF column. The bound molecules were eluted with a continuous 0.15–2.00 M NaCl gradient in 0.01 M Tris–HCl, pH 7.4. (*A*) ●, 4B peak I; ○, 4B peak II. (*B*) ▲, 4B peak I; △, 4B peak II.

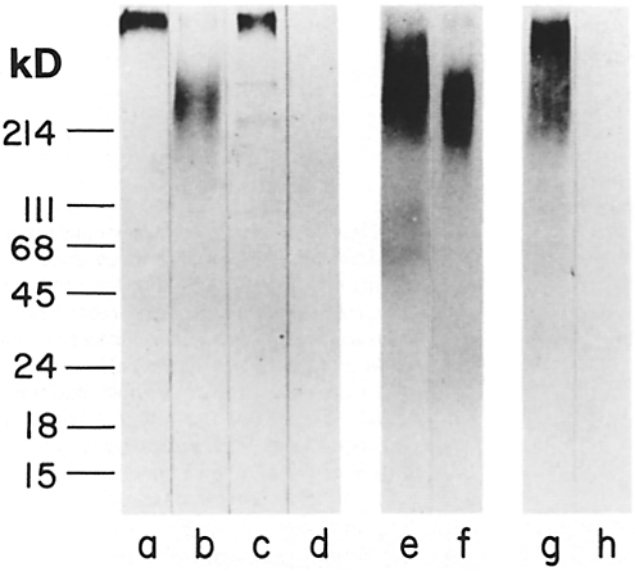

Figure 5. PAGE of BCE cell-secreted bFGF-binding molecules. Metabolically labeled molecules which eluted from immobilized bFGF between 0.5 and 2.0 M NaCl were analyzed in a reducing 3–16% continuous polyacrylamide gel followed by autoradiography. The bFGF-binding molecules from the Sepharose 4B peak I consisted of high molecular mass material labeled both with $^{35}SO_4$ (*a*) and with [^{35}S]methionine (*c*). The bFGF-binding molecules from peak II consisted of molecules labeled with $^{35}SO_4$ (*b*), but no [^{35}S]methionine-labeled material from peak II remained bound to the bFGF column after washing with 0.5 M NaCl (*d*). Lanes *e* and *f* are the same as lanes *a* and *b* except that the samples were treated with 0.05% trypsin for 6 h before binding to immobilized bFGF. Treatment of $^{35}SO_4$-labeled BCE cells with 0.05% trypsin for 5 min results in release of high molecular mass material, which binds to immobilized bFGF (*g*). Treatment with heparinase (5 IU/ml for 6 h) abolishes the binding (*h*).

In addition to these major bFGF-binding molecules, the absorption of the first peak from the Sepharose 4B column to immobilized bFGF resulted in binding of [^{35}S]methionine-labeled 230- and 280-kD proteins (Fig. 5 *c*). The two molecules appeared similar to those observed to bind to bFGF in the unfractionated conditioned medium (Fig. 1 *a*).

Extracellular bFGF-binding Heparan Sulfate in BCE Cells

The cell-associated heparan sulfate proteoglycans were studied after solubilizing the molecules by proteolytic digestion. Trypsin degrades the protein core mediating the binding of heparan sulfate proteoglycans to the cell membrane (14). A short trypsinization of metabolically labeled BCE cells resulted in the release from the cell layer of $^{35}SO_4$-labeled material. After isolation of the DE 52 binding fraction of the trypsin-solubilized material, it was bound to antibody-immobilized bFGF (Fig. 5 *g*). The trypsin releasate contained a major heparan sulfate species that migrated in SDS gel electrophoresis slightly faster than the larger molecule obtained from the conditioned medium. The ability of these molecules to bind to bFGF was abolished by heparinase treatment (Fig. 5 *h*).

Quantitation of the bFGF-binding Heparan Sulfate Secreted by BCE Cells

To quantitate the amount of bFGF-binding heparan sulfate secreted by the BCE cells, the amount of immobilized bFGF required to bind all the $^{35}SO_4$-labeled heparan sulfate in a sample of BCE cell-conditioned medium was determined. Excess amounts of antibody and Protein A–Sepharose were used to insure that the limiting factor in the absorbent was bFGF. After a 24-h incubation in serum-free medium, a 60-mm dish containing 0.6 mg cellular protein had secreted enough heparan sulfate to bind ~500 ng of added bFGF (data not shown).

To quantitate the proportion of the heparan sulfate that is capable of binding to immobilized bFGF from the total amount of heparan sulfate secreted by the cells, the following experiments were performed (Fig. 6). First, the material from each of the two peaks obtained after Sepharose 4B chromatography was digested with heparinase. Both digested and control samples were repurified using DE 52 (Fig. 6 *A*, columns *1*). This treatment was sufficient to degrade 52% of the sulfated molecules present in peak I and 20% of the molecules in peak II. In the second experiment, when the $^{35}SO_4$-labeled molecules from peak I were applied to the bFGF column (Fig. 6 *A*, columns *2*), 50% of the radioactivity bound to bFGF, while only 22% of the radioactivity in peak II bound to the column. When the material was pretreated with heparinase, >90% of the binding from each peak was lost. Similar results were obtained when the [^{35}S]-

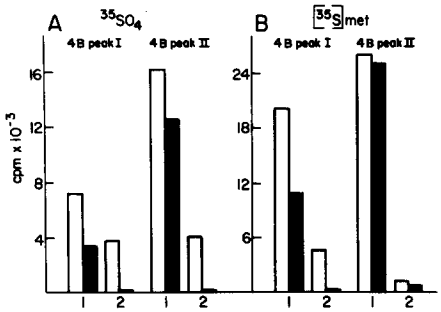

Figure 6. Effect of heparinase treatment on the binding of the BCE cell-secreted $^{35}SO_4$-labeled (*A*) or [^{35}S]methionine-labeled (*B*) molecules to DE 52 (columns *1*) or immobilized bFGF (columns *2*). Pooled fractions from the two peaks of the Sepharose 4B column were diluted to 0.15 M NaCl and incubated either with DE 52 or with bFGF immobilized to protein A–Sepharose by affinity-purified antibodies (*open columns*). This binding was compared to the binding of heparinase-treated samples (*shaded columns*) to the absorbents. The difference between open and shaded columns indicates the amount of heparinase-sensitive metabolically labeled molecules in the samples. The decrease in the $^{35}SO_4$-labeled material binding to DE 52 (*A*, columns *1*) equals the decrease in the material binding to immobilized bFGF (*A*, columns *2*). This suggests that almost all of the BCE cell-secreted heparan sulfate molecules bind to immobilized bFGF at 0.15 M NaCl. A slight difference in the binding of heparinase-sensitive [^{35}S]methionine-labeled molecules from Sepharose 4B peak I to DEAE-cellulose (*B*, columns *1*) compared to their binding to bFGF (*B*, columns *2*) was observed. Only a small proportion of the [^{35}S]methionine-labeled molecules in peak II bound to antibody-immobilized bFGF. *Open columns*, before heparinase digestion; *shaded columns*, after heparinase digestion.

Table I. Effect of Purified Heparan Sulfate on the Binding of ^{125}I-bFGF to BCE Cells

	fmol bFGF bound to	
	low affinity sites	high affinity sites
^{125}I-bFGF (2.5 ng in 1 ml)	28.0 ± 1.0	3.1 ± 0.3
^{125}I-bFGF + heparan sulfate (10,800 cpm)	10.4 ± 1.5	2.6 ± 0.3
^{125}I-bFGF + heparinase (2.0 U)	10.0 ± 2.0	ND
^{125}I-bFGF + heparinase (boiled 5 min)	27.2 ± 1.0	ND
^{125}I-bFGF + heparan sulfate (boiled 5 min)	12.2 ± 1.5	ND
^{125}I-bFGF + heparan sulfate (heparinase treated, boiled 5 min)	20.4 ± 2.0	ND

^{125}I-bFGF was bound to cultures of confluent BCE cells (4 × 10^5) grown on 60-mm dishes (reference 25). The purified heparan sulfate preparation tested for inhibitory effect on bFGF binding was preincubated with ^{125}I-bFGF for 15 min. The samples were added to the cells and incubated at 4°C for 2 h. The unbound label was removed and ^{125}I-bFGF bound to low affinity sites was released by 2 M NaCl at neutral pH and quantitated. ^{125}I-bFGF bound to the high affinity sites was solubilized with detergent and quantitated.

methionine-labeled proteoglycans from Sepharose 4B peak I were treated with heparinase (Fig. 6 *B*). Thus, it appears that essentially all of the heparinase-sensitive molecules present in the two Sepharose 4B peaks are capable of binding to bFGF at low-salt concentrations.

Biological Activity of bFGF-binding Heparan Sulfates

To assess the potential biological role of the bFGF-binding heparan sulfate, several experiments were performed. First, the ability of heparan sulfate to compete with the binding of ^{125}I-bFGF to low- and high-affinity sites on BCE cells was measured. Increasing amounts of a crude heparan sulfate preparation effectively decreased the amount of ^{125}I-bFGF bound to the cells. The preparation inhibited only the low-affinity binding of ^{125}I-bFGF to the cells and had no effect on binding of the label to high-affinity sites (Table I).

The low-affinity binding of ^{125}I-bFGF could be reduced up to 70% by the addition of purified heparan sulfate glycosaminoglycans eluted from the immobilized bFGF column. To demonstrate that the inhibition was indeed mediated by heparan sulfate molecules, the purified heparan sulfate was treated with heparinase and tested for inhibition of ^{125}I-bFGF binding. The heparinase by itself reduced the binding of ^{125}I-bFGF to low-affinity sites presumably because it degraded the cell-associated heparan sulfate molecules which constitute the low-affinity binding sites (25). The heparinase activity could be inhibited by boiling, which only slightly affected the inhibitory activity of the heparan sulfate. After heparinase digestion of purified heparan sulfate followed by boiling to inactivate the enzyme, only 20% of the inhibitory activity remained. The nature of this remaining activity is unknown. It does not appear to be due to undigested heparan sulfate molecules, since the inhibitory activity did not bind to DE 52 (data not shown).

To support the finding that heparan proteoglycans do not prevent the binding of bFGF to the high-affinity receptors that mediate the stimulation of the cells by bFGF (25, 29, 30), we tested the effect of the purified heparan sulfate on the stimulation of cellular PA activity by bFGF. Increasing amounts of bFGF were mixed with a constant amount of the purified heparan sulfate preparation from the Sepharose 4B peak II, and the mixture was incubated with BCE cells for 16 h. The amount of heparan sulfate used was equal to the concentration needed to cause a 50% reduction in the low-affinity binding of 2.5 ng of ^{125}I-bFGF to the cells. As shown in Fig. 7, the heparan sulfate preparation alone caused a slight but consistent decrease in the level of the cell-associated PA activity. The reason for this decrease is unknown. However, the addition of heparan sulfate did not affect the increase of PA activity caused by increasing amounts of bFGF. This result is consistent with the inability of the heparan sulfate to block bFGF binding to its high-affinity receptor.

Other studies have described a protective effect of heparin on the inactivation of bFGF by pH or temperature (10). Since active proteolysis may occur both at regions of angiogenesis and wound repair, we investigated whether heparan sulfate protected bFGF from proteolytic degradation. Purified and iodinated bFGF was diluted in 0.01 M PO_4, pH 7.4, containing 20 mM Hepes and 0.05% gelatin. The labeled growth factor was degraded during a 6-h incubation at 37°C by 5 μg/ml of plasmin (Fig. 8, *a* and *b*). This degradation is effectively prevented by adding either heparin (250 ng/ng bFGF; Fig. 7 *c*) or purified BCE cell-secreted heparan sulfate (Fig. 8 *e*) to the ^{125}I-bFGF preparation. The protective effect is greatly decreased by a 6-h pretreatment of the heparin or the heparan sulfate preparations with heparinase (5 IU/ml at

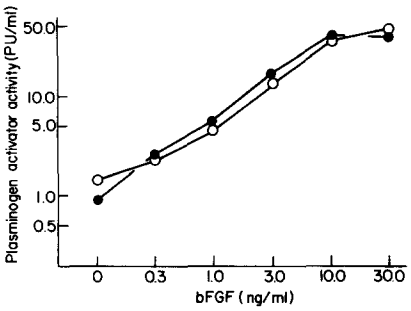

Figure 7. Effect of purified BCE cell-secreted heparan sulfates on the biological activity of bFGF. Increasing concentrations of bFGF were added to cultures of BCE cells either in the absence (○) or in the presence (●) of a constant amount of BCE cell-secreted purified heparan sulfate. The concentration of heparan sulfate used was sufficient to cause >50% inhibition of the low-affinity binding of ^{125}I-bFGF to BCE cells. After the incubation, the cells were washed, extracted in 0.5% TX-100, and the plasminogen activator activity of the lysate was determined by the ^{125}I-fibrin degradation assay.

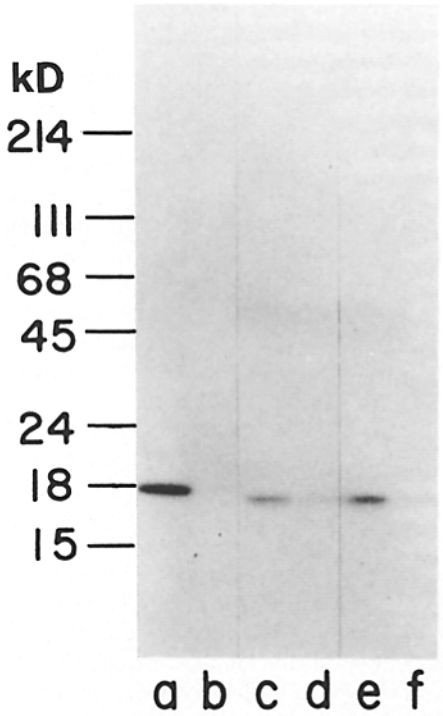

Figure 8. Protection of ^{125}I-bFGF from proteolytic degradation by BCE cell-secreted heparan sulfates and heparin. ^{125}I-bFGF (1 ng/ml; *a*) was rapidly degraded when incubated for 6 h with 5 μg/ml plasmin (*b*). Addition of heparan sulfate (*c*) or heparin (*e*) resulted in protection of the growth factor from proteolysis. Pretreatment of the glycosaminoglycans with heparinase before addition of plasmin resulted in loss of the protective effect (*c* and *f*).

37°C; Fig. 8, *d* and *f*). Neither the heparin nor the purified heparan sulfate had any effect on the plasmin activity as measured by the ^{125}I-fibrin degradation assay (data not shown).

The effect of plasmin treatment on bFGF activity was tested in the following way. bFGF was incubated with or without plasmin, aprotinin was added to both samples to inhibit the enzyme, and the bFGF preparations were tested for their biological activity (Table II). A significant stimulation of cell-associated PA activity in cultures of BCE cells was seen only by the control bFGF preparation, whereas no stimulation by the plasmin-treated bFGF was observed, indicating that the bFGF had been degraded. In contrast, when the plasmin treatment was carried out in the presence of heparin or purified BCE secreted heparan sulfate, the bFGF preparation retained its activity. In fact, both the heparan sulfate and heparin increased the stimulatory activity of bFGF recovered after the incubation. The difference in the recovered activities did not appear to be due to stimulation caused by heparan sulfate or heparin. Rather, it was due to partial loss of activity in the control bFGF preparation during the incubation. This can be seen by comparing the stimulation caused by bFGF after the incubation to the stimulation shown in Fig. 7. In addition, Fig. 7 indicates that when heparan sulfate and bFGF are added to the cells without prior incubation, there is no effect of heparan sulfate on the stimulation by bFGF. The results are consistent with the above data and confirm that bFGF bound to heparan sulfate is not degraded under these conditions and that the complex retains its biological activity.

Discussion

The strong binding to heparin is a characteristic feature of the fibroblast growth factors. Complex formation protects both acidic fibroblast growth factors and bFGFs from heat and acid inactivation (10). However, the biological significance of the interaction is not clear as heparin is rarely present in vivo. Endothelial cells do synthesize and release heparan sulfate, which has the disaccharide structure and anticoagulant activity of heparin (24, 28). The interaction with heparan sulfate or heparan sulfate proteoglycans rather than with heparin is more likely to be relevant in the action of bFGF. In this study we have characterized some of the properties of the bFGF-binding heparan sulfate molecules produced by BCE cells.

The bFGF-binding heparan sulfate secreted by BCE cells consists of two different classes of molecules. The larger molecule contains a protein core, whereas the existence of a core protein for the smaller heparan sulfate is not certain since it is not labeled by methionine (or cysteine) and is resistant to trypsin degradation. The smaller heparan sulfate elutes from columns of immobilized growth factor at slightly

Table II. Protection of the Biological Activity of bFGF from Plasmin Degradation by Purified Heparan Sulfate and Heparin

	Plasminogen activator activity in cell extracts
	Ploug units/ml
no additions	0.9
bFGF (5 ng/ml)	3.0
bFGF + plasmin (5 μg/ml)	0.8
bFGF + plasmin + heparan sulfate (10,800 cpm/ml)	5.0
bFGF + plasmin + heparin (1 μg/ml)	20.0
bFGF (15 ng/ml)	12.0
bFGF + plasmin (5 μg/ml)	1.1
bFGF + plasmin + heparan sulfate (10,800 cpm/ml)	25.0
bFGF + plasmin + heparin (1 μg/ml)	28.0

Basic FGF was diluted in a 0.01 M PO_4 buffer, pH 7.4, containing 0.04% gelatin, and incubated alone with purified heparan sulfate or with heparin for 1 h at 20°C. Plasmin was added as indicated and the incubation was continued for 3 h at 37°C, after which the enzyme was inhibited by aprotinin (10 μg/ml) and 1-ml aliquots of the samples were added to BCE cells grown on 24-well plates. The plasminogen activator activity of the cell cultures was measured after 16 h incubation. The values shown represent mean activities from four parallel measurements.

higher NaCl concentration than the larger heparan sulfate. This may reflect a structural difference between the bFGF-binding portions of the molecules, such as a difference in the degree of sulfation, or may simply be due to steric interference in the case of the larger proteoglycan.

An interesting feature of the isolated bFGF-binding heparan sulfate proteoglycan fraction was the copurification of some unidentified protein components. These proteins did not copurify with the smaller heparan sulfate species. It also appears that these proteins did not bind directly to bFGF, since their binding to immobilized bFGF was sensitive to heparinase treatment. Heparan sulfate proteoglycans have affinity towards several secreted and matrix molecules, such as collagens (35), fibronectin (17, 35), laminin (4, 17), and vitronectin (1). The binding of heparan sulfate proteoglycans to fibronectin may also involve the protein core of the proteoglycan (17). The proteins observed to copurify with the heparan sulfate proteoglycan, therefore, may represent components of high molecular mass aggregates of structural proteins secreted by the cells (33). The identification of these proteins is currently in progress.

Heparan sulfate glycosaminoglycans are a common constituent of extracellular matrices (14). In cultures of endothelial cells, they account for the majority of extracellular sulfated glycosaminoglycans (20). These extracellular heparan chains are thought to be a major bFGF-binding component in cultured BHK cells (25), since the binding can be competed with heparin and the sites are sensitive to heparinase treatment. The present finding that the endothelial cell-secreted heparan sulfate competes with binding of bFGF to the low-affinity sites is in agreement with previous results suggesting that extracellular matrix heparan sulfate can bind the growth factor (2, 25). Indeed, such a heparan sulfate proteoglycan was released from the cell surface by mild trypsinization of the cells.

Extracellularly located proteoglycans are either membrane-bound through their hydrophilic protein core or are bound to other membrane-attached molecules of the matrix (cf. references 13, 14). The release of these pericellular glycosaminoglycans may occur through limited proteolysis of the membrane-embedded protein core, as suggested for the integral membrane heparan sulfate in cultured colon carcinoma cells (15, 16) or in cultured rat hepatocytes (18). In the present study, we demonstrated the release of a bFGF-binding heparan sulfate from the extracellular structures of endothelial cells by trypsin. The relationship between cell surface heparan sulfate and the secreted heparan sulfate in BCE cell cultures was not studied, but preliminary pulse labeling experiments demonstrated that the bFGF-binding molecules appear in the conditioned medium and on the cell surface with the same rate. This suggests that the molecules may not be directly related; i.e., that the soluble heparan sulfate is not exclusively derived from the cell-associated form, or that the cell-associated molecules are not primarily taken up from the conditioned medium (unpublished observations).

Binding to heparin considerably increased the resistance of bFGF to degradation by plasmin. The less sulfated heparan proteoglycans can also effectively protect bFGF from proteolytic degradation. In spite of the effective protection, heparan sulfate does not inhibit the biological activity of bFGF. This was shown by the ability of the heparan sulfate-bound bFGF to interact with the high-affinity sites on BCE cells and by the stimulation of PA activity by bFGF in the presence of heparan sulfate proteoglycans. It is an intriguing possibility that pericellular hydrolytic activities may release bFGF located in extracellular complexes (2, 36). Two possibilities seem to exist for this process. In one, cell-derived heparanases would degrade the heparan sulfate releasing free bFGF, while in the other, proteinases would degrade the protein core of the heparan sulfate proteoglycans releasing bFGF–heparan sulfate glycosaminoglycan complexes. We favor the latter possibility, since heparinase treatment of bFGF–heparan sulfate complexes resulted in subsequent enhanced inactivation of the growth factor by proteinases. Thus, bFGF released by heparinase from the matrix (2) may be relatively short lived in vivo if proteolytic activity is present. At sites of tumor growth, neovascularization, and wound healing, high levels of proteolytic enzymes are produced that would limit the availability of bFGF. Conversely, the release of bFGF from heparan sulfate proteoglycan complexes by proteolytic degradation of the protein core would result in the formation of bFGF–glycosaminoglycan complexes. In this form the glycosaminoglycans might serve as carriers for the growth factor by protecting it from inactivation by proteinases. In addition, complex formation may facilitate bFGF diffusion. Since free bFGF would probably have an extremely limited diffusion capability because of its effective binding to the abundant matrix proteoglycans, this may be another important aspect of the in vivo function of bFGF–heparan sulfate complexes.

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