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Heparin Is Required for Cell-Free Binding of Basic Fibroblast Growth Factor to a Soluble Receptor and for Mitogenesis in Whole Cells

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Heparin is required for the binding of basic fibroblast growth factor (bFGF) to high-affinity receptors on cells deficient in cell surface heparan sulfate proteoglycan. So that this heparin requirement could be evaluated in the absence of other cell surface molecules, we designed a simple assay based on a genetically engineered soluble form of murine FGF receptor 1 (mFR1) tagged with placental alkaline phosphatase. Using this assay, we showed that FGF-receptor binding has an absolute requirement for heparin. By using a cytokine-dependent lymphoid cell line engineered to express mFR1, we also showed that FGF-induced mitogenic activity is heparin dependent. Furthermore, we tested a series of small heparin oligosaccharides of defined lengths for their abilities to support bFGF-receptor binding and biologic activity. We found that a heparin oligosaccharide with as few as eight sugar residues is sufficient to support these activities. We also demonstrated that heparin facilitates FGF dimerization, a property that may be important for receptor activation.

Heparin or heparan sulfate is required for basic fibroblast growth factor (bFGF) high-affinity receptor binding (38) and for bFGF-induced fibroblast growth and myoblast differentiation (29). These observations suggest that heparan sulfate proteoglycans (HSPGs) may be important regulators of bFGF biologic activity, acting directly at the level of the cell surface receptor (32). Not surprisingly, heparin and HSPGs are important regulators of cell growth (37). Depending on the tissue or cell type, heparin can either stimulate or inhibit cell proliferation (reviewed in references 14 and 31). Some of these effects may be mediated by FGF; however, other growth factors (including granulocyte-macrophage colony-stimulating factor, [GM-CSF], interleukin 3 [IL-3], pleiotrophin, platelet factor 4, keratinocyte autocrine factor or amphiregulin, and heparin-binding-epidermal growth factor [EGF]) are also known to interact with heparin and may mediate some of these effects (6, 16; reviewed in reference 32). With the exception of FGF and amphiregulin, it is not known that heparin modulates growth factor activity at the level of a cell surface receptor. Nevertheless, heparin decreases the binding of amphiregulin to its receptor (6) and is required for bFGF-receptor binding (38). In addition, bFGF and the hematopoietic growth factors GM-CSF and IL-3, are biologically active when bound to HSPG (26, 30, 36).

bFGF is known to reside in the extracellular matrix (ECM) of a wide variety of cells and tissues. This reservoir for bFGF serves to limit the diffusibility of the growth factor and thus to regulate its bioavailability. Biologically active bFGF can be displaced from the ECM by heparin or be released from the ECM by heparin-degrading enzymes (reviewed in references 14 and 20). Recently, syndecan, an HSPG, has been identified as a low-affinity binding site for bFGF (18). The observation that HSPGs serve as low-affinity binding sites for bFGF has been confirmed by comparing low-affinity FGF binding to wild-type and to cell surface heparan sulfate-deficient CHO cells (38). Additionally, treating cells with inhibitors of sulfation or with heparin-degrading enzymes decreases their ability to bind bFGF (25, 29), while heparin and heparan sulfate protect bFGF from degradation by heat, acid, and proteolysis (reviewed in reference 20). Taken together, these observations strongly support a major role for heparinlike molecules in the biologic activity of bFGF.

A model for FGF-receptor binding suggests that binding involves the formation of a trimolecular complex between receptor, ligand, and heparin (38). Peptide binding studies (1) suggest that the presumed heparin and receptor binding regions of bFGF lie in separate functional domains, while X-ray crystallographic studies show that heparin and receptor binding regions are on different faces of the bFGF molecule (8). In this study, we demonstrated that all three of these surface molecules are required to form a stable complex in the absence of other cell surface molecules. By creating a cell line that is dependent on both heparin and FGF for growth, we found that the biologic activity of bFGF is dependent on the presence of heparin. We also found that in the presence of bFGF, small heparin oligosaccharides of defined sizes can activate the mitogenic potential of bFGF on appropriate target cells and are active in the binding of bFGF to a soluble bFGF receptor.

MATERIALS AND METHODS

Materials. Recombinant human bFGF was a gift from Creative Biomolecules, Hopkinton, Mass. ^{125}I-bFGF was generously provided by B. Olwin (4). Heparin was obtained from Hepar Inc., Franklin, Ohio. Heparin oligosaccharides of defined sizes and high sulfate content were prepared by nitrous acid depolymerization of heparin and fractionation as

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described by Sudhalter et al. (33). Size homogeneity of the heparin oligosaccharides was determined by high-performance liquid gel permeation chromatography, and sulfur content was determined by elemental analysis (33).

Soluble FGF receptor construction. A soluble FGF binding protein was constructed by cloning the extracellular region of murine FGF receptor 1 (mFR1) into the APtag expression vector (9), which codes for a secreted form of placental alkaline phosphatase (AP) (3). The Mo/mFR1/SV plasmid (Fig. 1A and reference 38) was restricted with *Cla*I (5′ of the Moloney murine leukemia virus long terminal repeat [LTR]) and *Hin*cII to produce a DNA fragment containing the Moloney murine leukemia virus LTR and most of the sequence of mFR1 5′ of the transmembrane domain (Fig. 1A). To complete the extracellular region of mFR1 and link it to APtag, a polymerase chain reaction (PCR) fragment was synthesized by using a 5′ primer (GGAGATCTCCCAT CACTCTGCATGGTTG) just upstream of the *Hin*cII site and a 3′ primer (CGGA**AGATCT**CTCCAGGTAGAGCG) corresponding to the juxtamembrane region of mFR1 and containing a *Bgl*II restriction site (boldface) (Fig. 1B) in frame with the *Bgl*II site in APtag. The LTR-mFR1 fragment and the PCR fragment, restricted with *Hin*cII and *Bgl*II, were ligated into the APtag vector (cut with *Cla*I and *Bgl*II) to yield the plasmid (FRAP) shown in Fig. 1B. Sequencing confirmed that the PCR fragment contained no errors and was in frame with AP.

Tissue culture and transfections. NIH 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% bovine calf serum. The FRAP (FGF receptor alkaline phosphatase) plasmid was cotransfected by electroporation (960 μF, 300 V; Bio-Rad Gene Pulser) with a selectable neomycin resistance gene. Colonies were selected in G418 (600 μg/ml) and then screened for secreted AP enzyme activity in conditioned medium (9). Clone FRAP-A2, which produced a high level of AP activity (2 to 4 optical density units per min per ml; see below) was then used to produce conditioned medium for binding assays. [^{35}S]methionine labeling of this cell line followed by immunoprecipitation of conditioned medium with anti-AP-Sepharose (see below) and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis yielded a predominant band with an M_r of approximately 130,000.

BaF3 cells (24, 28) were grown in RPMI 1640 medium, supplemented with 10% bovine calf serum, 10% IL-3 conditioned medium (X63-IL3 cell line [17]) and L-glutamine. These cells were transfected by electroporation (960 μF, 220 V) with Mo/mFR1/SV (Fig. 1A and reference 38) and selected either with G418 (600 μg/ml; for a cotransfected neomycin resistance gene) plus IL-3 conditioned medium or with bFGF plus heparin (0.5 μg/ml) in the absence of IL-3. Both of these procedures yielded colonies which express mFR1 mRNA (see Fig. 5A). Clone F32 (selected in FGF and heparin) is a subclone derived from pool F3 by limiting dilution.

Proliferation assays. BaF3 cells and transfected clones were washed twice with RPMI 1640 medium lacking IL-3. Cells (2×10^4 per well) were plated in 96-well microtiter plates. Appropriate growth factors and heparin were added in a total volume of 200 μl. Thirty-six hours later, 1 μCi of [^{3}H]thymidine was added in a volume of 50 μl of RPMI 1640 medium. After 4 to 6 h, cells were collected with a PHD Cell Harvestor (Cambridge Technologies, Inc.). Incorporated thymidine was determined by liquid scintillation counting (LKB).

Binding assays. The components of the soluble receptor binding reaction mixture included FRAP conditioned medium (0.24 OD units per min), ^{125}I-bFGF (specific activity, 800 to 3,000 cpm/fmol), and heparin or heparin oligosaccharides. The bound complex was either immunoprecipitated with 20 μl of a 2× slurry of anti-AP monoclonal antibodies coupled to Sepharose (described in reference 9) or precipitated with heparin-Sepharose (Pharmacia CL-6B diluted 1:2 with Sepharose). All components were mixed at room temperature. The total volume was brought to 250 μl with DMEM–0.1% bovine serum albumin. The binding reaction was then allowed to proceed for 2 to 3 h at 4°C. Bound receptor or ligand was recovered by centrifuging for 10 s at 6,000 rpm at 4°C in a microcentrifuge (~2,000 × *g*) and washing two times with 500 μl of ice-cold phosphate-buffered saline (PBS) or PBS containing 0.5 M NaCl (for heparin-Sepharose). ^{125}I-bFGF binding was determined by counting tubes directly in a gamma counter (LKB). Nonlinear curve fitting to the binding equation [$B = B_{max} \times F/(K_d + F)$, where B is bound ligand and F is free ligand (22)] was performed with the program KaleidaGraph on a Macintosh computer.

AP enzyme activity was determined by transferring the heparin-Sepharose-bound receptor to a flat-bottom microtiter plate in a volume of 50 μl of PBS and adding 50 μl of 2× AP assay solution (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM homoarginine, 12 mM *p*-nitrophenyl phosphate [Sigma]) and measuring the A_{405} in a kinetic microplate reader (Molecular Devices).

Binding to BaF3 and F32 cells was set up as described above, except that 2×10^5 to 4×10^5 cells were used instead of Sepharose and FRAP conditioned medium. Binding to CHO cells was as described by Yayon et al. (38).

Cross-linking. FGF cross-linking experiments were carried out in a volume of 20 μl in siliconized 0.5-ml microcentrifuge tubes. All reactions took place at room temperature. Each reaction mixture contained 6.4 to 640 nM unlabeled bFGF, 0 to 300 ng of heparin oligosaccharide per ml, 1.6 nM ^{125}I-bFGF, 20 mM $NaPO_4$ (pH 7.4), and 140 mM NaCl. These reagents were incubated for 30 to 60 min. Next, 1 μl of disuccinimidyl suberate (Pierce) dissolved in dimethyl sulfoxide was added at a final concentration of 0.15 mM, and the mixture was incubated for an additional 30 min. The cross-linking reaction was quenched with 1 μl of 200 mM ethanolamine-HCl (pH 8.0) for 30 min and then diluted 1:1 with 2× SDS-polyacrylamide gel electrophoresis loading buffer and electrophoresed on an SDS–12% polyacrylamide gel. Cross-linked bFGF was visualized by autoradiography on Kodak XAR film.

RESULTS

Binding properties of a soluble FGF receptor. The ligand-binding properties of mFR1 were studied with a cell-free system in the absence of potentially interacting cell surface molecules. A soluble form of mFR1 was engineered by fusing the 5′ and coding regions of the receptor, up to but not including the transmembrane domain, to the secreted human placental AP cDNA (APtag vector [9] [Fig. 1]). This construct, FRAP (Fig. 1B), was then introduced into NIH 3T3 cells, and colonies secreting AP activity were analyzed for FGF binding properties. Monoclonal antibodies to placental AP coupled to Sepharose could quantitatively precipitate AP activity from FRAP conditioned medium (data not shown).

On the basis of our previous work, we predicted that heparin would be required for ligand binding. However, the possibility that other cell surface molecules would also be

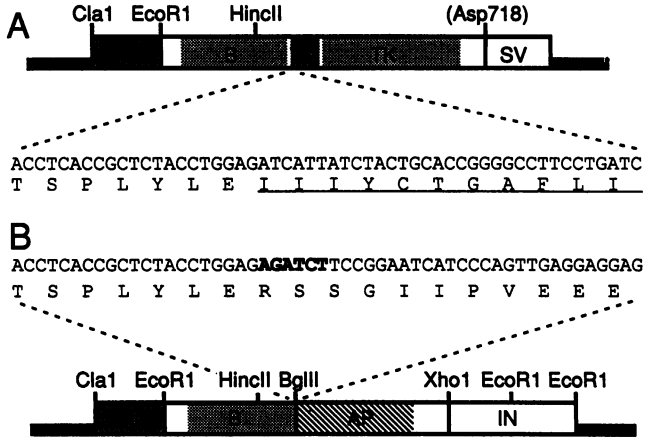

FIG. 1. FGF receptor and FRAP expression vectors. (A) mFR1 expression vector (38) showing the sequence at the 5′ end of the transmembrane domain (underlined). (B) FRAP expression vector showing the sequence across the *Bgl*II site (boldface) junction between mFR1 and AP. Mo, Moloney murine leukemia virus LTR; B, mFR1 extracellular ligand binding domain; TM, transmembrane domain; TK, tyrosine kinase domain; SV, simian virus 40 splice and polyadenylation sequence; IN, insulin splice and polyadenylation sequence.

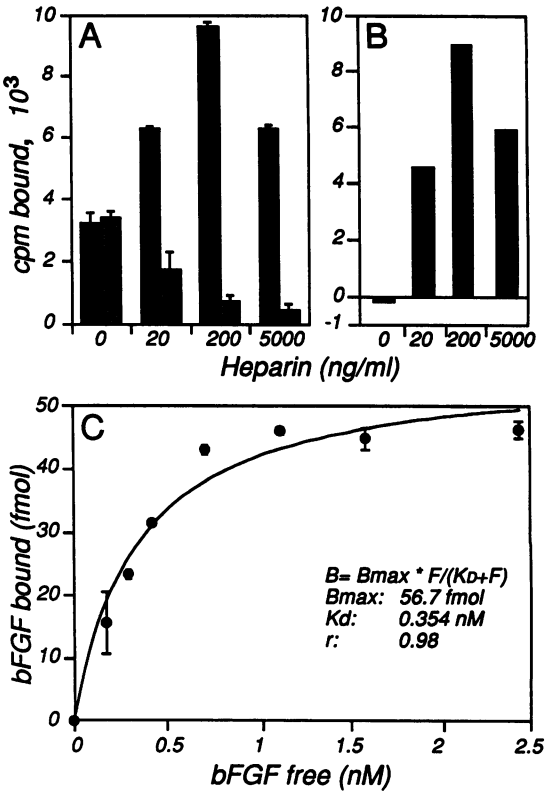

FIG. 2. Binding of bFGF to a soluble FGF receptor. (A) Total binding of ^{125}I-bFGF to FRAP (solid bars) and control APtag-KIT (checked bars). (B) Specific binding of bFGF to FRAP (binding to FRAP − binding to APtag-KIT). (C) Binding of ^{125}I-bFGF to FRAP. Labeled FGF was diluted to a specific activity of 144 cpm/fmol with unlabeled FGF and added in increments in the presence of 200 ng of heparin per ml. Each point in this and subsequent experiments represents the mean ± standard deviation from two binding reactions. All experiments were performed at least two times.

required for FGF binding remained to be determined. As predicted, in the absence of heparin, immunoprecipitated FRAP failed to specifically precipitate ^{125}I-bFGF (Fig. 2A), while with the addition of heparin to the binding medium ligand binding occurred. Compared with a control fusion protein consisting of the c-*kit* receptor extracellular domain fused to AP (APtag-KIT [9]), heparin decreases nonspecific binding while significantly increasing specific binding to FRAP. Plotting specific binding (binding to FRAP − binding to APtag-KIT [Fig. 2B]) demonstrates that there is no detectable interaction between bFGF and its high-affinity receptor in the absence of heparin. Moreover, the concentration of heparin required to saturate this binding is similar to that required for binding of bFGF to the cell surface (approximately 100 ng/ml [38]). Note, however, that at very high heparin concentrations (5 μg/ml [Fig. 2A and B]), binding to FRAP is significantly decreased (see discussion below).

The affinity of bFGF for its soluble receptor was studied by direct and competition binding assays. The results of a direct binding experiment are shown in Fig. 2C. The data, plotted as bound (B) versus free (F) bFGF, were fitted to the equation $B = B_{max} \times F/(K_d + F)$ (see Materials and Methods) to yield K_ds of 354 pM in the experiment, whose results are shown in Fig. 2C and 556 pM in a second experiment. Two independent competition binding experiments yielded K_ds of 742 pM and 1.38 nM (data not shown). These values are in agreement with values obtained by Scatchard and Hill analysis of these data. The dissociation constant for the soluble receptor is higher than that of cell surface receptors expressed on HSPG-deficient CHO cells (20 pM [data not shown]). This difference may reflect both a low-affinity FGF-heparin interaction and a high affinity FGF-heparin-receptor interaction. Unlike the FGF-dependent binding of FRAP to heparin-Sepharose (see below), this binding interaction is sensitive to salt concentrations of >350 mM, and we were not able to distinguish high- and low-affinity binding components.

FGF, heparin, and FRAP form a stable trimolecular complex. The model proposed for FGF-receptor binding (38) invokes the formation of a trimolecular complex. The data presented thus far demonstrate that iodinated FGF binds to cell surface (38) or soluble FGF receptors in a heparin-dependent manner; however, the question of how heparin molecules participate in this binding reaction has not been addressed. For example, heparin or heparan sulfate may convert FGF into a conformation that binds its receptor and only be required for the initial FGF-receptor binding interaction. Alternatively, heparin may interact directly with the receptor to permit ligand binding. To distinguish between these possibilities, we tested the binding of FRAP to heparin immobilized on Sepharose. When AP activity was used as an assay for FRAP, no soluble FGF receptor bound to heparin-Sepharose unless bFGF was added to the binding medium (Fig. 3). Note that the binding reaction is blocked by heparin and is specific for FRAP in that APtag-KIT does not bind even in the presence of bFGF. Additionally, FRAP is not eluted with 0.5 M NaCl, suggesting that this interaction is a high-affinity interaction similar to that between bFGF and its cell surface receptor.

Minimum-size heparin requirements for receptor binding. Commercially available heparin is a heterogeneously sulfated glycosaminoglycan (M_r, 5,000 to 30,000) consisting of

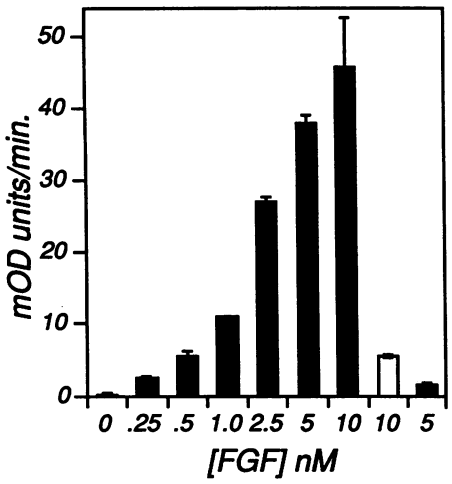

FIG. 3. FGF dependence for FRAP to bind to heparin-Sepharose. Shown is AP enzyme activity (in thousandths of optical density units at 405 nm (mOD_{405}) per minute retained on heparin-Sepharose incubated with FRAP and increasing FGF concentrations (solid bars). The open bar shows competition for FRAP binding by 80 μg of heparin per ml in the presence of 10 nM bFGF. The checked bar shows AP enzyme activity retained on heparin-Sepharose incubated with APtag-KIT in the presence of 5 nM bFGF.

repeating disaccharide subunits of hexuronic acid and D-glucosamine (23). Heparan sulfate is the endogenous sulfated glycosaminoglycan that constitutes the low-affinity binding site for bFGF (reviewed in reference 20). It qualitatively has the same structure as heparin; however, it shows differences in saccharide composition and is also less sulfated (12, 13). Heparan sulfates have been shown to contain blocks of 8 to 14 monosaccharide subunits similar to heparin which are rich in iduronic acid and high in sulfate content (27, 34). To determine the minimum saccharide size of heparin or heparan sulfate required for bFGF binding, we have tested the binding properties of heparin oligosaccharides of defined length.

HSPG-deficient CHO cells that overexpress mFR1 bind ^{125}I-bFGF only in the presence of heparin oligosaccharides containing at least eight sugar residues (Fig. 4A). Similarly, the soluble FGF receptor also requires heparin oligosaccharides for ligand binding. Twelve- and 16-residue heparin oligosaccharides allow detectable binding to the soluble receptor, whereas 6- and 8-residue oligosaccharides do not (data not shown). We chose to study this binding reaction in more detail with a highly sulfated hexadecasaccharide (HS-16).

FGF binding to FRAP in the presence of HS-16 saturates at concentrations between 20 and 50 ng/ml (Fig. 4B), which are similar to the concentration seen in the presence of heparin. Competition binding experiments demonstrate an initial increase in binding after addition of cold bFGF,

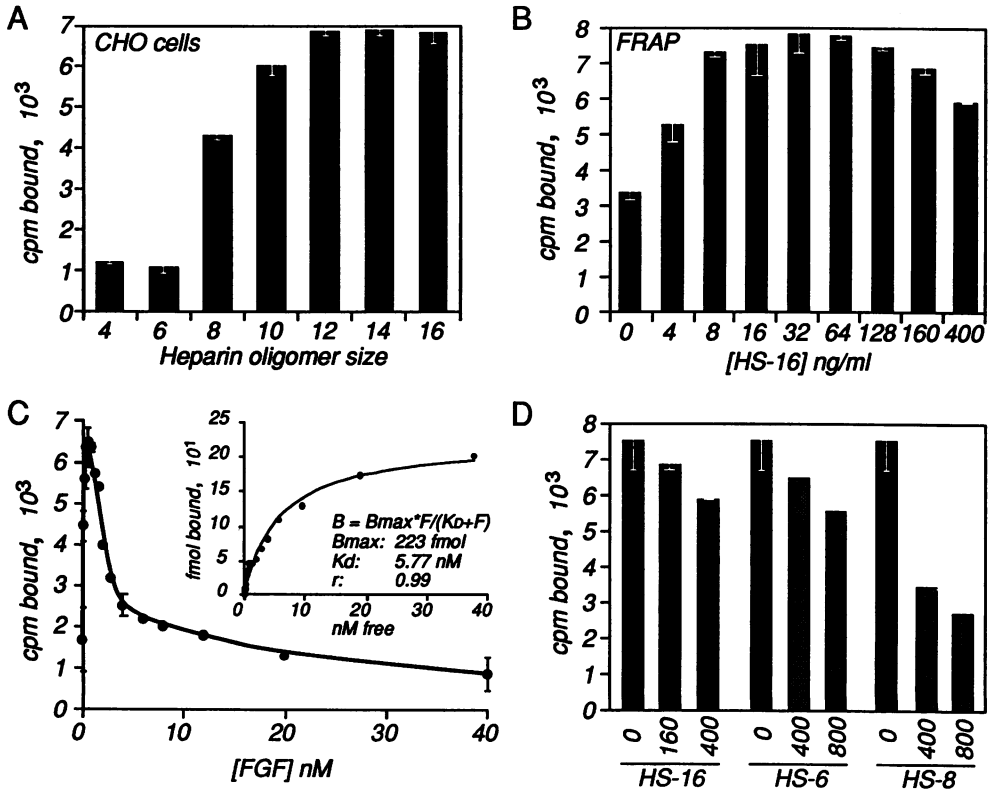

FIG. 4. FGF binding to CHO cells and to FRAP in the presence of heparin oligosaccharides. (A) ^{125}I-bFGF binding to CHO mFR1 cells (38) in the presence of 1 μg of heparin oligosaccharides per ml. These oligosaccharides were prepared by fractionation of alkali-treated heparin methyl ester (2). (B) Total binding of ^{125}I-bFGF to FRAP in the presence of increasing concentrations of HS-16. (C) Competition binding curve for ^{125}I-bFGF and FRAP in the presence of 20 ng of HS-16 per ml and increasing concentrations of unlabeled bFGF. The inset shows a replotting of the data as bound versus free ligand, using the binding equation as described in Materials and Methods. (D) Inhibition of ^{125}I-bFGF binding to FRAP by high concentrations of heparin oligomers. All of the mixtures used in these reactions contained 16 ng of HS-16 per ml plus the indicated concentrations of HS-6, HS-8, and HS-16.

followed by an exponential decrease in binding (Fig. 4C). Binding constants calculated as described above for heparin yield a dissociation constant of 3.2 to 5.8 nM (two independent experiments [Fig. 4C inset and data not shown]). This binding constant, in the low-affinity range for FGF binding (25), may reflect differences arising from either the heparin or receptor motifs because clearly both components have been altered. At high HS-16 concentrations of (>100 ng/ml), ^{125}I-bFGF binding decreases. Although a highly sulfated octasaccharide (HS-8) does not allow measurable binding of ^{125}I-bFGF to FRAP, it effectively competes for binding induced by HS-16 at concentrations of >100 to 200 ng/ml (Fig. 4D). A highly sulfated hexasaccharide (HS-6) also shows a small but reproducible decrease in binding in the presence of HS-16 (Fig. 4D and data not shown).

Heparin requirement for bFGF biologic activity. We have designed a mitogenic assay for FGF using the IL-3-dependent lymphoid cell line BaF3 (24). This cell line can be made to respond mitogenically to EGF, erythropoietin, GM-CSF, and IL-2 when transfected with their respective receptors (5, 7, 15, 19). Because this cell line grows in suspension culture, we hypothesized that it would synthesize minimal extracellular matrix and cell surface HSPG. Additionally, these cells do not express detectable levels of syndecan, the putative low-affinity receptor for FGF (32a). bFGF binding and RNA blot analysis demonstrate that parental BaF3 cells do not express detectable FGF receptors (Fig. 5A and B). Therefore, we transfected these cells with Mo/mFR1/SV, an mFR1 expression vector (Fig. 1A and reference 38) and selected either with G418 (for a cotransfected neomycin resistance gene) plus IL-3 conditioned medium or with bFGF plus heparin in the absence of IL-3. Both of these procedures yielded colonies which express mFR1 mRNA and bind ^{125}I-bFGF (Fig. 5A and B).

The mitogenic response of these transfected cells was tested in the absence of IL-3 and in the presence of various concentrations of bFGF and heparin (Fig. 5C). Parental BaF3 cells do not show any mitogenic response to FGF or heparin. Cells transfected to express mFR1 demonstrate a dose-dependent response to bFGF with an absolute requirement for heparin. Heparin alone (500 ng/ml) has no effect on the growth of these cells, and bFGF (500 pM) has no effect on these cells in the absence of heparin. This mitogenic response is consistent with the heparin-dependent binding of ^{125}I-bFGF to these cells (Fig. 5B).

To examine the biologic activities of size-selected heparin oligosaccharides, BaF3/mFR1 cells (clone F32) were grown in the presence of subsaturating bFGF (30 pM) and treated with various concentrations of heparin oligosaccharides (Fig. 5D). The mitogenic response to HS-16 and HS-8 is dose dependent; however, there is no mitogenic response to HS-6. Thus, the smallest biologically active heparin oligosaccharide appears to contain eight sugar residues. These results are consistent with the oligosaccharide-dependent binding on the surface of HSPG-deficient CHO cells expressing mFR1 (Fig. 4A). Because high concentrations of HS-6 and HS-8 inhibit binding to the soluble receptor, we tested these preparations for growth-inhibitory activity on F32 cells. HS-6 at a concentration of 5 μg/ml failed to inhibit the mitogenic response to HS-8 over a concentration range of 20 to 5,000 ng/ml (data not shown).

Heparin oligosaccharides cause bFGF oligomerization. A common theme with growth factors that interact with receptor tyrosine kinases is that receptor activation involves ligand-induced receptor dimerization (35). In several cases, ligand dimerization itself may be involved in either inducing

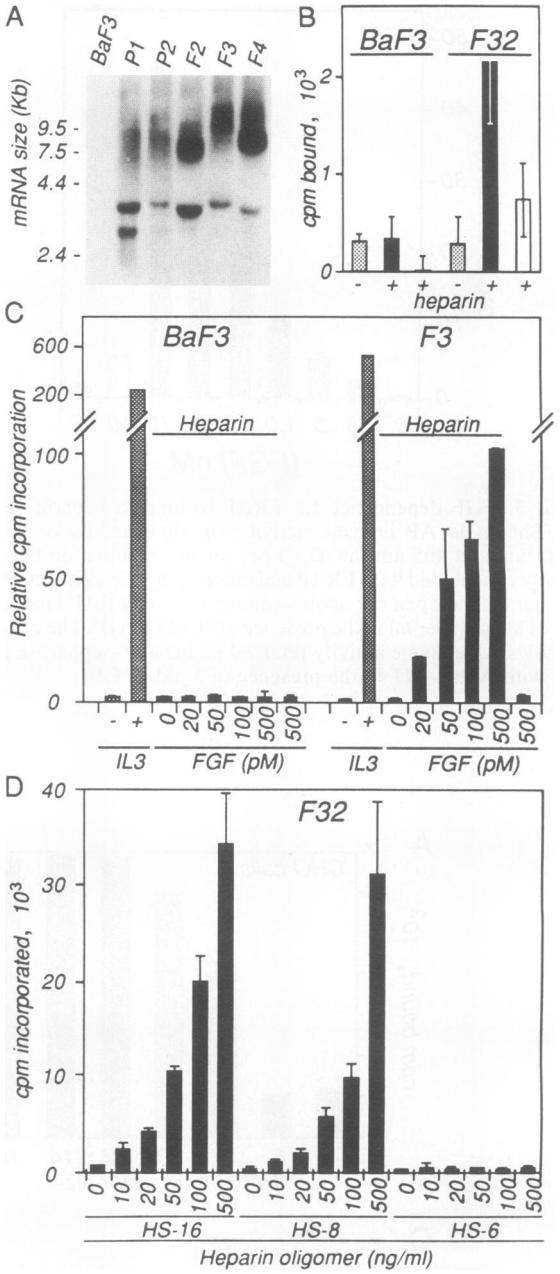

FIG. 5. Factor-dependent growth of mFR1-transfected BaF3 cells. (A) RNA blot of parental BaF3 cells and cells transfected with Mo/mFR1/SV hybridized to an mFR1-specific ^{32}P-labeled DNA probe. Lanes P1 and P2 are BaF3 cells cotransfected with a neomycin resistance gene and selected in 600 μg of G418 per ml in the presence of IL-3. F2, F3, and F4 were selected with 200 pM bFGF and 100 ng of heparin per ml. High-molecular-weight bands probably arise from incomplete RNA processing of the transfected plasmids. (B) ^{125}I-bFGF binding to BaF3 cells and F32 cells. Stippled bars, binding in the absence of heparin; solid bars, binding in the presence of 1 μg of heparin per ml; open bars, binding in the presence of 1 μg of heparin per ml and 4 nM unlabeled bFGF. Nonspecific binding (counts bound to parental cells in the presence of competitor) was subtracted from each point. (C) Relative [^{3}H]thymidine incorporation in parental BaF3 cells and F3 cells. Checked bars, untreated cells (−) and cells treated with 1% IL-3 conditioned medium (+); solid bars, cells treated with 500 ng of heparin per ml (where indicated) and the indicated FGF concentrations. (D) [^{3}H]thymidine incorporation in F32 cells (subcloned from F3 cells) treated with 30 pM bFGF and heparin oligomers at the indicated concentrations.

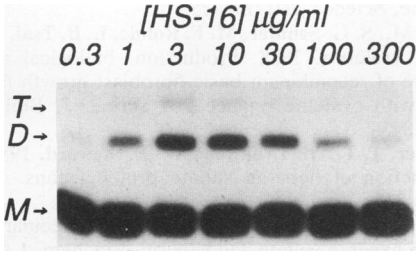

FIG. 6. FGF cross-linking to itself in presence of heparin oligomers. The concentration of HS-16 was varied from 0.3 to 300 μg/ml in the presence of 640 nM bFGF and 1.6 nM ^{125}I-bFGF. FGF molecules were crosslinked with disuccinimidyl suberate. M, monomer; D, dimer; T, trimer.

or stabilizing receptor dimers. We were therefore interested in the possibility that bFGF can itself form a functional dimer that may in turn facilitate receptor dimerization. Fox et al. (11) observed sulfhydryl-linked dimers in recombinant bFGF. However, the significance of these dimers is not clear because when the involved cysteines were changed to serines by site-directed mutagenesis, the modified FGF was fully active. To test the hypothesis that heparin can allow FGF dimers to form, we attempted to covalently cross-link bFGF to itself at various concentrations of heparin oligosaccharide (Fig. 6). The binding and cross-linking reactions were carried out at FGF concentrations ranging from 6.4 to 640 nM and at a physiologic salt concentration (140 mM). In the absence of heparin, no significant FGF oligomers could be detected; however, in the presence of heparin oligosaccharide HS-6, HS-8, or HS-16, cross-linked FGF oligomers could easily be detected at the higher FGF concentrations (Fig. 6 and data not shown). The mobilities of the cross-linked FGF oligomers are consistent with the formation of dimers and trimers. The formation of FGF dimers is dependent on the ratio of heparin to FGF, and at molar ratios exceeding 1:2 the amount of dimer formation decreases. At 10-fold lower FGF concentrations (64 nM), the maximum level of heparin fragment-mediated FGF dimer formation occurs at a 10-fold lower heparin fragment concentration (data not shown). Thus, for optimal dimer formation, the absolute ratio of FGF to heparin oligosaccharide seems essential.

DISCUSSION

We have addressed the question of whether cell surface molecules in addition to HSPGs are required for FGF-receptor binding by characterizing the ligand binding properties of FRAP, a soluble FGF receptor-AP fusion protein. The FRAP protein binds bFGF only in the presence of heparin, thus demonstrating that other cell surface molecules are not necessary for bFGF-receptor binding. To demonstrate that heparin is essential for FGF biologic activity, we have constructed a cell line that requires both FGF and heparin for growth. The normally IL-3-dependent cell line BaF3 has also been used to investigate a number of other cell surface receptors (5, 7, 15, 19), and in no case was there a requirement for added polysaccharide for mitogenicity. Thus, BaF3 cells do not have an inherent requirement for heparin for non-IL-3 factor-dependent growth. The correlation between the heparin requirement for FGF binding to mFR1 on the cell surface or in solution and the absolute requirement for heparin for FGF-dependent growth strongly suggests that a heparin-FGF or heparan sulfate-FGF complex is the biologically active form of bFGF.

The mechanism by which bFGF binds to its receptor was addressed in two ways in this study. First, iodinated ligand requires heparin in order to bind to its receptor, and second, the soluble receptor, FRAP, requires FGF in order to bind to heparin. These observations suggest that bFGF, receptor, and heparin form a stable trimolecular complex. Because heparin sulfate polysaccharides are large heterogeneous molecules, we have begun to characterize biologically active heparin oligosaccharides by determining the minimum-length heparin oligosaccharide required for bFGF-receptor binding and for bFGF biologic activity. Binding to receptor-expressing, HSPG-deficient CHO cells and mitogenicity assays with BaF3/mFR1 (clone F32) cells indicate that eight sugar residues constitute the minimum-length heparin oligosaccharide that is biologically active. The soluble receptor also binds ligand in the presence of small heparin oligosaccharides. Compared with cell surface mFR1, which is diffusion limited in two dimensions, the soluble receptor may have more stringent binding requirements, reflecting its requirement for slightly longer heparin oligosaccharides. In vivo, the ligand-receptor complex may be stabilized by cell surface receptor dimerization and possibly by ligand dimerization (see below).

It is of interest to note that the endogenous ligand, heparan sulfate, although generally much less sulfated than heparin, contains regions with length, saccharide composition, and sulfate content similar to those of heparin (27, 34). While future studies will address the importance of specific heparin and heparan sulfate structures in the receptor binding and biologic activity of bFGF, a recent observation of Folkman et al. (10) is of particular interest. They showed that, in the absence of steroids, heparin stimulates tumor angiogenesis whereas heparin-derived hexasaccharides do not. Our observation that an octasaccharide is required for bFGF biologic activity may explain this finding.

The binding affinity that we have measured with respect to the binding of bFGF to its soluble FGF receptor is 10- to 20-fold lower than that of the binding of bFGF to its cell surface receptor. A similar phenomenon has been observed with a soluble form of the EGF receptor. This soluble receptor also has a lower affinity for EGF than its cell surface form (21). It is possible that iodination alters FGF such that it preferentially binds heparin rather than its receptor. If this were the case, specific binding would nevertheless occur because multiple FGF molecules (labeled and unlabeled) could bind a single heparin molecule and link labeled FGFs to their receptors via heparin and unlabeled FGF. The consequence of this possible interaction is that the apparent K_d of iodinated FGF for the soluble receptor would at least in part reflect the K_d of heparin-FGF interactions. This may be the case because the observed K_d (350 pM to 1.4 nM) lies between the high- and low-affinity FGF binding constants (reference 25 and our unpublished data). Alternatively, the reduced K_d might be due to inherent differences between the soluble and cell surface receptors. Nevertheless, the FGF-dependent binding of FRAP to heparin-Sepharose demonstrates that the molecule recognized by the receptor is an FGF-heparin complex.

Another interesting observation is that competition FGF binding to the soluble receptor in the presence of heparin or heparin oligosaccharides shows an initial increase in counts bound after addition of small amounts of unlabeled FGF (up to 900 pM). While this phenomenon suggests positive cooperativity between ligand and receptor, it may also reflect

different binding properties of iodinated and unlabeled ligand (22).

The possibility that heparin itself stabilizes FGF dimers and thus promotes and stabilizes receptor dimers is suggested by several observations. First, heparin oligosaccharides allow ligand association and cross-linking which is dependent on the ratio of FGF to heparin oligosaccharide. Second, the soluble receptor and presumably the cell-surface receptor recognize heparin-bound bFGF and form a complex which could be linked by a heparin molecule with multiple FGF binding sites. Third, high heparin or heparin oligosaccharide concentrations inhibit ligand binding to the soluble receptor, perhaps by creating conditions that favor formation of monomeric FGF. Fourth, unlabeled FGF increases binding of labeled FGF at low concentrations and competes for binding at higher concentrations, suggesting either positive cooperativity or linkage of labeled FGF to receptor-bound unlabeled FGF by heparin. These observations suggest that heparin oligosaccharides of sufficient length may stabilize FGF dimers under physiologic conditions. Stable FGF dimers may in turn facilitate receptor dimerization and subsequent activation.

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