Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 450-458

DISTINCT ROLE OF 2-*O*-, *N*-, and 6-*O*-SULFATE GROUPS OF HEPARIN IN THE FORMATION OF THE TERNARY COMPLEX WITH BASIC FIBROBLAST GROWTH FACTOR AND SOLUBLE FGF RECEPTOR-1

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Received July 8, 1994

SUMMARY: Interaction of basic fibroblast growth factor (bFGF) with heparan sulfate proteoglycans (HSPGs) plays an important role in the binding of bFGF to its tyrosine kinase receptor (FGFR). The molecular bases of this interaction were investigated by evaluating the capacity of conventional and selectively desulfated heparins i) to affect the binding of bFGF to FGFR and HSPGs of NIH 3T3 cells transfected with FGFR-1/*flg* cDNA, ii) to facilitate the interaction of bFGF with a recombinant soluble form of the extracellular domain of FGFR-1/*flg* (xcFGFR-1), and iii) to protect xcFGFR-1 from tryptic cleavage. 6-*O*-desulfated (6-*O*-DS) heparin, but not 2-*O*-desulfated (2-*O*-DS) and *N*-desulfated/*N*-acetylated (N-DS/*N*-Ac) heparins, retains the capacity to bind bFGF, as assessed by its ability to inhibit bFGF-binding to cell-associated FGFR-1 and HSPGs. On the other hand, at variance with conventional heparin, 2-*O*-DS, *N*-DS/*N*-Ac, and 6-*O*-DS heparins are all ineffective in potentiating the binding of bFGF to xcFGFR-1 and protecting xcFGFR-1 from tryptic cleavage. The data indicate that 6-*O*-sulfate groups are not essential for the interaction of heparin with bFGF but are involved in the interaction with xcFGFR-1. Our findings support the hypothesis that HSPGs modulate the binding of bFGF to FGFR through the formation of a ternary complex in which the glycosaminoglycan chains interact with bFGF *via* 2-*O*- and *N*-sulfate groups and with FGFR also *via* 6-*O*-sulfate groups.

Basic fibroblast growth factor (bFGF) is a 18 kDa mitogen which belongs to the heparin-binding growth factor family. bFGF plays an important role in angiogenesis, wound healing, and embryonic development and it has been implicated in the proliferation or differentiation of a variety of cell types (1).

bFGF interacts with two distinct classes of receptors. The first class is represented by high affinity tyrosine-kinase receptors (FGFRs) responsible for the transduction of the signal (2). The second class is represented by low affinity heparan sulfate proteoglycans (HSPGs) present on the cell surface and extracellular matrix (3). The interaction of bFGF with HSPGs modulates the binding of the growth factor to FGFRs (4-6). Several data suggest that the glycosaminoglycan (GAG) chain of HSPG participates to the assembling of a ternary complex with bFGF and FGFR (4-7). Indeed, a direct

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association of FGFR with heparin has been reported and a heparin-binding domain has been identified in the NH_2-terminus of IgG-like domain II of FGFR-1/*flg* (8).

bFGF binds also sulfated GAGs in solution. This interaction protects bFGF from proteolytic degradation (9-12) and prevents the binding of the growth factor to cell-associated HPSGs and FGFRs (13, 14). On the other hand, heparin promotes the binding of bFGF to a soluble form of FGFR (15, 16) or to cell-associated FGFR when HPSGs are absent or undersulfated (4, 5, 7).

The molecular bases of the interaction of bFGF with HPSGs have been investigated. bFGF-binding sequences have been characterized in heparin and heparan sulfate from different sources (17, 18). These sequences contain disaccharide units composed of IdoA(2-OSO_3)-GlcNSO_3 or IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3), suggesting that *N*- and 2-*O*-sulfate groups, but not 6-*O*-sulfate groups, are essential for bFGF recognition. However, even though a pentasaccharide containing this structure is the minimal bFGF-binding sequence (18), a dodesaccharide represents the minimal requirement for promoting growth factor activity (19). Also, 6-*O*-desulfated (6-*O*-DS) heparin, despite the capacity to bind bFGF (18, 20), is ineffective in restoring bFGF mitogenic activity in chlorate-treated fibroblasts with impaired synthesis of HSPGs (19). Thus, the bulk of data suggest that GAG chains of HSPG contain distinct but adjacent sequences involved in bFGF- and FGFR-binding and that different structural features, including sulfation pattern, are responsible for the interaction with the two molecules.

In the present paper the capacity of selectively desulfated heparins to affect the binding of bFGF to a recombinant soluble form of the extracellular domain of human FGFR-1/*flg* (xcFGFR-1) was investigated. The results indicate that 2-*O*- and *N*-sulfate groups, but not 6-*O*-sulfate groups, are required for bFGF binding. Nevertheless, all sulfate groups are essential for the formation of bFGF/heparin/xcFGFR-1 complex, suggesting that 6-*O*-sulfate groups interact directly with FGFR.

MATERIALS AND METHODS

Chemicals. Human recombinant bFGF and the extracellular domain of FGFR-1/*flg* (xcFGFR-1) were expressed and purified from transformed *E. coli* cells (21, 22). Native and selectively desulfated heparins were provided by B. Casu, G. Ronzoni Institute of Chemistry and Biochemistry, Milan, Italy. ^{13}C-NMR spectra (23-25) indicated 95% 2-*O*-desulfation and 0% 6-*O*-desulfation, 100% 6-*O*-desulfation and 15% 2-*O*-desulfation, 100% *N*-desulfation/*N*-acetylation and 0% *O*-desulfation for 2-*O*-desulfated (2-*O*-DS), 6-*O*-desulfated (6-*O*-DS), and *N*-desulfated/*N*-acetylated (*N*-DS/*N*-Ac) heparins, respectively.

Cell transfection. NIH 3T3 cells (provided by G. Parmiani, Istituto Nazionale dei Tumori, Milan, Italy) were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum. Cells were transfected by calcium phosphate precipitation with a murine FGFR-1/*flg* cDNA (26) cloned in the pZip-NeoSV(X)1 expression vector (27). After G418 selection, the clone NIH-*flg*-Z2 (40,000 receptor/cell; Kd = 67 pM) was used for further studies.

^{125}I-bFGF binding assay. bFGF was labeled with ^{125}I to a specific radioactivity of 800 cpm/fmol using Iodogen (Pierce Chemical Co., Rockford, IL), as described (28). For ^{125}I-bFGF binding assay, NIH-*flg*-Z2 cells were seeded at 70.000 cell/cm^2 in 24-well dishes. After 24 h, cultures were washed with ice-cold phosphate buffered saline (PBS) and were incubated at 4°C in serum-free medium containing 3 ng/ml of ^{125}I-bFGF, 0.15% gelatin, 20 mM Hepes buffer (pH 7.5) in the absence or in the presence of increasing concentrations of heparin. After 2 h, the amount of ^{125}I-bFGF bound to low and high affinity sites was evaluated as described (29). Briefly, the medium was removed and cells were washed once with PBS, twice with 2 M NaCl in 20 mM Hepes buffer (pH 7.5), and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0). Radioactivity released by neutral and acidic 2 M NaCl washes represent ^{125}I-bFGF

bound to low and high affinity sites, respectively. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabeled bFGF and was subtracted from all the values.

^{125}I-bFGF cross-linking. 3 ng of ^{125}I-bFGF were incubated for 2 h at 37°C in PBS with 30 ng of xcFGFR-1 in the absence or in the presence of increasing concentrations of heparin. Then, xcFGFR-1/^{125}I-bFGF complexes were cross-linked by adding 1 mM bis[2-(succinimido-oxycarbonyloxy)-ethyl]sulfone (BSOCOES, Pierce Chem. Co.). After 30 min, samples were added with reducing sample buffer, boiled, and loaded onto a SDS-10% polyacrylamide gel. Gels were dried and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at -70°C. The 63 kDa band, corresponding to xcFGFR-1/^{125}I-bFGF complex, was quantified by computerized image analysis of the autoradiography using a Magiscan Image Analyzer (Joyce-Loebl LTD, England) with the Genias 3.0 software package.

Proteolytic digestion of xcFGFR-1. One μg-aliquots of xcFGFR-1 (45 kDa) were equilibrated at 37°C for 10 min in 70 μl of PBS containing 4 μg of bFGF and the indicated concentrations of heparin. Then, 60 ng of trypsin (Sigma, St. Louis, MO) were added. After 2 h of incubation at 37°C, samples were added with 20 μl of reducing sample buffer and boiled. Proteins were analyzed on SDS-10% polyacrylamide gels and visualized by silver staining. The protective effect of heparin was evaluated by quantification of the integrated density of the band corresponding to the protected 33 kDa xcFGFR-1 fragment by computerized image analysis of the gels.

Amino acid sequence analysis. Heparin-protected 33 kDa xcFGFR-1 fragment was transferred from SDS-10% polyacrylamide gel to PVDF membrane for 50 min at 300 mA in 10 mM CAPS buffer, 10% methanol. Then, the PVDF membrane was stained for 1 min with 0.1% Coomassie R 250 in 40% methanol, 1% acetic acid and destained for 10 min with 50% methanol, 1% acetic acid. The band corresponding to the xcFGFR-1 fragment was cut out with a lancet and analyzed in a pulsed liquid-phase sequencer model 477A (Applied Biosystem, Foster City, CA) using standard manufacturer's programs.

RESULTS

Complete desulfation of heparin prevents its interaction with the growth factor, indicating that sulfate groups play an important role in the recognition of bFGF (3). bFGF-binding sequences in heparin and heparan sulfate contain disaccharide units composed of IdoA(2-OSO_3)-GlcNSO_3 or IdoA(2-OSO_3)-GlcNSO_3(6-OSO_3) (15, 17, 18, 30, 31). This suggests that *N*- and 2-*O*-sulfation are essential for bFGF recognition, while 6-*O*-sulfation is irrelevant. On this basis, to study the effect of selective desulfation on the interaction of bFGF with heparin, the capacity of modified heparins to prevent the binding of ^{125}I-bFGF to low and high affinity sites in FGFR-1/*flg* cDNA-transfected NIH 3T3 cells was evaluated.

Conventional and 6-*O*-DS heparins displaced ^{125}I-bFGF from cell-associated FGFR-1 in a dose-dependent manner, with ID_{50} equal to 700 μg/ml and 30 μg/ml, respectively (Fig. 1A). 2-*O*-DS and *N*-DS/*N*-Ac heparins were ineffective. Accordingly, conventional and 6-*O*-DS heparins prevented with high potency the binding of ^{125}I-bFGF to low affinity HSPGs (ID_{50} equal to 3 μg/ml for both molecules), while 2-*O*-DS and *N*-DS/*N*-Ac heparins were much less effective (ID_{50} equal to 300 μg/ml and 150 μg/ml for the two molecules, respectively) (Fig. 1B). These data demonstrate that heparin retains the capacity to bind bFGF and to sequester it in solution only when both 2-*O*- and *N*-sulfate groups are present, while 6-*O*-sulfate groups are not required for bFGF interaction.

Recent observations have shown the presence of an essential heparin-binding domain in FGFR-1 (8), raising the hypothesis that a ternary bFGF/heparin/FGFR complex may exist in which the same GAG molecule can bind both the growth factor and its receptor. On this basis, we have investigated the role of the different sulfate groups of heparin in the *in vitro* formation of this ternary complex by

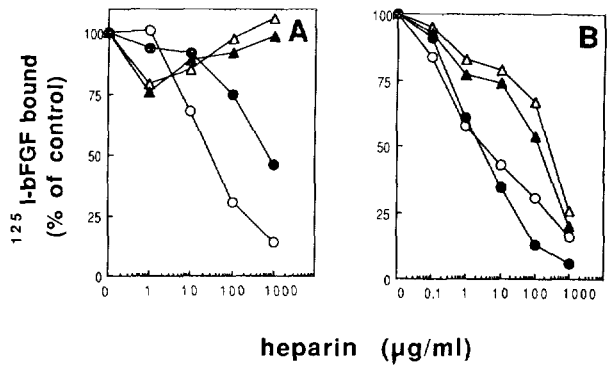

Fig. 1. Effect of conventional and selectively desulfated heparins on the binding of bFGF to NIH-*flg*-Z2 cells. Cells were seeded at 70.000 cells/cm^2. After 24 h, cell cultures were incubated for 2 h at 4°C with binding medium containing ^{125}I-bFGF (3 ng/ml) in the absence or in the presence of increasing concentrations of conventional (●), 6-*O*-DS (o), 2-*O*-DS (△), or *N*-DS/*N*-Ac (▲) heparin. At the end of incubation, radioactivity associated with high (*panel A*) and low (*panel B*) affinity sites was measured. Data are the mean of 3 experiments. S.E.M. is lower than 10% of the mean.

utilizing selectively desulfated heparins and a soluble recombinant form of the extracellular domain of FGFR-1/*flg* (xcFGFR-1) produced in *E. coli*. To this purpose, ^{125}I-bFGF was incubated with xcFGFR-1 in the absence or in the presence of increasing concentrations of conventional heparin. Samples were then chemically cross-linked and analyzed by SDS-PAGE followed by autoradiography. As shown in Fig. 2, a 63 kDa band, corresponding to the 18 kDa bFGF/45 kDa xcFGFR-1 complex (22), was detectable. Conventional heparin increases the amount of ^{125}I-bFGF cross-linked to xcFGFR-1 in a dose-dependent manner, with an ED_{50} equal to 50 ng/ml. Maximal effect, corresponding to 3-fold increase in the amount of ^{125}I-bFGF/xcFGFR-1 complex, was observed at 100 ng/ml of heparin (Fig. 3). When 6-*O*-DS, 2-*O*-DS, and *N*-DS/*N*-Ac heparins were assessed for the capacity to affect the interaction of ^{125}I-bFGF with xcFGFR-1, all selectively desulfated heparins were ineffective in potentiating the binding of bFGF to the soluble receptor also when tested at 10 μg/ml (Fig. 3).

Our data suggest that 6-*O*-DS heparin does not promote the formation of the bFGF/xcFGFR-1 complex despite its capacity to bind the growth factor. This raises the hypothesis that, at variance with 2-*O*- and *N*-sulfate groups, 6-*O*-sulfate groups may play a role in mediating the interaction of heparin with FGFR rather than with bFGF. To investigate this possibility, we compared the capacity of conventional and selectively desulfated heparins to protect xcFGFR-1 from tryptic cleavage *in vitro* (8). To this purpose, xcFGFR-1 was added with 10-fold molar excess of bFGF and incubated with 70 μg/ml of conventional heparin in the presence of trypsin. After 2 h at 37°C, samples were analyzed by SDS-PAGE followed by silver staining. As shown in Fig. 4A, intact xcFGFR-1 migrates as a single 45 kDa band. No bands were detectable when xcFGFR-1 was incubated with trypsin in the absence of heparin, either with or without bFGF. On the contrary, xcFGFR-1 was partially protected from trypsin digestion in presence of heparin, originating a 33 kDa fragment. The protective effect of heparin was strictly dependent on the presence of native bFGF. In the absence of bFGF or in the presence of heat-inactivated bFGF, heparin did not protect xcFGFR-1 even when tested at 1.5 mg/ml Fig. 4A). The protective capacity of heparin

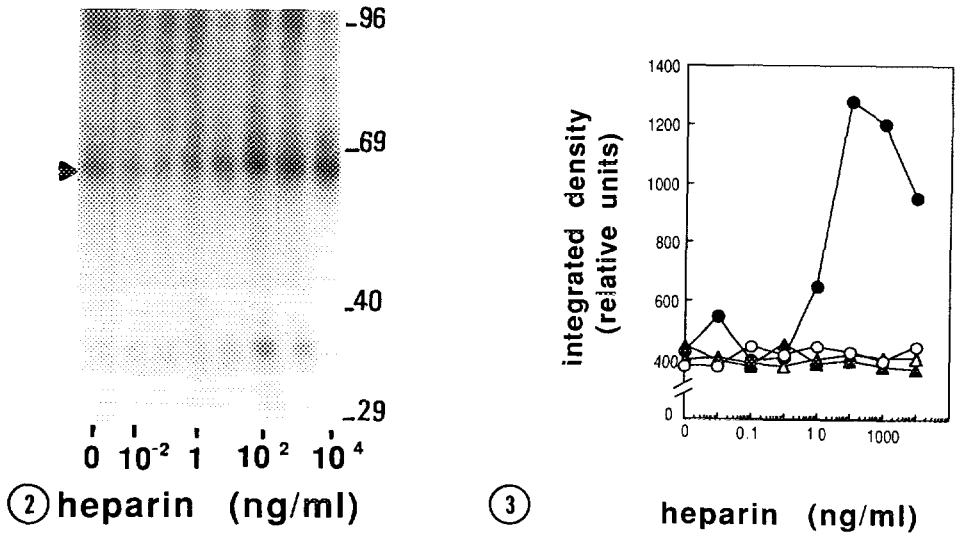

Fig. 2. Effect of conventional heparin on the binding of bFGF to xcFGFR-1. 3 ng of ^{125}I-bFGF were incubated for 2 h at 37°C with 30 ng of xcFGFR-1 in the absence or in the presence of increasing concentrations of conventional heparin. Then, samples were cross-linked with BSOCOES and analyzed by SDS-PAGE followed by autoradiography of the gel. Molecular weights are in thousands. Arrowhead: bFGF/xcFGFR-1 complex.

Fig. 3. Effect of selectively desulfated heparins on the binding of bFGF to xcFGFR-1. 3 ng of ^{125}I-bFGF was incubated for 2 h at 37°C with 30 ng of xcFGFR-1 in the absence or in the presence of increasing concentrations of conventional (●), 6-*O*-DS (o) , 2-*O*-DS (△), or *N*-DS/*N*-Ac (▲) heparin. Then, samples were cross-linked with BSOCOES and analyzed by SDS-PAGE followed by autoradiography of the gel. For each sample, the amount of ^{125}I-bFGF/xcFGFR-1 complex was estimated by computerized image analysis of the autoradiography. Data are the mean of 2-4 independent experiments. S.E.M. is lower than 20% of the mean.

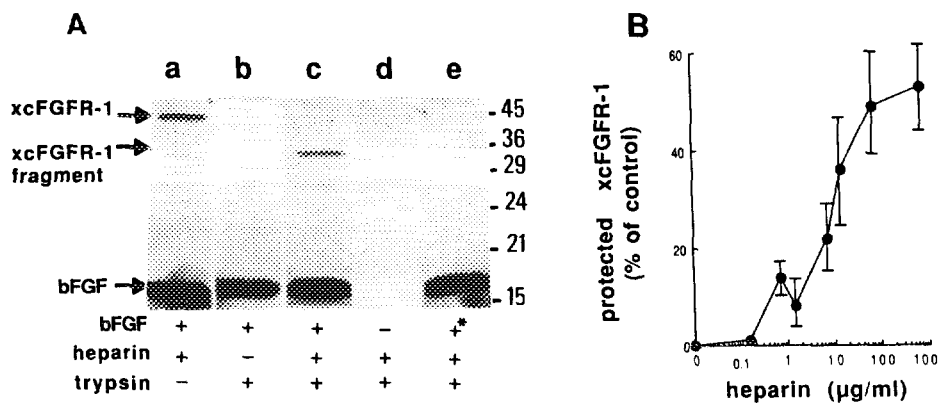

Fig. 4. Characterization of heparin protection of xcFGFR-1 from trypsin digestion. *Panel A:* One µg-aliquots of xcFGFR-1 were incubated for 10 min at 37°C in the absence (-) or in the presence of 4 µg of native bFGF (+) or of 4 µg of heat-inactivated bFGF (+*). Conventional heparin was added to lanes *a*, *c* and *e* at 700 µg/ml and to lane *d* at 1.5 mg/ml. Then, 60 ng of trypsin was added to all the samples except for lane *a*. After 2 h of incubation at 37°C, samples were analyzed by SDS-PAGE followed by silver staining. Molecular weights are in thousands. *Panel B:* Aliquots of xcFGFR-1 were incubated with increasing concentrations of native heparin in presence of bFGF and added with trypsin as described for panel A. After 2 h of incubation at 37°C, samples were analyzed by SDS-PAGE followed by silver staining and the amount of heparin-protected 33 kDa xcFGFR-1 fragment was estimated by computerized image analysis of the gel. Data are the mean ± S.E.M. of 2-5 experiments.

Table 1. Amino-terminal sequence analysis of the heparin-protected xcFGFR-1 fragment

Cycle:	1	5	10	15	20
Observed sequence:	I-T-G-E-E-V-E-V-Q-D-S-V-P-A-D-S-G-L-Y-A				
Deduced sequence*:	I-T-G-E-E-V-E-V-Q-D-S-V-P-A-D-S-G-L-Y-A				
Amino acid residue*:	81	85	90	95	100

*Amino acid sequence and numbering were deduced from human FGFR-1 cDNA (33).

was dose-dependent, with an ED_{50} equal to 10 μg/ml. Maximal effect, corresponding to 53% protection of the amount of xcFGFR-1 originally added, was obtained with 75 μg/ml of heparin (Fig. 4B). Amino acid sequence analysis of the NH_2-terminus of the heparin-protected fragment of xcFGFR-1 is shown in Tab. 1. The 33 kDa fragment initiates at residue Ile^{81}, within IgG-like domain I (8), and its size indicates that the heparin-protected region of xcFGFR-1 spans through the entire IgG-like domains II and III.

In agreement with ^{125}I-bFGF/xcFGFR-1 cross-linking experiments, 2-*O*-DS, 6-*O*-DS, and *N*-DS/*N*-Ac heparins were all ineffective in preventing trypsin digestion of xcFGFR-1 also when tested at 700 μg/ml (Fig. 5). These data confirm the hypothesis that 6-*O*-DS heparin does not mediate the interaction of the growth factor with xcFGFR-1 despite its capacity to bind bFGF.

DISCUSSION

In the present paper, we investigated the role of the different sulfate groups of heparin in mediating the binding of bFGF to a soluble form of xcFGFR-1 produced in *E. coli*. This non-glycosilated form of FGFR-1 and the recombinant glycosilated counterpart expressed in eukaryotic cells binds bFGF in the absence of heparin with similar affinity (15, 16, 22). Our results demonstrate that conventional heparin potentiates the binding of ^{125}I-bFGF to xcFGFR-1 in a dose-dependent manner, with an ED_{50} equal to 50 ng/ml. This value is close to that obtained for the glycosilated form of FGFR-1 [10-20 ng/ml

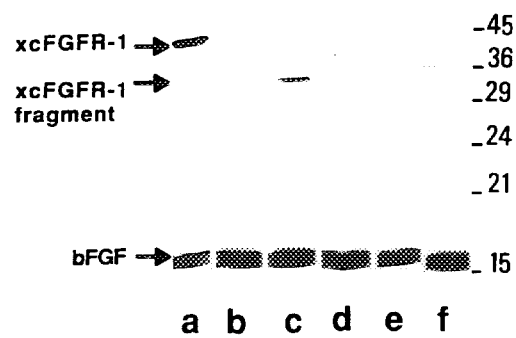

Fig. 5. Effect of selectively desulfated heparins on the protection of xcFGFR-1 from trypsin digestion. Samples containing 1 μg of xcFGFR-1 and 4 μg of native bFGF were incubated for 10 min at 37° in the absence (*lane b*) or in the presence of 700 μg/ml of conventional (*lane c*), 6-*O*-DS (*lane d*), 2-*O*-DS (*lane e*), or *N*-DS/*N*-Ac heparin (*lane f*). At the end of incubation, 60 ng of trypsin was added to all the samples. After 2 h of incubation at 37°C, samples were analyzed by SDS-PAGE followed by silver staining. *Lane a*: undigested xcFGFR-1. Molecular weights are in thousands.

(7, 15)]. Taken together, the results indicate that the glycosilation of FGFR-1 is not required for bFGF-binding and for the formation of bFGF/heparin/FGFR ternary complex.

In agreement with previous observations (17, 18, 20), selective 2-*O*- or *N*-desulfation, but not 6-*O*-desulfation, reduces significantly the bFGF-binding capacity of heparin. This was demonstrated by the incapacity of 2-*O*-DS and *N*-DS/*N*-Ac heparins to prevent the binding of ^{125}I-bFGF to high affinity sites present in FGFR-1/*flg* cDNA-transfected NIH 3T3 cells. Accordingly, when compared to conventional and 6-*O*-DS heparins, 2-*O*-DS and *N*-DS/*N*-Ac heparins show a 50-100 fold reduced ability to prevent the binding of ^{125}I-bFGF to HSPGs in these cells. It is interesting to note that 6-*O*-DS heparin displaces ^{125}I-bFGF from high affinity sites in FGFR-1-overexpressing cells with an efficiency which is 20 times higher than that of conventional heparin. The two molecules are instead equipotent in preventing the interaction of the growth factor with HSPGs. Since 6-*O*-DS heparin, at variance with conventional heparin, does not promote the interaction of bFGF with xcFGFR-1 (see below), these results suggest that the ability of heparin to prevent bFGF/FGFR-1 interaction in intact cells results not only from its bFGF-binding capacity but also from its ability to affect bFGF/FGFR interaction.

Selective 2-*O*-, *N*-, or 6-*O*-desulfation abolishes the capacity of heparin to potentiate the binding of bFGF to xcFGFR-1. These results could be anticipated for 2-*O*-DS and *N*-DS/*N*-Ac heparins, since they interact poorly with bFGF, but not for 6-*O*-DS heparin which retains the bFGF-binding capacity.

Recently, a direct association of FGFR with heparin has been reported and a heparin-binding domain has been identified in the NH_2-terminus of IgG-like domain II of FGFR-1/*flg* (8). Also, heparin by itself was able to protect FGFR-1 from proteolytic cleavage. At variance with these observations, we found that heparin requires the presence of bFGF in order to protect xcFGFR-1 from tryptic digestion. 50% of xcFGFR-1 was protected by a molar excess of heparin when incubated with trypsin in the presence of 3 nM bFGF, a concentration of the growth factor close to the Kd for the soluble receptor (5-10 nM, ref. 22). 100% protection was observed in the presence of 15 nM bFGF (data not shown). This confirms that in our experimental conditions heparin binds preferentially to the bFGF/xcFGFR-1 complex rather than to the soluble receptor by itself. Accordingly, previous observations had shown that xcFGFR-1 binds to heparin-Sepharose only when the resin beads are pre-adsorbed with bFGF (32). It is interesting to note that also Ornitz et al. (7) reported an absolute requirement for bFGF to induce the binding of FGFR-1/alkaline phosphatase fusion protein to heparin-Sepharose. Discrepancies about the requirement for bFGF in heparin/FGFR interaction may be explained on the basis of the different experimental procedures adopted, source, and/or structural differences of the soluble FGFR utilized by the different laboratories. Also, it must be pointed out that the reported FGFR/heparin interaction which occurs in the absence of bFGF is weak, being disrupted by salt concentrations equal to 0.6 M NaCl (8), a concentration that does not affect the binding of the GAG to bFGF.

As observed for chemical cross-liking studies, selective 2-*O*-, *N*-, or 6-*O*-desulfation abolishes the capacity of heparin to prevent tryptic cleavage of xcFGFR-1. Thus, 6-*O*-DS heparin does not protect xcFGFR-1 from tryptic digestion and does not enhance bFGF/xcFGFR-1 interaction despite its capacity to bind the growth factor. These observations rule out the possibility that the interaction between heparin and xcFGFR-1 is mediated by their independent binding to bFGF which acts as a bridge between the two

molecules. On the contrary, they suggest that 6-*O*-sulfate groups are involved in a direct interaction between heparin and soluble receptor. On the other hand, 2-*O*-DS and *N*-DS/*N*-Ac heparins do not enhance bFGF/xcFGFR-1 interaction because of their incapacity to bind bFGF, even though 2-*O*- and/or *N*-sulfate groups may play a direct role not only in bFGF binding but also in FGFR interaction (19).

Even though the minimal bFGF-binding sequence in heparin is a pentasaccharide structure (18), a dodecasaccharide represents the minimal requirement for promoting growth factor activity (19). Also, 6-*O*-DS heparin, despite its capacity to bind bFGF, is ineffective in restoring bFGF mitogenic activity in chlorate-treated fibroblasts with impaired synthesis of HSPGs (19). These data have been interpreted as the indication that GAG chains of HSPG contain distinct but adjacent sequences involved in bFGF- and FGFR-binding. We provide experimental evidence that bFGF, FGFR, and heparin assemble a ternary complex in which the GAG interacts with the growth factor *via* its 2-*O*- and *N*-sulfate groups and with FGFR also *via* its 6-*O*-sulfate groups.

ACKNOWLEDGMENTS. We wish to thank Miss M. D'Adda for her help in performing trypsin digestion experiments and Dr. G. Orsini for making instrumentation for amino acid sequencing available. This work was supported by grants from C.N.R. (Progetto Finalizzato Biotecnologie e Biostrumentazioni, Sottoprogetto Biofarmaci e grant n° 94.00316.CT14), MURST 60%, and Associazione Italiana per la Ricerca sul Cancro to M. Presta.

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