The Acidic Domain and First Immunoglobulin-Like Loop of Fibroblast Growth Factor Receptor 2 Modulate Downstream Signaling through Glycosaminoglycan Modification

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Fibroblast growth factor receptors (FGFRs) are membrane-spanning tyrosine kinases that have been implicated in a variety of biological processes including mitogenesis, cell migration, development, and differentiation. We identified a unique isoform of FGFR2 expressed as a diffuse band with an unusually large molecular mass. This receptor is modified by glycosaminoglycan at a Ser residue located immediately N terminal to the acidic box, a stretch of acidic amino acids. The acidic box and the glycosaminoglycan modification site are encoded by an alternative exon of the FGFR2 gene. The acidic box appears to play an important role in glycosaminoglycan modification, and the presence of this domain is required for modification by heparan sulfate glycosaminoglycan. Moreover, the presence of the first immunoglobulin-like domain encoded by another alternative exon abrogated the modification. The high-affinity receptor with heparan sulfate modification enhanced receptor autophosphorylation, substrate phosphorylation, and ternary complex factor-independent gene expression. It also sustained mitogen-activated protein kinase activity and increased eventual DNA synthesis, a long-term response to fibroblast growth factor stimulation, at physiological ligand concentrations. We propose a novel regulation mechanism of FGFR2 signal transduction through glycosaminoglycan modification.

Various types of growth factors bind to heparin and heparan sulfate. It has been suggested that fibroblast growth factors (FGFs) are stored and protected from proteolytic degradation in the extracellular matrix and on the cell surface by interaction with heparan sulfate proteoglycans, which thereby serve as a reservoir of the growth factors (9, 26, 30, 33, 44, 45). Heparan sulfate proteoglycans not only play such a modulatory role but are involved in the binding of FGF to its high-affinity receptors as an essential component. Thornton et al. (42) demonstrated that heparin potentiates the mitogenic activity of crude preparations of FGF-1 with heparin suggested that the potentiation of mitogenic activity is caused by the ability of heparin to increase the affinity of FGF-1 for its receptor (35). It has recently been shown that free heparin or heparan sulfate glycosaminoglycan (HSGAG) is required for high-affinity binding of FGF-2 to FGF receptor (FGFR) 1 expressed in heparan sulfate-deficient CHO cells (50). Based on these findings, a dual-receptor model has been proposed, in which a complex composed of a classical protein-type receptor and a low-affinity glycosaminoglycan-type receptor mediates the cellular responses to FGF (15).

The FGF family of growth factors currently consists of 15 members: FGF-1 (acidic FGF [aFGF]), FGF-2 (basic FGF), FGF-3 (INT-2), FGF-4 (HST; kaposi-FGF), FGF-5, FGF-6 (HST-2), FGF-7 (keratinocyte growth factor [KGF]), FGF-8 (AIGF), FGF-9 (GAF), FGF-10, FGF-11 (HHF-1), FGF-12 (HHF-2), FGF-13 (HHF-3), FGF-14 (HHF-4), and FGF-15 (1, 37, 47). FGFs have been shown to function as mitogens, an- giogenic factors, neurotrophic factors, oncogenes, metagons, and key factors for several developmental processes. FGFs show differential binding to FGFRs (28). FGFRs are members of a transmembrane tyrosine kinase receptor superfamily. Four distinct FGFR genes, FGFR1, FGFR2, FGFR3, and FGFR4, have been identified. A variety of isoforms are generated by alternative splicing of these FGF genes, which contributes to the functional diversity of this receptor family (13). While the extracellular domain of FGFRs determines ligand-binding specificity (24), the C-terminal domain regulates the basal activity of the receptor (20). The extracellular domain is composed of two or three immunoglobulin (Ig)-like loops. In FGFR1 and FGFR2, Ig-2 and Ig-3 loops have been shown to bind FGF-1 and FGF-2, respectively. The KGF receptor, an isoform of FGFR2 generated by alternative splicing, binds KGF/FGF-7 at its Ig-3 loop and FGF-1 at its Ig-2 loop, but not FGF-2. The KGF receptor is referred to here as FGFR2-K, while FGFR2, which binds FGF-1 and FGF-2 with high affinity but not KGF/FGF-7, is designated FGFR2-B to distinguish it from the KGF receptor. FGFR2-B and FGFR2-K differ only in the second half of their Ig-3 domains, which are encoded by alternative exons (13, 24). While both Ig-2 and Ig-3 loops play major roles in FGF binding, no growth factors are known to bind to the Ig-1 loop. In addition to these Ig domains, both the FGFR2-B and FGFR2-K isoforms have variants containing or lacking an acidic box, a domain consisting of a stretch of acidic amino acids, and surrounding sequences in their ligand-bind- ing domains. The functional significance of the acidic box in FGFRs was not known.

We report here the identification of a unique isoform of FGFR2 which exhibited a diffuse band with a much larger molecular mass than other isoforms. This receptor was modified by glycosaminoglycans, and the modification was regulated by alternative splicing of the receptor mRNA that encodes the Ig-1 loop and the acidic domain. Moreover, we show that both
FIG. 1. FGFR2 constructs used in the present studies. The transmembrane and intracellular domains are the same in all of the constructs and the same as those of the clones previously reported (41). FGFR2-B and FGFR2-K contain different sequences only in the second half of the Ig-3 loop, which is shown by thick arcs. SSG, AD, or SAG is the common amino acid sequence immediately N-terminal to the acidic box; SSG represents the wild type sequence, while the other two were generated by localized mutagenesis. The thick lines at the ends of the N termini represent the common sequence including the signal peptide. TM, transmembrane domain; A, acidic box; AD, acidic domain (an amino acid sequence that is encoded by an exon and contains both SSG and the acidic box).

SAG, GATGGGATCTCAGGTGGAGATGCAC; \( \Delta \) AD, CACTTTGAA CCAGAAAGGACGAGACG.

SAG, CGTCATCTCCAGCTGAGATGG; \( \Delta \) AD, GTCTTCGGAGCTTCCAGATGAGATGGCATC; ASG, CGTCATCTCCAGCTGAGATGG.

Material and Methods

Engineering of FGFR2 cDNA constructs. A rat FGFR2-B isomorph containing two Ig loops and an acidic domain has been designated WT (21) and used to construct FGFR2-B variants. A rat FGFR2-K isomorph (41) was used to construct FGFR2-K variants. Deletions and mutations were created by three steps of PCR. The vector used for expression of all the constructs was pCEV27 (25). In the first step, a forward primer containing the sequence 5′ to the BamHI site of pCEV27 (5′-GGATCCATTTAGGACACTAT-3′) and a reverse primer containing the desired deletion or mutation (primer 1R [described below]) were used. The second step utilized a forward primer containing the desired deletion or mutation (primer 2F [described below]) and a reverse primer containing the sequence downstream of the BamHI site of pCEV27 (5′-TTCCAGCCTATGACTACT-3′). The third step employed the forward primer of the first step and the reverse primer of the second step with equal amounts of the products from the first and second steps as a template. PCRs were 35 cycles of 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C. The products of these three steps were digested with BamHI and ligated with the BamHI-digested original construct containing pCEV27 and a part of FGFR2. The orientations of FGFR2 variants in the vector were determined by several restriction enzyme digestions. The nucleotide sequences of the final products were confirmed by DNA sequencing. The following FGFR2-B and FGFR2-K constructs were generated and designated as shown in Fig. 1. 3 Loop, FGFR2 with three Ig domains and an acidic domain; WT, FGFR2 with two Ig domains (Ig-2 and -3) and the acidic domain with an acidic box (a short stretch of acidic amino acids [Fig. 1]) and a normal Ser-Ser-Gly amino acid sequence at the N terminus of the acidic box; \( \Delta \) A, FGFR2 with two Ig domains and the acidic domain lacking the acidic box; ASG, FGFR2 with two Ig domains and the acidic domain containing a mutated Ala-Ser-Gly sequence at the N terminus of the acidic box; SAG, FGFR2 with two Ig domains and the acidic domain, containing a mutated Ser-Ala-Gly sequence at the N-terminus of the acidic box; and \( \Delta \) AD, an FGFR2 isomorph without the acidic domain. The primers 1R and 2F for each construct are listed below in a 5′-3′ orientation. Primers 1R: 3 Loop, ATCTGGTCTGCTCTCGATCTC (template, three-loop FGFR2-K); \( \Delta \) A, GTCTTGGATCTCAGGTGGAGATGCAC; SAG, CGTCATCTCCAGCTGAGATGGCATC; and \( \Delta \) AD, TACCTGTTCTTCTTGAGGAAAGTGT, Primers 2F: 3 Loop, ATCTGGTCTGCTCTCGATCTC (template, three-loop FGFR2-K); \( \Delta \) A, GTCTTGGATCTCAGGTGGAGATGCAC; SAG, CGTCATCTCCAGCTGAGATGGCATC; and \( \Delta \) AD, TACCTGTTCTTCTTGAGGAAAGTGT.

Detection of cfos mRNA expression. A mutant CHO-K1 cell line carrying the pga-745 mutation, which is deficient in glycosaminoglycan modification initiation, was provided by J. D. Esko and designated CHO-745 (48). CHO-745 cells were stably transfected with pCEV27 expression vector alone or a construct containing the FGFR2-K isomorph with two Ig domains (\( \Delta \) AD [Fig. 1]) as described previously (41). CHO-745 transfecants were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 450 \( \mu \)g of G418/ml. The cells were first plated in the same medium in six-well plates for time course studies of cfos mRNA expression, and the medium was changed overnight to Ham’s F-12 containing 3% fetal bovine serum. The cells were stimulated with pCEV27 expression vector alone or a construct containing the FGFR2-K isomorph with two Ig domains (\( \Delta \) AD [Fig. 1]) as described previously (41). CHO-745 transfecants were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum and 450 \( \mu \)g of G418/ml. The cells were first plated in the same medium in six-well plates for time course studies of cfos mRNA expression, and the medium was changed overnight to Ham’s F-12 containing 3% fetal bovine serum. The cells were stimulated by FGF-1 (final concentration, 10 ng/ml) for the indicated period in the presence (final concentration, 0.1, 1, or 10 \( \mu \)g/ml) of absence of heparin. The stimulation by FGF-1 was terminated by lysing the cells, and total RNA was extracted by using RNA STAT-60 (TEL-TEST B, Inc., Friendswood, Tex.) according to the company’s protocol. Total RNA (10 \( \mu \)g) was fractionated by 1% formaldehyde agarose gel, and Northern blotting analysis was performed by conventional methods (34). The DNA probes used for detecting cfos and cyclophilin mRNAs were kind gifts from T. Curran (5) and P. E. Danielson (7), respectively. A PhosphorImager (Molecular Dynamics) was used for autoradiography, and the image was imported into NIH Image software for quantitative analysis of band intensities.

Affinity-labeling of FGFR2 with \(^{125}\text{I}-\text{FGF-1}.\) Affinity labeling was performed as described previously (32). Cells were incubated at 4°C for 3.5 h with 10 ng of \(^{125}\text{I}-\text{FGF-1}\) in the presence or absence of an excess amount of unlabeled FGF-1 (R&D Systems Inc., Minneapolis, Minn.) in 1 ml of NIH Eagle’s no. 2 medium (without bicarbonate) containing 20 mM HEPES, pH 7.3, 0.3% bovine serum albumin, 0.5 mM MgSO_4, and 1 mM CaCl_2. FGF receptors were then
cross-linked with radiolabeled ligand by exposing the cells to 0.3 mM disuccinimidyl suberate (Pierce Chemical Co., Rockford, Ill.) in cold HEPES-buffered saline (HBS) for 20 min, and the reaction was terminated by adding 250 μl of quenching buffer containing 1 mM Tris-HCl, 10 mM EDTA, 10 μg of antipain/ml, 10 μg of pepstatin/ml, 10 μg of leupeptin/ml, and 10 μg of aprotinin/ml (Calbiochem, San Diego, Calif.). The cells were washed three times in cold HBS and harvested in HBS containing 1 mM phenylmethylsulfonyl fluoride (Pierce), 1 mM N-ethylmaleimide, 0.1 mM EDTA, 10 μg of anti-FGFR2 antibody, and a 1:500 dilution of 125I-protein A as described previously (21) or membrane was sequentially incubated with a 1:500 dilution of the anti-FGFR2 antibody in the presence of GammaBind G-plus immune complex or agarose-conjugated immune complex was washed 3 times with 1 ml of lysis buffer three times by mixing them, centrifuged at 14,000 rpm for 10 min at 4°C in an Eppendorf S417C microcentrifuge. The supernatant. The final pellet was resuspended in 2 μl of cold lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 10 μg of protein was incubated with an anti-FGFR2 antibody (SA571; 1:100), a rabbit antiphosphotyrosine monoclonal antibody (no. 16-101C; Chemicon) at 4°C for 1 h. For immunoprecipitation, the supernatant containing 1 mg or 500 μg of protein was incubated with an anti-FGFR2 antibody (SA571; 1:100), a rabbit polyclonal antibody raised against the intracellular domain of FGFR2, or 10 μl of agarose-conjugated anti-phosphotyrosine monoclonal antibody (no. 16-101C; UBI) at 4°C for 2 h. For immunoprecipitation of FGFR2, the mixture incubated with an anti-FGFR2 antibody was then incubated with 40 μl of GammaBind G-plus (Pharmacia) at 4°C for 1 h with constant rotation. The GammaBind G-pluses were washed, and the immune complex was incubated with 1 ml of lysis buffer three times by mixing them, centrifuging at 14,000 rpm for 2 to 3 min in an Eppendorf SI471C microcentrifuge, and aspirating the supernatant. The final pellet was resuspended in 2× sample buffer, and the supernatant was boiled and fractionated by discontinuous SDS–7% PAGE under reducing conditions. The fractionated protein was incubated with an anti-FGFR2 antibody (SA571; 1:100), a rabbit antiphosphotyrosine monoclonal antibody (no. 16-101C; Chemicon) at 4°C for 1 h. In the case of treatment with heparitinase alone, the control experiment without heparitinase digestion to minimize the effect of contaminating chondroitinases in the heparitinase preparation. The enzyme-digested samples were dialyzed against water overnight at 4°C and concentrated under vacuum before being loaded onto a gel. For immunoblotting studies, whenever appropriate, treatment with heparitinase and/or chondroitinase ABC digestion. Cell lysates from affinity-labeling samples were diluted at least 50-fold in 0.1 M Tris acetate buffer and treated with heparitinase (code 100703; lot no. E95601; Seikagaku America, Rockville, Md.) and/or chondroitinase ABC (code 100332; lot no. KE95702; Seikagaku America) (final concentration, 10 μl/ml) in 0.1 M Tris acetate buffer, pH 7.3, at 37°C. In the case of treatment with heparitinase alone, the control experiment without heparitinase digestion was also included. The enzyme-digested samples were dialyzed against water overnight at 4°C and concentrated under vacuum before being loaded onto a gel. For immunoblotting studies, whenever appropriate, treatment with heparitinase and/or chondroitinase ABC digestion was performed after the receptors were immunoprecipitated by the antibody in the presence of GammaBind G-pluses; the immunoprecipitated receptors captured by GammaBind G-pluses were incubated with the enzymes in 0.1 M Tris acetate buffer, pH 7.3, at 37°C for 30 to 60 min. The control experiment without heparitinase digestion was also included. For performing heparitinase digestion to minimize the effect of contaminating chondroitinases in the heparitinase preparation.

Analysis of MAP kinase activation. For detecting extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase, NIH 3T3 cells were treated with heparin in the presence of 10 μg of heparin/ml. The cells were exposed to 10 ng of FGF-1/ml for the periods indicated and lysed in the same lysis buffer used for immunoprecipitation of FGFR2. The cell lysates (50 μg of protein) were fractionated by discontinuous SDS–12.5% PAGE under reducing conditions and blotted onto Immobilon-P (Millipore) membrane. For protein, either rabbit anti-phosphorylated ERK MAP kinase (1:1000, no-phosMAPK), which recognizes tyrosine 204-phosphorylated MAP kinase, or rabbit anti-MAP kinase polyclonal antibody (no-MAPK) was used as the first antibody, followed by an alkaline phosphatase-conjugated anti-rabbit IgG according to the manufacturer's instructions (PhosphoPlus MAPK antibody kit [catalog no. 9100]; New England Biolabs) Chemiluminescence was detected after the addition of a substrate of alkaline phosphatase (CDP-Star).

Induction of DNA synthesis. DNA synthesis was measured as reported previously (21). Ninety-six-well microtiter plates (no. 3596; Falcon) were precoated with human fibronectin (Collaborative Research) at 1 μg/cm² prior to being seeded with NIH 3T3 cells. The cells were incubated without serum for 48 h before samples were added. Incorporation of [3H]thymidine into DNA was monitored during a 6-h period beginning 16 h after the addition of the samples.

Saturation binding. Saturation binding studies with 125I-FGF-1 were performed according to the methods described previously (2), and the binding data were analyzed with Ligand software (27). 125I-FGF-1, which was prepared by the Bolton-Hunter method and purified by heparin-Sepharose affinity chromatography, was purchased from DuPont NEN (North Billerica, Mass.). The biological activity of the 125I-FGF-1 as measured by the thymidine incorporation assay described above always showed more than 95% of the potency of the unlabelled ligand.

RESULTS

Heparin or heparan sulfate glycosaminoglycan is required for high-affinity binding of FGF to FGFR2 and c-fos mRNA induction. The addition of heparin is required for high-affinity binding of FGF to FGFR2 and c-fos mRNA induction, which is not observed in mutant CHO cells defective in HSGAG synthesis (30). To examine whether FGF-1 and FGFR2 show the same requirements in similar systems, we stably expressed an isoform of FGFR2 containing Ig-2 and Ig-3 loops and the K exon sequence in the Ig-3 domain (FGFR2-K; AAD [Fig. 1]) in a mutant cell line, CHO-745, deficient in glycosaminoglycan chain initiation (8). Scatchard analysis of the FGF-1 saturation binding to the cells expressing FGFR2-K showed curvilinear plotting in the presence of heparin (Fig. 2A), suggesting that multiple forms of complex with different affinities can be formed between the ligand and the receptor by adding heparin, as reported previously (29). To assess the effect of heparin on the dissociation constant (Kₐ), we applied linear regression to the Scatchard plots. Heparin increased the binding affinity of FGF-1 to FGFR2-K in a dose-dependent fashion with Kₐ of 2250, 450, and 200 pM at 0, 1, and 10 μg of heparin/ml, respectively. The increase of binding affinity by the addition of 10 μg of heparin/ml was more than 10-fold higher than the basal level (Fig. 2A). Addition of chondroitin sulfate (up to 10 μg/ml) in place of heparin did not affect the binding affinity. Cells expressing vector alone showed barely detectable levels of FGF-1 binding either with or without heparin (data not shown).

To further examine the effect of heparin on the biological function of FGF, we tested c-fos mRNA induction after exposing the cells to FGF-1. While FGF-1 stimulation did not induce significant levels of c-fos mRNA expression in CHO-745 cells transfected with the vector alone (CHO-745-Vector), adding FGF-1 with 1 μg of heparin/ml induced a significant expression of the mRNA (Fig. 2B and D, left). This c-fos mRNA expression in CHO-745-Vector was not induced in the native FGF-1 expressing in CHO-745 cells, which can be activated by FGF-1 in the presence of heparin. In CHO-745 cells expressing FGFR2-K, significant induction of c-fos mRNA expression was observed in response to FGF-1 alone, and this induction was further enhanced by the addition of heparin in a dose-dependent manner (Fig. 2C and D, right). These findings suggest that activity of the expressed FGFR2-K to induce c-fos mRNA was stimulated by heparin. Addition of chondroitin sulfate (up to 10 μg/ml) in place of heparin did not affect c-fos mRNA induction. Therefore, heparin is required for high-affinity bind-
ing of FGF-1 to FGFR2-K and subsequent activation of the receptor.

A high-molecular-mass FGFR2 species is created by glycosaminoglycan modification. During the course of expression cloning of oncogenes activated in osteosarcoma cells, we identified an FGFR2 variant with a large molecular mass generated by C-terminal fusion to a novel protein, FRAG1 (21). We have converted this constitutively activated FGFR2 to a wild type FGFR2 by replacing the rearranged C-terminal domain with the normal sequence and designated it FGFR2-WT. While the cDNA for the same FGFR2-WT isoform had already been cloned from stomach cancer and HeLa cells (3, 11), neither the receptor expression nor the glycosaminoglycan modification has been characterized for this FGFR2 isoform. When expressed in NIH 3T3 cells and analyzed by SDS-PAGE following cross-linking with 125I-FGF-1, this wild type receptor still showed a diffuse band with a mass larger than that predicted from the primary amino acid sequence (Fig. 3A). Since the broad band was suggestive of glycosaminoglycan modification of the receptor, we digested the cell surface receptor with heparitinase and chondroitinase ABC, which degrades glycosaminoglycan moieties. The enzyme treatment shifted the molecular mass of the receptor to ~130 kDa, which after subtraction of the mass of the ligand, corresponded well to the reported size of FGFR2 with Ig-2 and Ig-3 loops (Fig. 3A). These results indicated that the diffuse high-molecular-mass band of FGFR2-WT in these transfectants arises from glycosaminoglycan modification.

Glycosaminoglycan modification is determined by an alternative exon encoding the acidic domain. The extracellular domain of the wild type FGFR2 modified by glycosaminoglycan contained the acidic domain and Ig-2 and Ig-3 loops, as shown in Fig. 1. Two FGFR2 isoforms, FGFR2-B and FGFR2-K, have different amino acid sequences in the second half of the...
constructed a deletion mutant, FGFR2-B-(acidic box) on the glycosaminoglycan modification, we considered that the B-exon-derived sequence, or the acidic domain, determines the modification. To test these possibilities, we constructed a set of FGFR2-K isoforms containing WT or lacking (ΔAD) the acidic domain (Fig. 1) and expressed these constructs in NIH 3T3 cells. As shown in Fig. 3B, FGFR2-K-WT showed mobility similar to that of FGFR2-B-WT by SDS-PAGE, suggesting that the B-exon-derived sequence is not responsible for the modification. Although we previously found that the Ig-3 loop of FGFR2-K (K-exon sequence) is weakly modified by glycosaminoglycans in a parathyroid cell line (41), this sequence was not involved in the major glycosaminoglycan modification detected here. In contrast, an FGFR2-K isoform lacking the acidic domain (FGFR2-K-ΔAD) was identified as a discrete band with a much smaller molecular mass. These results indicated that the acidic domain is involved in the glycosaminoglycan modification.

The Ser-Gly-acidic box motif is essential for glycosaminoglycan modification of FGFR2. Glycosaminoglycans modify core proteins at the Ser residue of a putative attachment site of Ser-Gly (8, 49). Close inspection of the amino acid sequence of the wild type two-loop FGFR2-B (FGFR2-B-WT) revealed a Ser-Ser-Gly sequence in the acidic domain. To examine whether this sequence is involved in the modification, we mutated the cDNA sequences of the amino acids in this site of FGFR2-B, as well as FGFR2-K (Fig. 1). The mutant receptors were stably expressed in NIH 3T3 cells, affinity-labeled with 125I-FGF-1, and subjected to treatments with heparitinase, an HSGAG-degrading enzyme, and/or chondroitinase ABC, a chondroitin sulfate glycosaminoglycan (CSGAG)-degrading enzyme. As shown in Fig. 3C, the broad band of FGFR2-B-WT was shifted to a smaller discrete band by the double digestion with heparitinase and chondroitinase ABC. Single digestions with either of the enzymes resulted in similar but incomplete shifts of the band, indicating that each FGFR2-B-WT molecule was modified by either HSGAG or CSGAG. Mutation of the first Ser residue in the Ser-Ser-Gly sequence did not alter the level of glycosylation by HSGAG or CSGAG (Fig. 3C and D). In contrast, mutation of the second Ser residue to Ala in the Ser-Ser-Gly sequence abolished the modification by glycosaminoglycans (Fig. 3C), indicating that this Ser residue is essential for the modification. Thus, an FGFR2 molecule can be modified by either HSGAG or CSGAG at the single Ser residue adjacent to Gly. The population of FGFR2 molecules modified with heparitinase and chondroitinase ABC. Single digestions with either of the enzymes resulted in similar but incomplete shifts of the band, indicating that each FGFR2-B-WT molecule was modified by either HSGAG or CSGAG. Mutation of the first Ser residue in the Ser-Ser-Gly sequence did not alter the level of glycosylation by HSGAG or CSGAG (Fig. 3C and D). In contrast, mutation of the second Ser residue to Ala in the Ser-Ser-Gly sequence abolished the modification by glycosaminoglycans (Fig. 3C), indicating that this Ser residue is essential for the modification. Thus, an FGFR2 molecule can be modified by either HSGAG or CSGAG at the single Ser residue adjacent to Gly. The population of FGFR2 molecules modified by CSGAG was predominant over that modified by HSGAG by about twofold.

To examine the effect of the acidic amino acid residues (acidic box) on the glycosaminoglycan modification, we constructed a deletion mutant, FGFR2-B-ΔA, which lacks the acidic box but retains the Ser-Ser-Gly sequence. The modification of this receptor was drastically reduced compared with FGFR2-B-WT (Fig. 3C). The glycosaminoglycan species attached to the FGFR2-B-ΔA disappeared after chondroitinase ABC digestion, but not after heparitinase digestion, indicating that most of the molecules were modified by CSGAG alone. These findings suggest that the presence of the acidic box is more critical for HSGAG modification than for CSGAG modification. Essentially the same results were obtained with FGFR2-K constructs containing these mutations (data not shown).

To confirm that the entity detected by FGF-1 affinity labeling is FGFR2 and not comigrating proteoglycan, which can bind FGF-1 at a low affinity, we used immunoblot analysis to detect FGFR2 before and after enzyme digestion. All of the receptor species were comparably expressed, as detected by immunoblotting with anti-FGFR2 antibodies (Fig. 3D). The Ser residue adjacent to Gly was responsible for modification by glycosaminoglycan. Enzyme digestion showed essentially the same results as those detected by affinity labeling studies (Fig. 3E). The higher-resolution separation in this experiment revealed faster-migrating species in every lane at a similar level regardless of the enzyme digestion. Presumably they represent immature non-carbohydrate-modified forms of FGFR2 which are not yet processed in the Golgi.

Based on the above findings, we concluded that the Ser residue of the Ser-Gly-acidic box motif in FGFR2 is covalently modified by either HSGAG or CSGAG, with a slight predominance of CSGAG, and that the acidic box is a prerequisite for modification by HSGAG.

The first Ig domain of FGFR2 inhibits glycosaminoglycan modification. The common Ig-2 loops of FGFR2-B and FGFR2-K are known to bind FGF-1, and their distinct Ig-3 loops bind FGF-2 and KGF/FGF-7, respectively (24). However, no ligand has been identified that binds to the Ig-1 loop. The FGFR2-B and FGFR2-K isoforms with three Ig domains contain the acidic domain between the Ig-1 and Ig-2 domains. To examine the effect of Ig-1 on glycosaminoglycan modification of the receptor, we performed similar enzyme digestion experiments after cross-linking the receptor with 125I-FGF-1. Interestingly, the FGFR2-B isoform with three Ig domains was not modified by either HSGAG or CSGAG (Fig. 3C), despite the presence of the modification site in its acidic domain. Immunoprecipitation and immunoblotting studies with anti-FGFR2 antibodies before and after enzyme digestion also showed essentially the same results (Fig. 3E). These results indicate that the presence of the Ig-1 loop inhibits the glycosaminoglycan modification of FGFR2-B at the site within the acidic domain. Similar results were obtained with the FGFR2-K isoform containing three Ig domains (data not shown). Introduction of a mutation in a site responsible for the disulfide bond of the Ig-1 loop essentially abolished this negative effect of the modification (data not shown). Therefore, the Ig-1 domain appears to act as an inhibitor of glycosaminoglycan modification of both FGFR2-B and FGFR2-K.

Glycosaminoglycan-modified FGFR2 increases and sustains ligand-induced phosphorylation of the receptor and its substrates. The presence of covalently linked glycosaminoglycan chains in a two-loop FGFR2 containing the acidic domain suggested that this receptor species might function as a high-affinity receptor without the requirement of other cell surface heparin-like molecules. We reasoned that, in wild type CHO cells or in NIH 3T3 cells, the glycosaminoglycan-modified FGFR2 may be more potently stimulated by FGFs than unmodified receptor species, which require cell surface heparin-like molecules to function as high-affinity receptors.

We first examined the effect of FGF-1 on the time course of receptor autophosphorylation in NIH 3T3 cells expressing vector alone or various forms of FGFR2, including WT, SAG, ΔA, or 3 Loop. To quantitatively estimate the phosphorylation levels of the receptor, samples were treated by enzymes which degrade glycosaminoglycans before electrophoresis. As shown in Fig. 4A, cells expressing FGFR2-B-WT, which is a glycosaminoglycan-modified form, showed increased phosphorylation of the receptor over those expressing other forms of FGFR2, and the stimulation was sustained for more than 6 h. The expression levels of the receptor did not change over a
indicate that the activation of the MAP kinase pathway is ing the glycosaminoglycan modification site. These findings those expressing the mutant two-loop FGFR2-B (SAG) lackified by glycosaminoglycan, exhibited the same time course as FGFR2-B with three Ig domains (3 Loop), which is not mod-

expression levels of all three FGFR2-B constructs were com-

compared to those expressing mutant (SAG) or natural (3 Loop) receptors lacking HSGAG modification. The protein expressed over the basal level after ligand exposure in FGFR2-B-AD isoform of FGFR2-K. Essentially the same results were obtained with the corresponding FGFR2-B constructs (data not shown). These results indicate that glycosaminoglycan modification of FGFR2 increases and sustains FGF-1-induced phosphorylation of the receptor and its substrates.

Glycosaminoglycan modification of FGFR2 results in sustained MAP kinase activation. Two parallel signaling pathways converge at the level of the serum-responsive element (SRE) to induce c-fos expression (12). Signals through Ras activate ERK MAP kinase pathways, which in turn activate TCF, which binds the SRE. Another signal through the Rho family of small GTPases results in activation of SRF, which binds the 3’ half of SRE, designated SRE.mutL. Therefore, we first investigated the effect of FGF-1 on MAP kinase activation in NIH 3T3 cells expressing FGFR2-B-WT, its glycosaminoglycan modification site mutant (SAG), or the three-loop form with anti-phosphorylated ERK MAP kinase antibody (Fig. 4A). All of these FGFR2-B transfectants showed increased ERK activity compared to cells transfected with vector alone. Interestingly, NIH 3T3 cells expressing the FGFR2-B isoform with glycosaminoglycan modification (WT) showed sustained ERK MAP kinase phosphorylation of both p42 and p44 over a 4-h period, compared to those expressing mutant (SAG) or natural (3 Loop) receptors lacking HSGAG modification. The protein expression levels of all three FGFR2-B constructs were comparable (Fig. 3D). It is noteworthy that the cells expressing FGFR2-B with three Ig domains (3 Loop), which is not modified by glycosaminoglycan, exhibited the same time course as those expressing the mutant two-loop FGFR2-B (SAG) lacking the glycosaminoglycan modification site. These findings indicate that the activation of the MAP kinase pathway is sustained in the FGFR2 isoforms with glycosaminoglycan modification.

TCF-independent transcription is also potentiated by glycosaminoglycan-modified FGFR2. Although it is known that FGFR2 can activate SRE through phosphorylation of TCF by ERK MAP kinase, whether FGFRs can activate TCF-independent transcription is still unknown. To examine TCF-independent activation of SRE, we transfected CHO-Ki cells with FGFR2-K isoforms either containing or lacking the acidic box together with a luciferase reporter DNA construct containing SRE.mutL (12), which can be activated by SRF but not by TCF. Exposure of the transfectants to FGF-1 resulted in a three- to fivefold increase of luciferase activity over the basal level (data not shown), indicating that FGFR2-K can activate TCF-independent transcription upon FGF-1 stimulation. Cells expressing the FGFR2-K isoform with the glycosaminoglycan modification domain (FGFR2-K-WT) showed a higher peak and a more sustained time course than those expressing an non HSGAG-modified FGFR2-K isoform without the glycosaminoglycan modification site (FGFR2-K-ΔAD) (Fig. 5C). In separate experiments, cells expressing FGFR2-K-WT showed a luciferase activity increased over the basal level for more than 4 h, while luciferase activity decreased to the basal level within 3 h in FGFR2-K-ΔAD or FGFR2-K-3Loop (data not shown). Similar amounts of receptor protein were expressed in these two transfectants, as assessed by Western blotting after immunoprecipitation from equal amounts of cell protein (Fig. 5D). Cells transfected with a vector lacking SRE.mutL did not show luciferase induction upon exposure to FGF-1 (data not shown). Cells expressing FGFR2-K with three Ig domains (3 Loop), which is not modified by glycosaminoglycan, exhibited a time course of luciferase induction similar to that of the cells expressing the ΔAD isoform of FGFR2-K. Essentially the same results were obtained with the corresponding FGFR2-B constructs (data not shown). These results indicate that glycosaminoglycan modification of FGFR2 promotes sustained activation of TCF-independent transcription as well as the ERK-mediated pathway.

Glycosaminoglycan modification potentiates induction of DNA synthesis by FGF-1. Since glycosaminoglycan-modified FGFR2 exhibited sustained activation of ERK activity and TCF-independent transcription, we examined the effects of the

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**FIG. 4.** Effect of HSGAG modification on FGF-1-induced phosphorylation of FGFR2 and FGFR substrates. (A and B) Receptor autophosphorylation. Cells stably transfect with vector alone (pCEV27) or various forms of FGFR2-B (WT, SAG, ΔA, or 3 Loop) were cultured and exposed to 5 ng of FGF-1/ml. Cell lysates containing 500 μg of protein were immunoprecipitated with anti-FGFR2 antibody and detected by either anti-phosphotyrosine (αPY) (A) or anti-FGFR2 (αFGFR2) (B) antibody as noted on the right. For experiments involving immunoprecipitation of FGFR2, treatments with heparitinase and chondroitinase ABC were performed after immunoprecipitation. The membrane was first used for detection of phosphotyrosine followed by detection of FGFR2 after the antibodies were stripped off. (C) Phosphorylation of receptor kinase substrates. Cell lysates containing 500 μg of protein were immunoprecipitated with αPY and detected by the same antibody. The locations of the molecular mass markers are shown on the left. The arrow denotes the major FGFR substrate of 95 kDa.
glycosaminoglycan modification of FGFR2 on a long-term biological effect of FGF by measuring DNA synthesis. NIH 3T3 cells expressing FGFR2-B with glycosaminoglycan modification (WT) showed a dose-dependent response of DNA synthesis upon FGF-1 stimulation between 0.1 and 20 ng/ml (Fig. 6A). This response was almost completely abolished in cells expressing FGFR2-B without glycosaminoglycan modification (SAG), especially at low concentrations of FGF-1, indicating that the receptor-linked glycosaminoglycan potentiates the long-term response upon FGF-1 stimulation. In cells expressing the FGFR2-B modified by CSGAG alone (ΔA), DNA synthesis was not induced by FGF-1 at concentrations lower than 1 ng/ml, and only moderate induction was observed at higher concentrations. Cells transfected with vector alone exhibited a response similar to that by SAG-transfected cells (data not shown). In contrast, platelet-derived growth factor (PDGF) induced DNA synthesis similarly in all of these transfectants. When the DNA synthesis stimulation by FGF-1 was compared with that by 3 ng of PDGF/ml in the same transfectants, the cells expressing FGFR2-B-WT showed significantly higher values than did those expressing FGFR2-B-SAG or FGFR2-B-ΔA at 1 and 1.5 ng of FGF-1/ml (Fig. 6B).
expression levels of all receptor species used in these studies were similar, as detected by immunoblotting with anti-FGFR2 antibodies following immunoprecipitation of the receptors from equal amounts of cell protein (Fig. 6C). All of these results indicate that HSGAG modification of FGFR2 promotes more potent FGF-1 stimulation of DNA synthesis and that cells expressing the modified receptor are stimulated even at low concentrations of the ligand.

**DISCUSSION**

A number of FGFR2 isoforms generated by alternative splicing have been reported (13). These include FGFR2-B and FGFR2-K, with three Ig loops, and those with two Ig loops containing or lacking the acidic domain. However, the functions of the Ig-1 loop and the acidic domain were not known. We have identified a major glycosaminoglycan modification site in both FGFR2-B and FGFR2-K molecules. The modification site has been localized to the Ser residue of the Ser-Gly sequence immediately N-terminal to the acidic box in these FGFR2 isoforms. The sequence requirement of core proteins for glycosaminoglycan modification, especially by HSGAG, has been studied in several proteoglycans (16, 36, 51, 52). A Ser-Gly motif flanked by acidic residues appears to be required for HSGAG modification (51, 52). The localization of the modification site in FGFR2 that we report here is consistent with these results. In FGFR2, the Ser-Gly sequence and the acidic box are encoded by a single exon (13, 48). The acidic box in FGFR2 appears to be a prerequisite for HSGAG modification of the Ser residue in this Ser-Gly motif immediately N-terminal to the acidic box, while it is not absolutely required for CSGAG modification. These results suggest that the Ser-Gly-acidic box motif is crucial in promoting glycosaminoglycan attachment to FGFR2.

We have shown that only the two-loop isoforms containing the acidic domain can be modified by glycosaminoglycan. It was surprising that FGFR2-B and FGFR2-K, with three Ig loops, showed no modification by either HSGAG or CSGAG, even though they contain the required amino acid structural motif. This finding strongly suggests that the Ig-1 domain has an inhibitory effect on the glycosaminoglycan modification. The Ig-1 loop appears to be encoded by a single exon (13). An isoform containing the Ig-1 loop but not the acidic domain is not known. Based upon these results, we have concluded that the glycosaminoglycan modification of FGFR2 at the Ser residue N-terminal to the acidic box is regulated by alternative splicing: positively and negatively by exons encoding the acidic domain and the Ig-1 domain, respectively.

Since the four known FGFRs are closely related, they may also contain possible glycosaminoglycan modification sites. FGFR3 is likely to be subject to glycosaminoglycan modification, since it has a sequence similar to that of FGFR2, including the Ser-Gly-acidic box motif. FGFR1 is unlikely to be modified by glycosaminoglycan, since the Ser-Gly motif is located more than 15 amino acid residues to the N-terminal side of the acidic box and is encoded by the same exon as that for the Ig-1 loop (46). FGFR4 does not have any Ser-Gly motifs in the region flanking the acidic box. These features of FGFRs are conserved in mice, rats, and humans.

We have shown that glycosaminoglycan-modified FGFR2 exhibited an increased and sustained autophosphorylation and...
substrate phosphorylation and sustained responses to the two pathways leading to c-fos induction. In addition to the sustained activation of ERK MAP kinase, which mediates the TCF-dependent pathway for SRE activation, we found TCF-independent activation of SRE by FGFR2. The activity of this TCF-independent pathway was also increased and sustained in the cells expressing glycosaminoglycan-modified FGFR2. These findings suggest that the two pathways are simultaneously activated by FGFR2. Whereas both the ERK-mediated and the TCF-independent pathways lead to c-fos induction, they may synergize to augment the mitogenic activity of FGFR2. FGFR2 may regulate pathways controlled by Rho proteins, such as actin reorganization, since Rho family GTPases are known to regulate TCF-independent transcription (12). There appear to be some differences in the degree of phosphorylation of FGFR2 and its substrates and MAP kinase activation between the HSGAG-modified and unmodified receptors. However, it is known that phosphorylation of different sites of FGFR transmits different downstream signals (40). We previously found that the ligand-independent transforming activity of FGFR2 mutants is not well correlated either with the overall phosphorylation level of the receptor or with their effect on MAP kinase activation (20). The glycosaminoglycan modification of FGFR2 might have differential effects on its downstream pathways. Furthermore, MAP kinases, receptors, and receptor substrates should have different feedback regulation mechanisms for reversing phosphorylation. This might explain the apparent discrepancy in phosphorylation levels along the signal transduction pathway.

In accordance with the sustained activation of ERK MAP kinase and TCF-independent transcription, glycosaminoglycan-modified FGFR2 greatly potentiated induction of DNA synthesis, a long-term response by FGF stimulation. The glycosaminoglycan-modified FGFR2 responded to FGF-1 at concentrations as low as 0.1 ng/ml, while the receptors without glycosaminoglycan modification did not show any increase in DNA synthesis in this concentration range. The mutant receptor with CSGAG modification alone showed no response at low FGF-1 concentrations, although a small increase in DNA synthesis was observed at higher concentrations. Therefore, it is confirmed that HSGAG, rather than CSGAG, is important to enhance and sustain the signal transduction of FGFR2.

The presence of a low-affinity receptor, HSGAG, covalently bound to a high-affinity receptor, FGFR2, appears to increase the binding affinity of FGF to FGFR and thereby to enhance downstream signal transduction. An alternative explanation for the enhancement of FGFR2 signal transduction might be differences in receptor turnover caused by glycosaminoglycan modification of FGFR2. However, this is much less likely, though not excluded completely, since the expression level of the modified receptor was similar to those of the unmodified receptors during the course of 12 h of ligand exposure. The high-affinity FGF binding induced by HSGAG does not exclude other reported functions of the glycosaminoglycan. The glycosaminoglycan-modified receptor appears to be subject to oligomerization through the ligand bound to the glycosaminoglycan (39). Since the glycosaminoglycan is covalently attached to the high-affinity receptor, ligand accessibility to the receptor and subsequent receptor oligomerization would probably be elevated compared to the case where glycosaminoglycan is attached to other cell surface proteoglycans. Furthermore, the HSGAG on the receptor might also protect the ligand from degradation by proteases and thereby prolong the activation of FGFR, as reported for the cell surface HSGAG (6, 10, 19, 30, 33, 38).

Based on the data described here, we propose a model for an FGF dual-receptor system (Fig. 7). In cells where proteoglycans are not present, FGF-1 binds marginally to an isoform of FGFR2 that contains two Ig loops but no acidic box and transmits weak downstream signals (Fig. 7A). In cells which have proteoglycans on their surfaces, FGF-1 binds to FGFR2 with a high affinity through interaction with the heparan sulfate glycosaminoglycan moiety and transmits moderate downstream signals. (C) When heparin is added to the system where no cell surface proteoglycans are available, heparin promotes high-affinity binding of FGF-1 to FGFR2. The strength of the signal transduction may be modulated by heparin concentration. (D) The two-loop form of FGFR2 containing the acidic domain can be modified by HSGAG and thus functions as a dual-receptor system in a single molecule. It binds to FGF-1 with a high affinity and transmits strong downstream signals. (E) A three-loop form cannot be modified by glycosaminoglycan despite the presence of a modification site and therefore cannot function as a high-affinity receptor unless heparin or proteoglycans are present.
seem to be shared between differentiation and proliferation (4). EGF stimulation of PC12 cells results in transient ERK activation and leads to cell proliferation, while NGF stimulation of the same cells results in sustained ERK activation and leads to differentiation to sympathetic neurons. Marshall (22) proposed a model in which quantitative differences in ERK activation are translated into qualitative differences in transcription factor activation. FGFR is known to be expressed in various stages of embryonic development (40). At certain stages, the two-loop form containing the acidic domain, which can be modified by glycosaminoglycans, might be selectively expressed and might thus play critical roles in decisions of differentiation. We propose a novel regulation of FGFR2 glycosaminoglycan modification by alternative splicing and thereby of the signal transduction via FGFR2.

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REFERENCES


