Two Hierarchies of FGF-2 Signaling in Heparin: Mitogenic Stimulation and High-Affinity Binding/Receptor Transphosphorylation†

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Received January 18, 1996; Revised Manuscript Received April 8, 1996⊗

ABSTRACT: FGF-2 activates multiple signaling pathways by a cell surface signaling complex assembled with FGF, its receptor tyrosine kinase, and heparan sulfate proteoglycan. Heparan sulfate binds to a site on the receptor and at least one site on the growth factor. Several models propose an important role for heparan sulfate not only in facilitating FGF-2 binding to its receptor tyrosine kinase but also in promoting signaling via formation of receptor dimers. Such dimers are capable of transphosphorylation of the cytoplasmic domain of the receptor, leading to the generation of phosphotyrosines that are important initiators of intracellular signaling pathways. To explore the participation of heparan sulfates in the formation of a signaling complex that activates these pathways, the binding and activity of FGF-2 on Swiss 3T3 fibroblasts and F32 lymphoid cells is examined with either native or modified forms of heparin. As shown previously, fibroblasts treated with chlorate, which inhibits the sulfation of heparan sulfate and its subsequent binding to FGF-2, display a dramatically reduced response to picomolar concentrations of FGF-2, but binding to receptors and a mitogenic response is restored by heparin. However, the restoration of high-affinity binding is seen only at an optimal concentration of heparin. Excess heparin competes for binding sites within the signaling complex such that high-affinity binding and receptor transphosphorylation are reduced. Despite this, mitogenic signaling is not diminished. A similar result is observed using heparin fragments that promote mitogenesis but not high-affinity binding. These results suggest that the high-affinity signaling complex that is necessary for stable receptor transphosphorylation differs from the signaling complex sufficient for triggering mitogenesis. We speculate that heparan sulfate *in vivo* participates in two hierarchies of receptor activation. In one, heparan sulfate participates in FGF-2 binding to its receptor tyrosine kinase and activation of mitogenic signaling, perhaps through monomeric receptors or the transient formation of receptor dimers. In the second hierarchy, heparan sulfate participates in the stabilization of a signaling complex that is likely to be comprised of receptor multimers that carry out effective receptor transphosphorylation. A further description of this mechanism may lead to an understanding of how heparan sulfate or its homologues can regulate specific signaling pathways within the cell.

The FGF family consists of nine related polypeptides that share sequence homology and an affinity for heparin and heparan sulfate glycosaminoglycans. These growth factors have multiple effects on numerous cell types and have emerging roles in early embryonic development [see Baird and Klagsbrun (1991), Burgess and Maciag (1989), Mason (1994), and Tickle and Eichele (1994)]. FGFs have also been implicated in several disease mechanisms, most notably being expressed as oncogenes or as tumor products that promote local angiogenesis and survival of solid tumors (Basilico & Moscatelli, 1992).

The FGFs bind and activate receptor tyrosine kinases (RTKs).[1] These consist of a family of four related molecules that contain intracellular tyrosine kinase domains. Alternatively spliced forms support differential binding of FGF family members (Avivi et al., 1993; Johnson & Williams, 1993; Werner et al., 1992). Signaling occurs through activation of the RTK and the phosphorylation of intracellular substrates leading to a signal transduction cascade (Coughlin et al., 1988; Friesel et al., 1989; Huang & Huang, 1986; Pasquale et al., 1988). This is believed to require receptor transphosphorylation (Bellot et al., 1991; Ueno et al., 1992; Schlessinger et al., 1995). A truncated version of FR1, lacking its tyrosine kinase domain, acts as a dominant negative mutant and blocks FGF signaling (Amaya et al., 1993; Ueno et al., 1992); this mutant presumably acts by forming an inactive complex with native receptor and provides circumstantial evidence that normal signaling involves receptor aggregation. However, the activation of discrete cellular responses by FGF is likely to occur via distinct signaling pathways, and responses induced within the same cell can often be uncoupled by mutating specific tyrosines within the receptor cytoplasmic domain or by modifying the FGF itself (Burgess et al., 1990; Isacchi et al., 1991; Mohammadi et al., 1992; Peters et al., 1992). This leads to the interesting possibility that the FGF receptors are capable of transmitting discrete signals dependent on the mechanism of their activation.

† Work supported by NIH Grant GM48850 and University of Wisconsin Graduate School Award 920642.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: FR1, FGF receptor 1; PLC, phospholipase C; RTK, receptor tyrosine kinase.

S0006-2960(96)00125-0 CCC: $12.00

FGF signaling is regulated by heparan sulfate (Rapraeger, 1995). Cells deficient in heparan sulfate or cells treated with sodium chlorate to block the sulfation of heparan sulfate chains show reduced binding and activation of the FGF RTK (Olwin & Rapraeger, 1992; Ornitz & Leder, 1992; Ornitz et al., 1992; Rapraeger et al., 1991; Yayon et al., 1991). Thus, the high-affinity FGF binding site demonstrated on a variety of cell surfaces is a complex of heparan sulfate proteoglycan, FGF, and RTK. The participation of heparan sulfate in FGF signaling may be via binding to at least two different sites in the signaling complex. Heparan sulfate clearly binds the FGFs via specific sulfation sequences. Analysis of heparan sulfate or heparin fragments that bind FGF-2 has identified a repeating unit of iduronosyl 2-*O*-sulfate−glucosaminyl *N*-sulfate (Habuchi et al., 1992; Maccarana et al., 1993; Turnbull et al., 1992), with the minimum of a pentasaccharide required for binding (Maccarana et al., 1993). Such studies have shown that FGF-2 binding and activation of its RTK is facilitated by fragments on the order of 10−12 sugars in length bearing iduronosyl 2-*O*-sulfates (Guimond et al., 1993). Although other oligosaccharides may bind FGF-2, they may be inappropriate for receptor binding and thus act to inhibit activity. A primary heparin-binding domain within FGF-2 has been described (Baird et al., 1988; Pantoliano et al., 1994; Thompson et al., 1994), although more than one may be present (Ornitz et al., 1995). In addition, a conserved heparin binding domain exists within the FGF receptor itself and evidence suggests that heparan sulfate binds directly and specifically to this site (Kan et al., 1993; Brickman et al., 1995).

A major uncertainty to be resolved in FGF signaling is the elucidation of the molecular nature of the signaling complex. Although FGF has a measurable affinity for its receptor in the absence of heparan sulfate, its activity in most assays is greatly enhanced by the participation of the glycosaminoglycan. This is likely to be due in part to the enhanced binding of the growth factor to the RTK but may also be due to the formation of a multimeric complex, probably involving more than one receptor, that triggers a strong intracellular signal. The importance of heparan sulfate occupying sites within this complex may relate to the stability of the complex and the strength of the signal. In this study, we examine FGF signaling on chlorate-treated Swiss 3T3 fibroblasts where the ability of exogenous heparin to restore FGF-2 binding to its receptor tyrosine kinase allows investigation of specific heparan sulfate requirements. The participation of heparan sulfate in formation of the FGF−RTK complex is investigated by manipulation of receptor occupancy and transphosphorylation. These findings demonstrate that (i) heparin participates in the formation of a high-affinity FGF-binding complex capable of activating receptor transphosphorylation, (ii) heparin also participates in formation of a signaling complex that triggers mitogenesis, and (iii) the mitogenic signaling is accomplished without the formation of the high-affinity complex that effectively stimulates receptor phosphorylation. These results suggest an important role for heparan sulfate in efficient receptor transphosphorylation but also suggest that mitogenic signals in Swiss 3T3 fibroblasts are generated by different signaling events.

MATERIALS AND METHODS

Materials. Human recombinant FGF-2 (fibroblast growth factor-2) was kindly provided by Brad Olwin, Department of Biochemistry, Purdue University. Desulfated heparin and heparin oligosaccharides were generously provided by Ulf Lindahl, Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden. Anti-phosphotyrosine monoclonal antibody, 4G10, was obtained from Upstate Biotechnology, Lake Placid, NY. Rabbit anti-mouse monoclonal antibodies were obtained from Jackson Immunoresearch, West Grove, PA. Epidermal growth factor (EGF) and porcine intestinal mucosa heparin were purchased from Sigma Chemical Co., St. Louis, MO. Heparin was prepared as a 10 mg/mL stock in phosphate-buffered saline, boiled for 5 min, and then diluted as needed. Sodium chlorate was purchased from J. T. Baker Inc., Phillipsburg, NJ. Sodium sulfate was purchased from EM Science, Cherry Hill, NJ.

Cell Culture. Swiss 3T3 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DME; Gibco, Grand Island, NY) and 10% calf serum (Hyclone, Logan, UT). For inhibition of glycosaminoglycan sulfation, cells were treated as described previously (Rapraeger et al., 1991). Briefly, cells were plated in low-sulfate, low-cystine (50 μM) DME supplemented with 10% dialyzed calf serum and 30 mM sodium chlorate for 48 h and then trypsinized and plated for experiments in low-sulfate, low-cystine DME supplemented with 10% dialyzed calf serum and 30 mM sodium chlorate. Alternatively, cells were treated with chlorate, as above, in the presence of 10 mM sulfate to allow restoration of glycosaminoglycan sulfation.

BaF3 cells transfected with FR1 (designated F32) were kindly provided by David Ornitz, Washington University, St. Louis, MO. F32 cells were maintained in RPMI (Gibco) containing 10% calf serum, 10% WEHI-3 cell-conditioned medium (as a source of IL-3), 0.00035% β-mercaptoethanol, 100 u/mL penicillin, and 50 μg/mL streptomycin sulfate.

Mitogenesis Assays. Sodium chlorate-treated Swiss 3T3 cells were trypsinized and plated in 24-well dishes at 50% confluence in low-sulfate, low-cystine DME + 10% dialyzed calf serum and 30 mM sodium chlorate and allowed to adhere overnight. Cells were then serum-starved for 24 h in low-sulfate, low-cystine DME + 0.1% bovine serum albumin (Sigma) and 30 mM sodium chlorate. Cells were stimulated with FGF-2 for 18 h and cultured an additional 6 h with 2 μCi/mL [^{3}H]thymidine (NEN Research Products, Boston, MA). Cells were incubated at room temperature with 5% trichloroacetic acid, washed, solubilized with 0.1 N NaOH, and suspended in Biosafe II scintillation fluid (Research Products International, Mount Prospect, IL). [^{3}H]Thymidine incorporated into DNA was detected by counting in a LS 5800 liquid scintillation counter (Beckman Instruments, Irvine, CA).

F32 cells were plated at a density of 1×10^5 cells/mL in RPMI lacking IL-3 and containing 0.1% BSA in the place of serum. Cells were cultured in the presence of FGF-2 and heparin for 36 h. [^{3}H]Thymidine (2 μCi/mL) was added to the cells for 8 h. Cells were then centrifuged, suspended in 5% TCA, and recentrifuged. Cell pellets were solubilized in 0.5 mL of 0.1 N NaOH and quantified by liquid scintillation counting.

Iodinations. Human recombinant FGF-2 was iodinated by the chloramine T method (Rapraeger et al., 1994). A minicolumn was prepared using 0.2 mL of heparin−agarose (Sigma) and prewashed with elution buffer [20 mM Hepes (pH 7.4), 3 M NaCl, and 0.2% BSA] followed by washing

with loading buffer [20 mM Hepes (pH 7.4), 0.4 M NaCl, and 0.2% BSA]. Following iodination, iodinated FGF-2 was bound to the heparin−agarose column, washed with loading buffer to remove any free ^{125}I, and then eluted with 1 mL of elution buffer. A specific activity ranging from 1500 to 3500 cpm/fmol was determined by comparison to unlabeled FGF-2 in Swiss 3T3 mitogenic assays.

Protein A was iodinated using iodobeads (Pierce Chemicals, Rockford, IL). Protein A (200 μg; Boehringer Mannheim, Indianapolis, IN) was incubated with 1 mCi ^{125}I and one iodobead for 10 min. A fresh iodobead was added to the solution and the mixture incubated for an additional 10 min. Labeled protein A was separated from free iodine on a 10 mL G-25 Sephadex (Pharmacia, Piscataway, NJ) column.

Binding Assays. Sodium chlorate-treated Swiss 3T3 cells were plated at a density of 10^5 cells per well in six well plates. After serum starvation, cells were placed on ice and washed twice with HEPES-buffered DME supplemented with 0.1% BSA. Iodinated FGF-2 was added along with heparin and/or heparin oligosaccharides and the mixture incubated for 2 h at 4 °C on a rotary shaker. Binding was quantified by washing the cells four times with ice-cold 2 M NaCl at pH 7.4 to remove FGF-2 not bound to high-affinity complexes and then washing twice with 2 M NaCl (pH 4.0) to release FGF-2 incorporated into high-affinity complexes. The initial wash conducted at neutral pH was not technically necessary on cells lacking heparan sulfate but was necessary for experimental controls that did express the glycosaminoglycan (e.g., chlorate treatment recovered with sulfate). Similar results were obtained in chlorate-treated cells regardless of this initial wash step. Radioactivity contained in 2 M NaCl (pH 4.0) washes was quantified on an LKB 1282 CompuGamma γ counter.

Cross-Linking of FGF-2 to Cell Surface Proteins. Swiss 3T3 cells were chlorate-treated and serum-starved as described for the binding assay. Cells were incubated with 300 pM iodinated FGF-2 at 4 °C in Hepes-buffered DME and 0.1% BSA (binding buffer) for 2.5 h. Cells were then incubated with 250 μM disuccinimidyl suberate (DSS) or 1 mM bis(sulfosuccinimidyl) suberate (BS3) (Pierce Chemicals) for 30 min in phosphate-buffered saline (PBS). Non-cross-linked protein was removed by a wash with 20 mM sodium acetate (pH 4), 2 M NaCl, and 1 mg/mL BSA. Cells were then rinsed with PBS, extracted in Laemmli sample buffer, and subjected to electrophoresis on 7.5% acrylamide SDS−PAGE gels (Laemmli, 1970). Gels were stained with Coomassie Blue R-250 (0.125% in 50% methanol and 10% acetic acid) and destained with 50% methanol and 10% acetic acid. Dried gels were exposed to X-ray film (X-Omat AR, Eastman Kodak Co., Rochester, NY).

Detection of Protein Tyrosine Phosphorylation. Swiss 3T3 cells were chlorate-treated for 48 h and then plated at 70% confluence on 60 mm plates. Cells were then serum-starved in low-sulfate, low-cystine DME containing 1 mg/mL BSA and 30 mM sodium chlorate for 24 h. FGF-2 (100 pM) or EGF (1 nM) was bound to cells in Hepes-buffered DME, 0.1% BSA, and 50 μM sodium orthovanadate at 4 °C for 2.5 h to maximize FGF-2 binding prior to receptor activation. Cells were then warmed to 37 °C for 15 min. The medium was aspirated, and the dishes were placed on a boiling water bath for 1 min. Cells were extracted with boiling 2× Laemmli sample buffer containing phosphatase and protease inhibitors (1 mM sodium orthovanadate, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, and 10 mM sodium pyrophosphate). Extracts were boiled, brought to 1× sample buffer with water, sonified, and subjected to SDS−PAGE on 7.5% acrylamide gels. Proteins were transferred for 3 h at 60 mA to Immobolin-P transfer membrane (Millipore Corp., Bedford, MA). Membranes were blocked with 2% gelatin in Tris-buffered saline (pH 7.4) for 1 h and probed with a 1:2000 dilution of anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology). Antibodies were detected with secondary rabbit anti-mouse IgG followed by iodinated protein A (2×10^6 cpm/mL). Blots were washed with PBS containing 0.05% NP-40 (Sigma) and exposed to X-ray film.

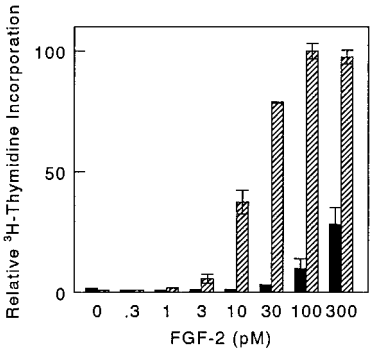

FIGURE 1: Growth of Swiss 3T3 cells in chlorate. Chlorate-treated cells (solid bars) or chlorate-treated cells incubated in the presence of 10 nM heparin (striped bars) were serum-starved, stimulated with FGF-2 for 18 h, and assayed for incorporation of [^{3}H]thymidine. Maximal incorporation is set to 100%.

RESULTS

Exogenous Heparin Can Substitute for Heparan Sulfate Proteoglycans and Support Mitogenesis in Chlorate-Treated Swiss 3T3 Cells

Swiss 3T3 fibroblasts cultured in sodium chlorate produce undersulfated heparan sulfate proteoglycans, reduced binding of FGF-2, and reduced mitogenic response (Rapraeger et al., 1991). However, addition of FGF-2 together with 10 nM (100 ng/mL) exogenous heparin generates a response, as measured by incorporation of [^{3}H]thymidine into new DNA (Figure 1). To begin to examine the mechanism by which heparin acts in FGF-2 signaling, fibroblasts cultured in 30 mM sodium chlorate were stimulated with a range of FGF-2 concentrations in the presence or absence of exogenous heparin and [^{3}H]thymidine incorporation into DNA was assessed as a measure of their mitogenic response. The chlorate-treated cells show little response to low levels of FGF-2 when stimulated in the absence of added heparin with a response only observed at FGF-2 concentrations of 100 pM or greater. Thus, reduction of the sulfation of heparan sulfate glycosaminoglycans reduces the FGF-2 dose response by at least 30-fold, with the mitogenic response in high levels of FGF-2 attributed either to low levels of sulfation remaining following chlorate treatment or to lower-affinity binding of FGF-2 to receptors in the absence of heparan sulfate. Addition of exogenous heparin restores the mitogenic response of chlorate-treated cells. Cells cultured in 10 nM heparin together with FGF-2 ranging in concentration from 0.3 to 300 pM reach maximal stimulation at 100 pM FGF-2, with half-maximal stimulation observed near 10 pM (Figure 1). This is consistent with the dose response shown by cells cultured in the absence of sodium chlorate, which

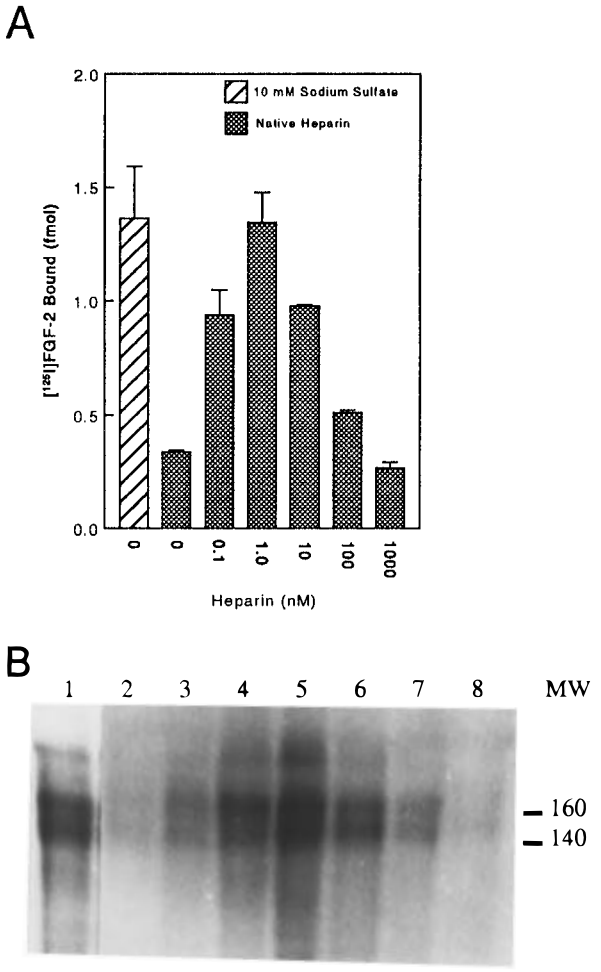

FIGURE 2: Cross-linking of iodinated FGF-2 to cell surface receptors. (A) Iodinated FGF-2 (100 pM) was incubated with chlorate-treated cells at 4 °C in the presence of various heparin concentrations, and then FGF bound to receptors was determined following washes in 2 M NaCl at pH 4.0. Sodium sulfate was added as a control to reverse the inhibition by chlorate. (B) Iodinated FGF-2 (300 pM) was bound to chlorate-treated cells at 4 °C and cross-linked to surface receptors with DSS and detected by autoradiography following SDS−PAGE: lane 1, chlorate treatment reversed by 10 mM Na_2SO_4; lane 2, no heparin; lane 3, 0.1 nM heparin; lane 4, 1 nM heparin; lane 5, 10 nM heparin; lane 6, 100 nM heparin; lane 7, 1000 nM heparin; and lane 8, 10 000 nM heparin.

bind FGF-2 to endogenous heparan sulfate proteoglycan rather than exogenous heparin (data not shown).

Occupancy of FGF Receptors of Chlorate-Treated Cells in the Presence of Heparin Is Biphasic

Binding of FGF-2 in high-affinity cell surface complexes was assessed by incubating 100−300 pM iodinated FGF-2 with either chlorate-treated cells in the presence of exogenous heparin or cells incubated with sodium sulfate to reverse the chlorate inhibition of glycosaminoglycan sulfation (Figure 2). Quantification of binding was performed by measuring [^{125}I]FGF-2 that is resistant to displacement by 2 M NaCl at pH 7.4, typically FGF bound to glycosaminoglycans, but released by 2 M NaCl at pH 4.0 (Figure 2A). Receptors that bind FGF-2 at the cell surface were identified by cross-linking iodinated FGF-2 to cell surface proteins with DSS. Examination of cell lysates by PAGE detects two predominant bands of 140 and 160 kDa, corresponding to receptors of ca. 120 and 140 kDa, respectively (Figure 2). Receptors of similar size have been described by others [reviewed by Jaye et al. (1992)] and correspond to two- and three-Ig loop forms of the FGF receptor tyrosine kinase.

Chlorate-treated fibroblasts show dramatic reduction of FGF-2-binding to receptor (Figure 2). This binding can be restored in two different ways, either by restoring the sulfation of endogenous heparan sulfate by the addition of 10 mM sulfate or by adding exogenous heparin; iodinated FGF-2 binding, measured as FGF present in high-affinity complexes or detected by the appearance of the 140 and 160 kDa protein bands upon cross-linking, is restored by heparin concentrations ranging from 0.1 to 1000 nM. Maximal occupancy, however, is observed at an intermediate concentration of 1−10 nM. Concentrations greater than 10 nM show reduced occupancy which becomes negligible at 1−10 μM (10−100 μg/mL), where binding appears to be no greater than in the absence of heparin alone. This suggests that the ratio of heparin to FGF may be important; indeed, 100 pM FGF used in Figure 2A shows maximal binding at 1 nM heparin, whereas 300 pM FGF used in Figure 2B shifts the optimal heparin concentration to 10 nM.

Receptor Activation of Tyrosine Phosphorylation in Chlorate-Treated Cells in the Presence of Heparin Is Biphasic

Two potential problems may exist in the measurement of binding using iodinated ligand as in Figure 2. First, the ligand may be altered by the iodination. Second, the efficacy of the cross-linking may be affected by the presence of heparin. To rule out these possibilities and to investigate the role of heparan sulfate in receptor transphosphorylation, FGF-2 activation of the receptor tyrosine kinase was assessed directly by examining tyrosine phosphorylation.

Since the activation of receptor phosphorylation is believed to require receptor transphosphorylation (Bellot et al., 1991), the appearance of protein bands newly phosphorylated on tyrosine is a marker for receptor activation. To measure this directly, chlorate-treated cells were serum-starved, incubated with growth factor in the presence or absence of heparin, and analyzed for newly phosphorylated proteins (Figure 3). Analysis of cells stimulated with 100 pM FGF-2 reveals three major phosphorylated proteins that are not observed in the absence of the growth factor. These are a doublet at 160 and 150 kDa, which is likely to represent phosphorylated receptor and/or PLC-γ, and a protein near 80 kDa that is likely to be the pp90 described by others in Swiss 3T3 cells (Coughlin et al., 1988). Similarly, stimulation with 1 nM EGF leads primarily to the phosphorylation of the EGF receptor at 180 kDa. As EGF binding to these cells does not require the presence of heparan sulfate or heparin, it is included as a positive control in all ensuing treatments and shows no change.

Cells treated with chlorate in the presence of 10 mM sulfate, which synthesize fully sulfated heparan sulfate proteoglycans, phosphorylate the same proteins as nontreated cells when stimulated with FGF-2. A major difference is seen in cells treated with chlorate alone, however (Figure 3). These cells, which show reduced binding of FGF-2 to surface receptors and reduced mitogenic response to FGF-2, do not phosphorylate these proteins. However, protein phosphorylation is restored by the inclusion of 10 nM heparin, a concentration shown to restore binding of iodinated FGF-2 to receptors. In contrast, the inclusion of heparin at

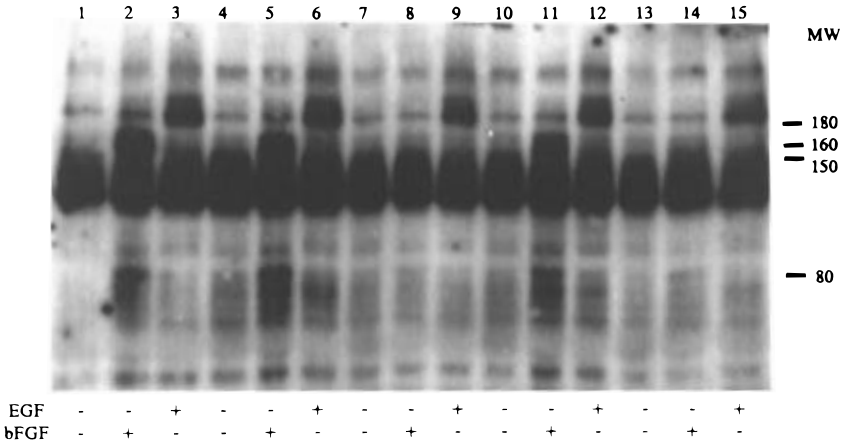

FIGURE 3: Detection of tyrosine-phosphorylated proteins in FGF-2- or EGF-stimulated Swiss 3T3 cells. Following 24 h of serum starvation, either 100 pM FGF-2, 1 nM EGF, or no growth factor as indicated was bound to the cell surfaces at 4 °C for 2.5 h in the presence or absence of heparin. Cells were incubated at 37 °C for 15 min and extracted in Laemmli sample buffer, and samples were subjected to SDS−PAGE and electrophoretically transferred to immobilon-P. Proteins phosphorylated on tyrosine were detected with a monoclonal anti-phosphotyrosine antibody: lanes 1−3, nontreated cells; lanes 4−6, chlorate treatment reversed by 10 mM Na_2SO_4; and lanes 7−15, chlorate-treated cells. The incubations with or without heparin were as follows: lanes 10−12, chlorate-treated cells incubated with 10 nM heparin; and lanes 13−15, chlorate-treated cells incubated with 10 000 nM heparin.

10 000 nM does not support FGF-2 receptor transactivation as measured by the low level of phosphorylation of these proteins. Thus, in the presence of an optimum concentration of heparin, FGF-2 binds its receptor and stimulates receptor transactivation. At higher heparin concentrations, this ability to support receptor transactivation is reduced. These results complement those obtained by measuring FGF-2 binding to cell surface receptors; the quantity of heparan sulfate may regulate both stable interaction of FGF-2 with its receptor and activation of receptor transactivation.

Binding of FGF-2 to FR1-Expressing Lymphoid Cells

BaF3 cells are IL-3 dependent lymphoid cells that do not express FGF receptors and fail to respond to FGF-2 (Ornitz et al., 1992). When transfected with FR1, the resulting F32 cell clone gains the ability to bind FGF-2, survives in FGF-2 in the absence of IL-3, and incorporates [^{3}H]thymidine in response to the growth factor (Ornitz et al., 1992). Similar to most lymphoid cells, however, the F32 cells lack endogenous heparan sulfate proteoglycans and require the addition of exogenous heparin or heparan sulfate to bind FGF-2 to FR1 and to support the FGF activity. Unlike the 3T3 fibroblasts which express multiple receptors, including splice variants of FR1, the F32 cells provide the opportunity to question the effect of heparin solely on the three-Ig loop form of FR1.

Iodinated FGF-2 (300 pM) was bound to the surface of parental BaF3 and FR1-expressing F32 cells in the presence of increasing concentrations of exogenously added heparin and cross-linked to cell surface proteins with BS3. In the presence of heparin, FGF-2 cross-linking to the surface of the F32 cells identifies FR1 as a protein of approximately 170 kDa (Figure 4). Binding to this protein is not detected in parental BaF3 cells. F32 cells fail to bind FGF-2 to FR1 in the absence of heparin. Furthermore, FGF-2 binding to FR1 occurs only in the presence of concentrations of exogenously added heparin from 1 to 100 nM, with practical abolition seen at 10 000 nM (Figure 4). Thus, like FGF-2 binding to its receptor on Swiss 3T3 cells, FGF-2 binding to FR1 on F32 cells is biphasic in the presence of heparin; an optimum heparin concentration is required for stable

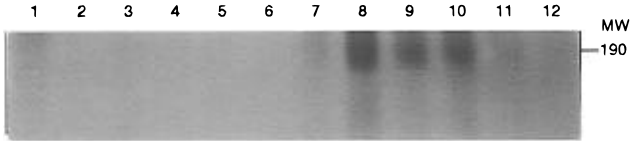

FIGURE 4: FGF-2 cross-linking to cell surface receptors of F32 cells. Iodinated FGF-2 was bound to BaF3 or F32 cells at 4 °C and cross-linked to cell surface receptors with BS3 and detected by autoradiography following SDS−PAGE: lanes 1−6, BaF3 cells; lanes 7−12, F32 cells; lanes 1 and 7, no heparin; lanes 2 and 8, 1 nM heparin; lanes 3 and 9, 10 nM heparin; lanes 4 and 10, 100 nM heparin; lanes 5 and 11, 1000 nM heparin; and lanes 6 and 12, 10 000 nM heparin.

FGF-2 binding to FR1, with concentrations either above or below that optimum reducing overall receptor occupancy.

FGF-Stimulated Mitogenesis Correlates with FGF Binding to Native Heparin, Not High-Affinity Receptor Occupancy

Mitogenesis of Swiss 3T3 Cells at Various Heparin Concentrations. The mitogenic potential of FGF-2 was investigated on chlorate-treated fibroblasts incubated with a range of heparin concentrations that either promote stable receptor binding and transactivation or result in reduced receptor occupancy. Quiescent serum-starved cells were stimulated with 10 pM FGF-2 for 18 h, and their response was assayed by incorporation of [^{3}H]thymidine into new DNA (Figure 5A). Chlorate-treated cells fail to respond to FGF-2 in the absence of exogenous heparin, but the response is restored to maximal levels by as little as 1 nM heparin, with half-maximal restoration seen at ca. 0.1 nM. Surprisingly, at high heparin concentrations (10 000 nM), where apparent occupancy of cell surface receptors is greatly reduced, the mitogenic response of the cells is not diminished. Equivalent results have been obtained with heparin concentrations as high as 300 μM (data not shown). As these experiments are conducted with quiescent cells that are stimulated with FGF at zero time to enter the cell cycle and initiate S phase 18 h later (data not shown), we conclude that FGF is equally effective at zero time whether at the optimal or high heparin concentrations.

Half-Maximal Stimulation Is Unchanged at High or Low Heparin Concentrations. This lack of correspondence

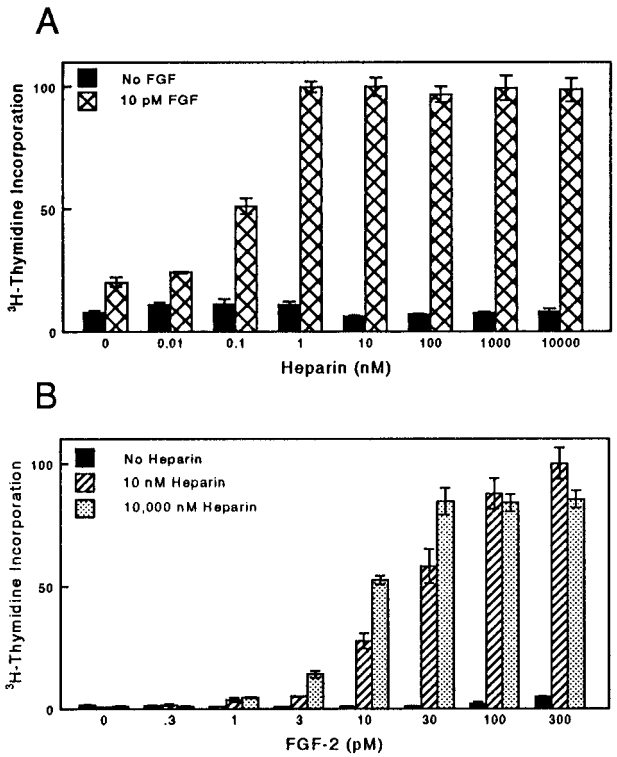

FIGURE 5: Growth of Swiss 3T3 cells in the presence of chlorate and heparin. (A) Serum-starved chlorate-treated cells were stimulated with 10 pM FGF-2 in various concentrations of heparin. The response was assayed by incorporation of [^{3}H]thymidine into DNA: no FGF-2 (solid bars) and 10 pM FGF-2 (hatched bars). (B) Serum-starved chlorate-treated cells were stimulated with various concentrations of FGF-2 in the presence or absence of heparin: no heparin added (solid bars), 100 ng/mL heparin (striped bars), and 100 μg/mL heparin (stippled bars).

between reduced receptor occupation and reduction of mitogenesis could reflect activation of a low level of FGF-2 receptors. Under test conditions, receptor binding may not be reduced below a critical level of receptor stimulation necessary for mitogenesis. This possibility was investigated by culturing 3T3 cells in levels of FGF that stimulate the cells submaximally to determine whether the FGF-2 dose response is altered when receptor occupancy is reduced. It would be expected that, at FGF-2 concentrations where the level of occupied receptors is sufficient for only half-maximal stimulation, dramatic changes in receptor binding would result in observable differences in growth stimulation.

To address the dose responsiveness of FGF-2, chlorate-treated 3T3 cells were stimulated with various concentrations of FGF-2 in the presence of either 10 or 10 000 nM heparin (Figure 5B). At the low heparin concentration, growth is restored as expected with a half-maximal response seen at 10−30 pM FGF. At the high heparin concentration, which apparently reduces receptor binding, the growth response is similar if not enhanced, with half-maximal stimulation seen at ca. 10 pM FGF. This contrasts with the expectation that such a reduction in binding would cause a major shift in the concentration required for half-maximal stimulation.

The half-maximal response to FGF mitogenic stimulation at high or low heparin concentrations was also explored in the F32 cells, using either 50 or 10 000 nM heparin (Figure 6). As previously described by Ornitz et al. (1992), F32 cells cultured in the presence of FGF-2 without the addition of exogenously added heparin fail to respond to the growth factor. However, the addition of either 50 or 10 000 nM heparin restores the cellular response. Despite the difference

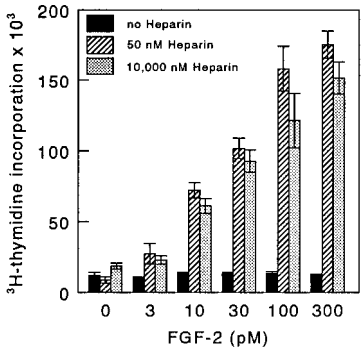

FIGURE 6: FGF-2 stimulation of FR1-expressing F32 cells. F32 cells were removed from serum and IL-3-containing medium and cultured in the presence of FGF-2 and different concentrations of heparin. The response was assayed by incorporation of [^{3}H]-thymidine into DNA: no heparin (solid bars), 50 nM heparin (striped bars), and 10 000 nM heparin (stippled bars).

in their ability to support detectable binding of FGF-2 to FR1, both concentrations of heparin support a similar FGF-2 dose response curve. The maximal level of incorporation is seen at 300 pM, with a half-maximal level of stimulation seen at 20 pM for both concentrations of heparin.

6-O-Desulfated Heparin Blocks both FGF-2 Binding to Its Receptor and FGF-2-Stimulated Mitogenesis. The failure to reduce mitogenic stimulation at heparin concentrations that appear to block FGF binding to its RTK suggests that binding is not actually inhibited. Rather, FGF bound to native heparin may interact with the RTK without forming a high-affinity complex. To demonstrate that an actual reduction in binding to receptors would indeed be manifested as a reduction in activity, we used a chemically 6-O-desulfated heparin. This heparin retains its ability to bind FGF-2 (Maccarana et al., 1993) but cannot support either FGF-2 binding to its receptor or FGF-2-stimulated mitogenesis (Guimond et al., 1993). It was reasoned that a reduction in binding using this inhibitor should result in a corresponding reduction in activity that would be measured as a shift in the half-maximal response to the growth factor.

Iodinated FGF-2 was incubated with chlorate-treated Swiss 3T3 cells in the presence of native heparin at 10 nM, which was then augmented either with additional native heparin or with the chemically 6-O-desulfated form, and the FGF-2 was bound and cross-linked to cell surface receptors with DSS (Figure 7A). The appearance of 140 and 160 kDa bands was detected by SDS−PAGE and autoradiography as shown earlier. As previously described, chlorate-treated cells lacking functional heparan sulfate show reduced binding of FGF-2 to cell surface receptors and 6-O-desulfated heparin alone at 10 000 nM is not capable of supporting FGF-2 binding. As expected, chlorate-treated cells incubated with FGF-2 in the presence of 10 nM heparin bind FGF-2 to these two proteins and augmentation by additional native heparin to 10 000 nM dramatically reduces binding. Similarly, augmentation of 10 nM native heparin to 1000 nM by the addition of 6-O-desulfated heparin leads to a major reduction in FGF binding.

To examine the effects of the reduced binding on mitogenic activity, [^{3}H]thymidine incorporation was examined in the presence of FGF-2 and 10 nM heparin either augmented with native heparin (e.g., 10 000 nM heparin) or augmented with 10 000 nM 6-O-desulfated heparin (Figure 7B). Either concentration of native heparin supported the same level of mitogenic response despite their differences

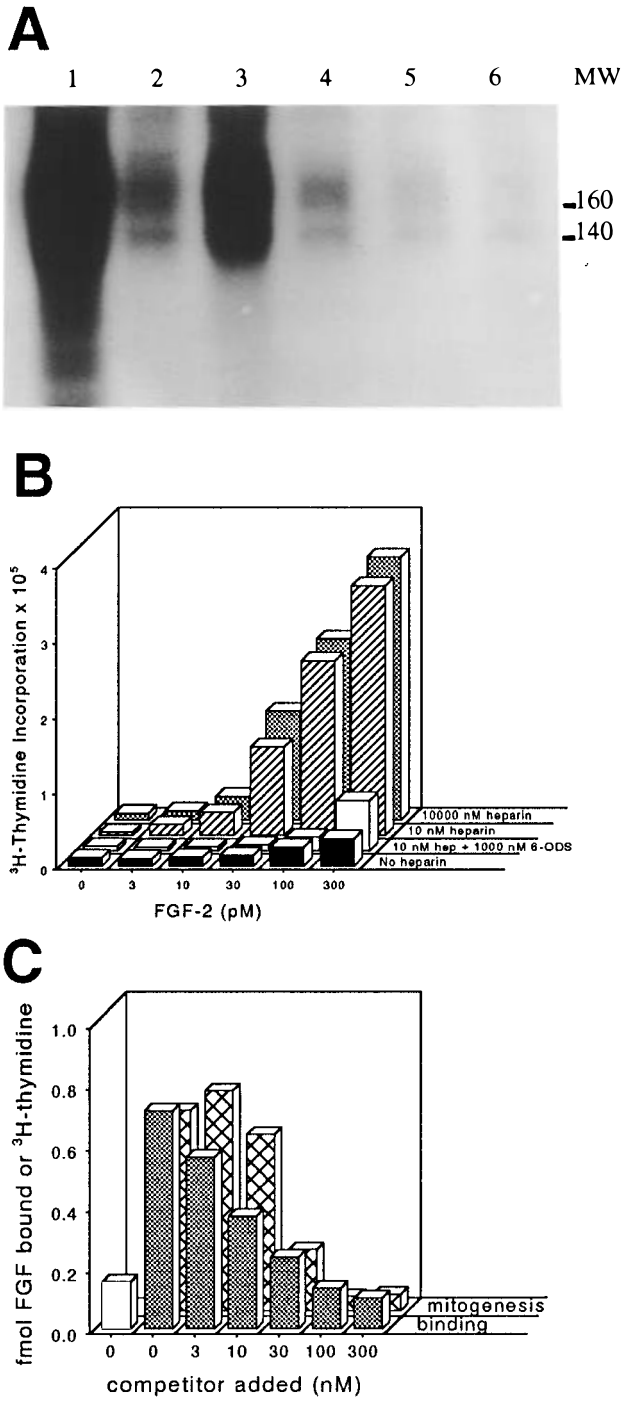

FIGURE 7: FGF-2 cross-linking to cell surface receptors and FGF-2-stimulated mitogenesis in the presence of 6-O-desulfated heparin. (A) Iodinated FGF-2 was bound to chlorate-treated cells at 4 °C and cross-linked to surface receptors with DSS and detected by autoradiograpy after SDS−PAGE: lane 1, nontreated cells; lanes 2−6, chlorate-treated cells; lane 2, no heparin; lane 3, 10 nM heparin; lane 4, 10 000 nM heparin; lane 5, 10 nM heparin and 1000 nM 6-O-desulfated heparin; and lane 6, 10 nM 6-O-desulfated heparin. (B) Serum-starved chlorate-treated cells were stimulated with FGF-2 in the presence of different concentrations of the indicated heparin type as shown in the legend. Response was assayed by incorporation of [^{3}H]thymidine into DNA. (C) A direct comparison of binding and activity. For binding, iodinated FGF (100 pM) was incubated with chlorate-treated cells in the absence (open bar) or presence of 3 nM native heparin (stippled bars) containing the indicated concentrations of 6-O-desulfated heparin as a competitor. Binding is expressed as femtomoles of FGF-2 bound. For activity, the mitogenic response of serum-starved cells to 100 pM FGF-2 in the absence or presence of 3 nM heparin containing 6-O-desulfated heparin is assessed by [^{3}H]thymidine incorporation (counts per minute × 10^5).

in the ability to support stable FGF-2 binding to its receptor. However, augmentation of 10 nM native heparin with 6-O-desulfated heparin reduces the mitogenic stimulation.

The reduction of mitogenic signaling when the 6-O-desulfated heparin is used as a competitor parallels the decrease in binding (Figure 7C). Quantification of the FGF-2 binding demonstrates that the 6-O-desulfated heparin competes effectively with native heparin, such that 10−30 nM concentrations of competitor combined with 10 nM native heparin cause greater than 50% reduction in binding. Measurement of [^{3}H]thymidine incorporation in response to FGF-2 binding is similarly reduced. Importantly, these results also show a direct correlation between binding measured using iodinated growth factor and activity using native FGF; this argues strongly that the behavior of iodinated FGF in these assays is identical to that of the noniodinated form.

It is emphasized again that the reduction in binding is similar to that seen when increasing concentrations of native heparin are used (cf. Figure 2), yet native heparin does not cause the reduction in mitogenic stimulation.

Binding and Signaling Mediated by Deca- and Dodecasaccharides. To explore this interaction further, binding and signaling were examined in the presence of heparin fragments that have different abilities to promote FGF signaling. A heparin decasaccharide (10-mer) binds FGF-2 but supports neither the binding of 300 pM [^{125}I]FGF-2 to cell surface receptors (data not shown) nor mitogenesis in the presence of 100 pM FGF (Guimond et al., 1993). For this reason, the 10-mer was used as a competitor with native heparin for FGF-2 binding and signaling. Increasing concentrations of 10-mer were added to a constant concentration of native heparin and FGF-2. In one set of experiments, 10-mer was used in the presence of 300 pM FGF-2 either in the presence or in the absence of 10 nM native heparin, conditions that promote a high degree of binding. 10-mer alone did not promote binding, as expected, but native heparin restores the binding of [^{125}I]FGF-2 to 3T3 fibroblasts (Figure 8A). However, as little as a 10-fold excess of 10-mer over native heparin is sufficient to reduce binding, with a 98% reduction seen at a 1000-fold excess of 10-mer.

A heparin dodecasaccharide (12-mer) binds FGF-2 with an affinity similar to that of the 10-mer (Maccarana et al., 1993). However, the 12-mer does support the mitogenic response of the cells in 100 pM FGF-2 (Guimond et al., 1993). Despite its activity at these FGF concentrations, however, the 12-mer does not promote the high-affinity binding of 300 pM [^{125}I]FGF-2 to cell surface receptors on chlorate-treated 3T3 cells and can also act as a competitor for 10 nM native heparin in FGF-2 binding studies. A 10-fold excess of 12-mer reduces the amount of FGF-2 binding in the presence of 10 nM native heparin by ca. 50%, while a 1000-fold excess reduces binding by 95% (Figure 8A). This reduction is identical to that seen with the 10-mer, which would be expected as the 10-mer and 12-mer bind to FGF-2 and displace native heparin to the same extent (Maccarana et al., 1993).

To explore the correlation between high-affinity binding and mitogenic stimulation, these studies were conducted at FGF concentrations that provide half-maximal stimulation, namely 30 pM FGF-2 and 1 nM heparin. The binding studies provide the same qualitative result; namely, both 10-mer and 12-mer compete with the native heparin in an identical fashion to reduce binding (Figure 8A). However,

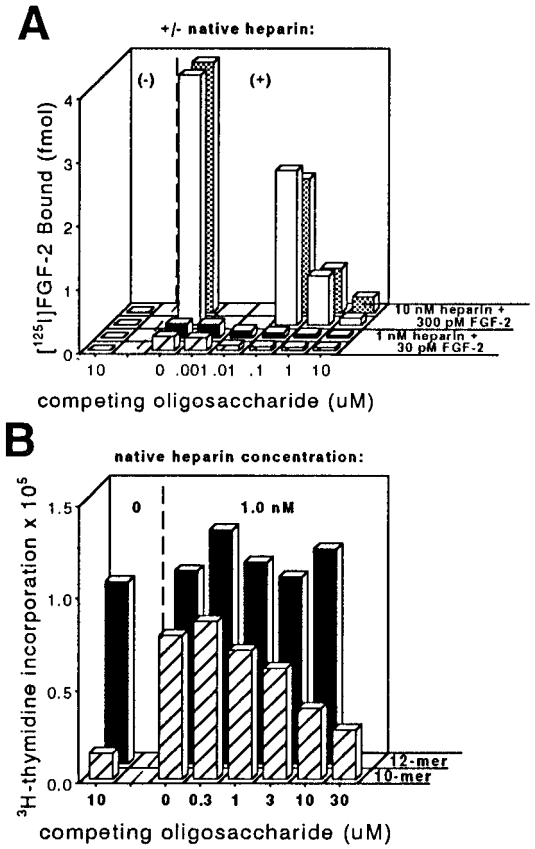

FIGURE 8: Binding of FGF-2 and induction of DNA synthesis in heparin decasaccharide or dodecasaccharide. (A) Binding. Chlorate-treated Swiss 3T3 fibroblasts were incubated for 2 h at 4 °C in 30 pM iodinated FGF-2 in the absence (−) or presence (+) of 1 nM heparin or in 300 pM iodinated FGF-2 in the absence (−) or presence (+) of 10 nM heparin. Also included were the indicated concentrations of either heparin decasaccharide (striped bars and open bars) or dodecasaccharide (solid bars and stippled bars). (B) Mitogenesis. Chlorate-treated, serum-starved fibroblasts were incubated for 24 h at 37 °C in 30 pM FGF-2 and either in the absence of native heparin or in 1.0 nM native heparin. In addition, the indicated concentrations of either decasaccharide (10-mer) or dodecasaccharide (12-mer) were added. The mitogenic response was assessed by [^{3}H]thymidine incorporation.

they differ dramatically in their abilities to block mitogenic stimulation (Figure 8B). Increasing concentrations of the 10-mer reduce stimulation, but increasing concentrations of the 12-mer do not. Indeed, as the 12-mer alone is capable of promoting FGF-2 activity, signaling is maintained despite the displacement of native heparin but in the face of declining high-affinity binding. These results parallel the effects of the 6-O-desulfated heparin which also reduces binding and activity simultaneously.

DISCUSSION

Numerous studies have shown that heparan sulfate proteoglycans bind members of the FGF family with a K_d of 1−10 nM and serve as coreceptors with RTKs by forming a high-affinity FGF-2−heparan sulfate−RTK complex. This functional role for heparan sulfate has been demonstrated for FGF-1, FGF-2, FGF-4, and FGF-5 (Guimond et al., 1993; Olwin & Rapraeger, 1992; Ornitz & Leder, 1992; Ornitz et al., 1992; Rapraeger et al., 1991). Cells either devoid of heparan sulfate or treated with an inhibitor that disrupts heparan sulfate synthesis are impaired in their ability to bind FGF-2 to cell surface RTKs; nonetheless, addition of exogenous heparin to these cells restores FGF binding to its receptor and FGF-mediated mitogenesis (Li & Bernard, 1992; Rapraeger et al., 1991; Yayon et al., 1991). This has led to the identification of sites on the FGF and receptor that bind heparan sulfate and enhance FGF-2 binding to its receptor. Investigation of the heparin concentration required for restoration of FGF-2 binding to the RTK demonstrates that there is an optimal concentration; heparin concentrations less than or greater than the optimum support only minimal high-affinity binding. This suggests that the mechanism of heparan sulfate involvement is more complex than binding to a single site (Rapraeger, 1995).

Role of Heparan Sulfate in FGF-2 Binding to Tyrosine Kinase Receptor. Identification of FGF receptors by cross-linking of iodinated FGF-2 to the cell surface of Swiss 3T3 cells results in two cross-linked bands of 140 and 160 kDa, indicative of cross-linking to cell surface proteins of 120 and 140 kDa, respectively. Since Swiss 3T3 cells express messenger RNA for FR1 (Reiland & Rapraeger, 1993) and smaller alternatively spliced forms lacking the first immunoglobulin domain of FR1 have been described (Reid et al., 1990; Jaye et al., 1992), the proteins cross-linked at the surface likely represent the full length FR1 receptor and a smaller alternatively spliced version. Receptor activation is demonstrated by the appearance of newly tyrosine-phosphorylated proteins. Although we have not attempted to identify these proteins, their appearance coincides with FGF-2 cross-linking to receptor at the cell surface, suggesting that they are a consequence of FR1 activation. Binding of FGF-2 to FR1 and receptor activation is biphasic over a range of heparin concentrations. Optimal binding and activation is seen at 1−10 nM heparin when 100−500 pM FGF is used. Assuming that the heparin has an average size of 10 kDa and binds FGF with a K_d of 1−10 nM, approximately 50−90% of the FGF would be bound by a heparin chain under these conditions. The correlation between binding to heparin and binding to receptor supports numerous published conclusions that binding of FGF-2 to heparin or heparan sulfate augments FGF binding to its RTK.

The reduction in high-affinity complex formation with the FGF receptor when the heparin concentration exceeds 10 nM suggests that the additional heparin chains are competing for a second important site (see below). This suggests either that a single heparin chain must occupy more than one site if high-affinity binding is to be achieved and that at high heparin concentrations these sites are occupied by distinct heparin chains rather than a single chain or that a second, previously unoccupied lower-affinity site now becomes occupied by another heparin chain that interferes with receptor binding.

Role of Heparan Sulfate in FGF-2-Stimulated Mitogenesis. Despite the identification of an optimal heparin concentration for stable FGF binding and receptor phosphorylation, the same requirement does not exist for the mitogenic response of either the 3T3 or F32 cells. Activation of RTK leading to mitogenesis appears to occur even when multiple binding sites are occupied by individual heparin chains, a condition that appears to block high-affinity binding. Mitogenesis in excess heparin, where receptor occupancy and tyrosine phosphorylation of intracellular substrates are greatly reduced, shows an identical FGF-2 dose response compared to that of mitogenesis at heparin concentrations that are optimal for stable receptor binding and transphosphorylation. A trivial explanation for this result on the 3T3 cells might be that mitogenic signaling is independent of the FGF RTK.

However, this is ruled out by the similar response of the F32 cells, which acquire the ability to respond to FGF-2 only through transfection with cDNA encoding FR1.

A second explanation for mitogenesis in the face of reduced receptor occupancy is that mitogenesis may be stimulated by occupancy of only a minor proportion of the receptors, a population so small that it defies detection by the binding methods used here. This also appears to be ruled out by several arguments. First, the half-maximal response to FGF-2, observed at ca. 3−10 pM FGF-2 on untreated 3T3 cells, does not change when the excess heparin concentrations are employed. Second, reducing receptor binding with modified heparins demonstrates that stepwise reduction in binding is in fact mirrored by reduction in mitogenic activity. The 6-O-desulfated heparin binds FGF-2 with nearly the same affinity as native heparin but fails to promote FGF binding and activity due to its desulfation, which quantitatively removed the glucosaminyl 6-O-sulfates and as much as 30% of the iduronosyl 2-O-sulfates. Reduction of FGF-2 binding to the RTK by competing with 6-O-desulfated heparin leads to a reduction in FGF-2-stimulated mitogenic response, as measured by a shift in the half-maximal dose response that is not seen when binding is reduced by competing with native heparin. This argument is also supported by the comparison of binding and activity seen using the decasaccharide to compete with native heparin. Again, reduction in FGF binding leads to a corresponding reduction in mitogenic activity. Interestingly, the dodecasaccharide, which supports the activity of FGF, also competes with native heparin to reduce binding; its ability to act as a binding competitor is identical to that of the decasaccharide, yet mitogenic activity is maintained. This suggests (i) that heparins the size of a 12-mer are sufficient to interact with FGF-2 and initiate activation of the RTK but that longer forms more effectively promote high-affinity binding and (ii) that FGF-2 binding to the RTK in the presence of excess native heparin is not actually inhibited but is altered in a manner that leads to reduced detection and reduced receptor phosphorylation.

Heparan Sulfate Binding Sites on FGF and Its RTK. These findings suggest that the activity of FR1 is regulated by the number of sites occupied by a single heparan sulfate chain. In the presence of 100−300 pM FGF-2 and the absence of heparan sulfate, effective receptor activation is not achieved. Participation of a single glycosaminoglycan chain, occupying one or more sites, leads to high-affinity binding, receptor phosphorylation, and mitogenesis. However, occupancy of multiple single sites by multiple individual chains alters binding and reduces phosphorylation but still activates a mitogenic response. The altered binding may lead to "transient" receptor binding, possibly a consequence of an increased "off" rate as described by Nugent and Edelman (1992). This would lead to a reduced steady state level of binding, which is measured here, but still activate the receptor.

Several sites for heparan sulfate binding have now been described. A major site is present on both the FGF-2 and the RTK. Three-dimensional modeling demonstrates a cationic site on the FGF comprising amino acids N27, R120, K125, and K135, among others, that binds to heparin (Eriksson et al., 1993; Ornitz et al., 1995; Pantoliano et al., 1994). A cationic heparin-binding domain in FR1 has been identified in the interloop region between Ig loops I and II (Kan et al., 1993). The heparin-binding domain within the FGF-2 is in close proximity to a primary receptor binding domain (Pantoliano et al., 1994) consisting of Y24, R44, N101, Y103, L140, and M142. Binding of heparan sulfate within these FGF and receptor heparin-binding domains is proposed to meld these cationic regions and allow the primary receptor binding domain within FGF-2 to interact with Ig loops II and III of the receptor (Pantoliano et al., 1994). Roghani et al. (1994), studying FGF-2 binding to cell surfaces, suggest that heparan sulfate increases the affinity of this binding by ca. 3-fold. Calorimetry data using purified, recombinant receptor suggest that FGF binds the RTK via this primary domain with a K_d of 41 nM (Pantoliano et al., 1994) and demonstrate that this affinity is increased approximately 10-fold when heparin participates (Pantoliano et al., 1994). Interestingly, these data were generated using a low-molecular weight heparin preparation, similar in size to the 10- and 12-mers used here. It is not clear whether longer heparins would lead to increased affinity at this site, although it is known that longer heparins bind FGF-2 with an affinity at least 10-fold greater than that exhibited by 10- and 12-mers (Maccarana et al., 1993).

The affinities of heparin and heparan sulfate for these heparin-binding sites remains unclear, largely because of variability in the size and sulfation of the heparin or heparan sulfate used in the studies. It is clear that maximal binding and activity requires a specific sulfation pattern and length. The affinity of native heparin binding to FGF-2 is estimated to be in the 1−10 nM range, depending on the methods used, whereas reports of heparin binding to the RTK are more variable, ranging from estimates in the 100 μM range when a low-molecular weight heparin is used (Pantoliano et al., 1994) to 130−340 nM for iodinated, full length heparin bound to FR1 highly expressed in baculovirus-infected Sf9 cells (Wang et al., 1995).

A second RTK binding site on the FGF comprising largely K110, Y111, and W114 on FGF-2 has been identified (Baird et al., 1988; Pantoliano et al., 1994; Springer et al., 1994), which is estimated to have a 250-fold lower affinity than the primary site. This latter site is proposed to bind a second receptor, an interaction that is dramatically enhanced by the presence of heparin in the primary site. Mutation of these amino acids curtails mitogenic signaling on a variety of cell types (Springer et al., 1994).

FGF Receptor Phosphorylation and Dimerization. Although it remains to be demonstrated which interactions are absolutely necessary for generating the high-affinity receptor binding, efficient receptor phosphorylation is proposed to be dependent upon receptor dimerization. The description of an optimal heparin concentration for the activation of receptor transphosphorylation suggests that heparan sulfate has a role in the process of stable dimer formation. Two models for receptor dimerization have been proposed. The first model, introduced above, proposes that heparan sulfate-mediated binding of FGF-2 to the RTK via the primary site on FGF-2 promotes interaction with a second receptor through the second site of the same FGF-2 (Pantoliano et al., 1994). A second model proposes that heparin simultaneously binds two FGFs, thus constituting a dimer (Ornitz et al., 1992, 1995; Spivak-Kroizman et al., 1994). Evidence for the latter model is supported by the demonstration that FGF can be cross-linked into dimers in the presence of optimal heparin concentrations; as would be predicted, excess heparin abolishes the dimer formation (Ornitz et al., 1992).

The results discussed here suggest that the two models may not be exclusive of one another. Initial binding of FGF to heparan sulfate may enhance binding to the RTK and recruitment of a second RTK, thus initiating signaling. The initial binding event can be accomplished with heparins on the order of 10−12 sugars in length (Guimond et al., 1993; Ishihara et al., 1993; Ornitz & Leder, 1992; Ornitz et al., 1992) but is disrupted by shorter oligosaccharides or heparins lacking the correct sulfation pattern, such as the 6-O-desulfated heparin used here. The formation of this complex may be transient unless it is further stabilized by the heparan sulfate bridging the receptors or bridging to another FGF−receptor. Such complexes would constitute the high-affinity complex typically observed upon FGF binding to native cells or observed here at heparin concentrations that promote maximal receptor phosphorylation. Excess heparin or oligosaccharides the size of the 12-mer would fail to carry out the bridging. An alternative explanation for these results is that excess heparin chains may bind to a secondary binding site on the FGF, thus inhibiting receptor dimerization altogether. In this scenario, however, the receptor would be expected to signal without dimerization. This cannot be ruled out as it remains to be demonstrated formally that the receptors are not activated as monomeric forms.

The requirement of heparan sulfate for FGF-2 receptor binding and its ability to regulate activation of the FGF receptor by interaction with distinct sites of the receptor complex suggest important regulatory roles for heparan sulfate proteoglycans. Heparan sulfate proteoglycans not only must be present at the cell surface to allow FGF receptor binding and activation, as shown here, but also must be decorated with a specific type of heparan sulfate chains capable of supporting a specific FGF. Specific heparan sulfate sequence requirements for FGF-2 receptor binding and mitogenesis indicate that both the length and composition of a heparan sulfate chain determine the ability of FGF to interact with its receptor and support mitogenesis (Guimond et al., 1993). In addition, developmental modulation of the type of heparan sulfate chains of a neuronal heparan sulfate proteoglycan dictates the mitogenic responsiveness of embryonic neural cells to basic and acidic FGF (Nurcombe et al., 1993; Brinkman et al., 1995). Furthermore, the potential ability of heparan sulfate to control the type of signal transmitted by the FGF receptor suggests another level of regulation by heparan sulfate proteoglycans and lends further insight into potential means of pharmaceutical intervention.

ACKNOWLEDGMENT

The authors thank Dr. D. Ornitz for the gift of the F32 cells and Dr. U. Lindahl for the heparin deca- and dodecasaccharides and the 6-O-desulfated heparin. Dr. B. Olwin, Department of Biochemistry, Purdue University, is thanked for numerous helpful discussions.

REFERENCES

Amaya, E., Stein, P. A., Musci, T. J., & Kirschner, M. W. (1993) *Development 118*, 477−487.

Avivi, A., Yayon, A., & Givol, D. (1993) *FEBS Lett. 330*, 249−252.

Baird, A., & Klagsbrun, M. (1991) *Cancer Cells 3*, 239−243.

Baird, A., Schubert, D., Ling, N., & Guillemin, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2324−2328.

Basilico, C., & Moscatelli, D. (1992) *Adv. Cancer Res. 59*, 115−165.

Bellot, F., Crumley, G., Kaplow, J. M., Schlessinger, J., Jaye, M., & Dionne, C. A. (1991) *EMBO J. 10*, 2849−2854.

Brickman, Y. G., Ford, M. D., Small, D. H., Bartlett, P. F., & Nurcombe, V. (1995) *J. Biol. Chem. 270*, 24941−24948.

Broad, T. E., & Ham, R. G. (1983) *Eur. J. Biochem. 135*, 33−39.

Burgess, W. H., & Maciag, T. (1989) *Annu. Rev. Biochem. 58*, 575−606.

Burgess, W. H., Shaheen, A. M., Ravera, M., Jaye, M., Donohue, P. J., & Winkles, J. A. (1990) *J. Cell Biol. 111*, 2129−2138.

Coughlin, S. R., Barr, P. J., Cousens, L. S., Fretto, L. J., & Williams, L. T. (1988) *J. Biol. Chem. 263*, 988−993.

Eriksson, A. E., Cousens, L. S., & Matthews, B. W. (1993) *Protein Sci. 2*, 1274−1284.

Friesel, R., Burgess, W. H., & Maciag, T. (1989) *Mol. Cell. Biol. 9*, 1857−1865.

Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., & Rapraeger, A. C. (1993) *J. Biol. Chem. 268*, 23906−23914.

Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., & Kimata, K. (1992) *Biochem. J.* 285, 805−813.

Huang, S. S., & Huang, J. S. (1986) *J. Biol. Chem. 261*, 9568−9571.

Isacchi, A., Bergonzoni, L., Statuto, M., Rusnati, M., Chiesa, R., Caccia, P., Sarmientos, P., Presta, M., & Ragnotti, G. (1991) *Ann. N. Y. Acad. Sci. 638*, 369−377.

Ishihara, M., Tyrrell, D. J., Stauber, G. B., Brown, S., Cousens, L. S., & Stack, R. J. (1993) *J. Biol. Chem. 268*, 4675−4683.

Jaye, M., Schlessinger, J., & Dionne, C. A. (1992) *Biochim. Biophys. Acta 1135*, 185−199.

Johnson, D. E., & Williams, L. T. (1993) *Adv. Cancer Res. 60*, 1−42.

Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., & McKeehan, W. L. (1993) *Science 259*, 1918−1921.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Li, M., & Bernard, O. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3315−3319.

Maccarana, M., Casu, B., & Lindahl, U. (1993) *J. Biol. Chem. 268*, 23898−23905.

Mason, I. J. (1994) *Cell 78*, 547−552.

Mohammadi, M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Jaye, M., & Schlessinger, J. (1992) *Nature 358*, 681−684.

Nugent, M. A., & Edelman, E. R. (1992) *Biochemistry 31*, 8876−8883.

Nurcombe, V., Ford, M. D., Wildschut, J. A., & Bartlett, P. F. (1993) *Science 260*, 103−106.

Olwin, B. B., & Rapraeger, A. (1992) *J. Cell Biol. 118*, 631−639.

Ornitz, D. M., & Leder, P. (1992) *J. Biol. Chem. 267*, 16035−16311.

Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., & Leder, P. (1992) *Mol. Cell. Biol. 12*, 240−247.

Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C. M., & Waksman, G. (1995) *Science 268*, 432−436.

Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van, D. D., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., & Sisk, W. P. (1994) *Biochemistry 33*, 10229−10248.

Pasquale, E. B., Maher, P. A., & Singer, S. J. (1988) *J. Cell. Physiol. 137*, 146−156.

Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, D., & Williams, L. T. (1992) *Nature 358*, 678−681.

Rapraeger, A. C. (1995) *Chem. Biol. 2*, 645−649.

Rapraeger, A. C., Krufka, A., & Olwin, B. B. (1991) *Science 252*, 1705−1708.

Rapraeger, A. C., Guimond, S., Krufka, A., & Olwin, B. B. (1994) *Methods Enzymol. 245*, 219−240.

Reid, H. H., Wilks, A. F., & Bernard, O. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1596−1600.

Reiland, J., & Rapraeger, A. C. (1993) *J. Cell Sci. 105*, 1085−1093.

Roghani, M., Mansukhani, A., Dell'Era, P., Bellosta, P., Basilico, C., Rifkin, D. B., & Moscatelli, D. (1994) *J. Biol. Chem. 269*, 3976−3984.

Spivak-Kroizman, T., Lemmon, M. A., Dikic, I., Ladbury, J. E., Pinchasi, D., Huang, J., Jaye, M., Crumley, G., Schlessinger, J., & Lax, I. (1994) *Cell 79*, 1015−1024.

Springer, B. A., Pantoliano, M. W., Barbera, F. A., Gunyuzlu, P. L., Thompson, L. D., Herblin, W. F., Rosenfeld, S. A., & Book, G. W. (1994) *J. Biol. Chem. 269*, 26879−26884.

Thompson, L. D., Pantoliano, M. W., & Springer, B. A. (1994) *Biochemistry 33*, 3831−3840.

Tickle, C., & Eichele, G. (1994) *Annu. Rev. Cell Biol. 10*, 121−152.

Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., & Gallagher, J. T. (1992) *J. Biol. Chem. 267*, 10337−10341.

Ueno, H., Gunn, M., Dell, K., Tseng, A. J., & Williams, L. (1992) *J. Biol. Chem. 267*, 1470−1476.

Wang, F., Kan, M., Yan, G., Su, J., & McKeehan, W. L. (1995) *J. Biol. Chem. 270*, 10231−10235.

Werner, S., Duan, D.-S. R., de Vries, C., Peters, K. G., Johnson, D. E., & Williams, L. T. (1992) *Mol. Cell. Biol. 12*, 82−88.

Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., & Ornitz, D. M. (1991) *Cell 64*, 841−848.

BI960125+