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Heparin can activate a receptor tyrosine kinase

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Heparin, a densely sulfated glycosaminoglycan produced by mast cells, is best known for its inhibitory effects on the blood coagulation system. Heparin or heparan sulfate proteoglycans are also essential co-factors for the interaction of fibroblast growth factors (FGFs) with their receptor tyrosine kinases (FGFRs). Here we show that heparin is a growth factor-independent activating ligand for FGFR-4. Heparin stimulates FGFR-4 autophosphorylation on transfected myoblasts, fibroblasts and lymphoid cells, and is most potent on cells lacking surface heparan proteoglycan. Two functional analogs of heparin, fucoidan and dextran sulfate, are also activators of FGFR-4, while neither heparin nor its analogs can stimulate FGFR-1 in the absence of FGF. A mutation in the FGFR-4 ectodomain which impairs receptor activation by FGFs does not interfere with activation by heparin, demonstrating that receptor domains required for heparin or FGF activation are not identical. Heparin activation of FGFR-4 or of a chimeric receptor bearing FGFR-4 ectodomain and FGFR-1 cytodomain triggers downstream tyrosine phosphorylation of several signaling proteins, and induces proliferation of cells bearing the chimeric receptor. Consistent with these findings, a soluble FGFR-4 ectodomain has strong FGF-independent affinity for immobilized heparin resin, while soluble FGFR-1 requires FGF for stable heparin interaction. Heparin activation of FGFR-4 is the first example of a mammalian polysaccharide serving as a signaling ligand.

Key words: fibroblast growth factor/heparin/receptor binding/tyrosine kinase

Introduction

Protein tyrosine phosphorylation induced by polypeptide ligand–receptor interaction is a fundamental mechanism for intercellular communication in vertebrates. Phosphorylation is accomplished by cytoplasmic kinases non-covalently associated with receptors (Stahl and Yancopoulos, 1993) or by kinases intrinsic to transmembrane receptors (receptor tyrosine kinases; Ullrich and Schlessinger, 1990). In both cases, ligands induce receptor clustering and autophosphorylation followed by recruitment of intracellular substrates to activated receptors (Ullrich and Schlessinger, 1990; Stahl and Yancopoulos, 1993).

Fibroblast growth factors (FGFs) are a family of structurally related ligands which play many roles in cell growth, differentiation and survival (Burgess and Maciag, 1989; Goldfarb, 1990; Basilico and Moscatelli, 1992). The FGF receptor tyrosine kinases (FGFRs) are homologous in overall structure (Basilico and Moscatelli, 1992), but differences in ectodomain sequence dictate the ligand specificities for each receptor (Miki *et al.*, 1991; Partanen *et al.*, 1991; Dell and Williams, 1992; Ornitz and Leder, 1992; Werner *et al.*, 1992) while differences in intracytoplasmic sequence determine the biochemical and functional responses mediated by each receptor (Vainikka *et al.*, 1992; Wang *et al.*, 1994). The mechanism by which FGFRs bind FGFs is seemingly unusual in that a glycosaminoglycan serves as an essential co-factor (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). FGF cannot efficiently bind and activate FGFR on cells lacking surface heparan sulfate proteoglycan (HSPG; Yayon *et al.*, 1991; Ornitz and Leder, 1992) or on cell surfaces where heparan sulfation is depleted by prior culture in the presence of chlorate (Rapraeger *et al.*, 1991). Soluble HSPGs or heparin, a densely sulfated heparan glycosaminoglycan secreted by mast cells, can replace surface heparan as co-factor for FGF-dependent receptor activation (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991; Ornitz and Leder, 1992; Nurcombe *et al.*, 1993). Heparin has strong affinity for FGFs (Burgess and Maciag, 1989) and weak affinity for FGFRs (Kan *et al.*, 1993) and may utilize distinct sulfated moieties along a dodecasaccharide segment to bring ligand and receptor together (Guimond *et al.*, 1993; Ishihara *et al.*, 1993).

We report here that FGFR-4 has strong FGF-independent affinity for heparin and can be activated by heparin or functionally related sulfated polysaccharides ectopically expressed on fibroblasts, myoblasts or lymphoid cells. FGF-independent activation by heparin is specific to the FGFR-4 ectodomain, since FGFR-1 is not similarly responsive, while a chimeric receptor bearing the ectodomain of FGFR-4 and the intracytoplasmic domain of FGFR-1 can be activated by heparin. Heparin initiates rapid characteristic biochemical responses in myoblasts expressing FGFR-4 or the chimeric receptor, and lymphoid cells expressing the chimeric receptor will proliferate in response to heparin, demonstrating that heparin-induced receptor activation can mediate short-term and long-term cellular events.

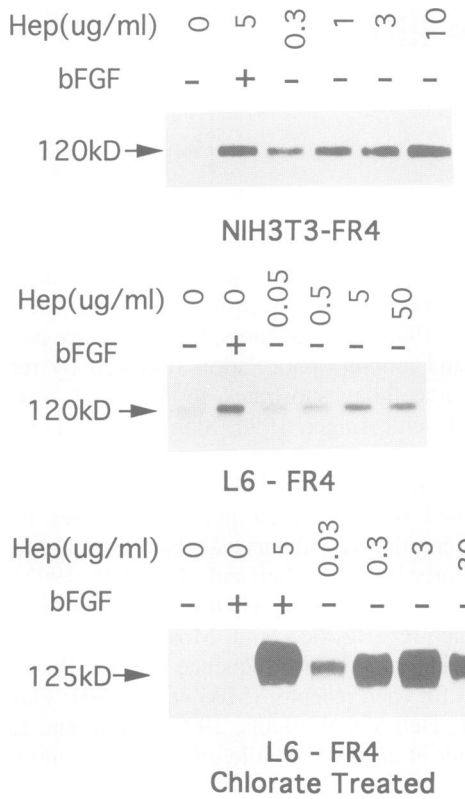

Fig. 1. Heparin stimulates FGFR-4 tyrosine phosphorylation on transfected 3T3 and L6 cells. FGFR-4-transfected NIH 3T3 fibroblasts (upper panel) or L6 myoblasts maintained in normal medium (middle panel) or chlorate-containing medium (lower panel) were serum-starved for 2 h, then challenged for 5 min with different concentrations of heparin (Hep) with or without 100 ng/ml bFGF. Receptors were immunoprecipitated from cell lysates with rabbit FGFR-4 antiserum, subjected to electrophoresis through SDS–7.5% polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes, and probed for receptor phosphorylation with 4G10 anti-phosphotyrosine monoclonal antibody, followed by detection with ECL reagents. The mol. wt of FGFR-4 (120 kDa in medium without chlorate, 125 kDa in chlorate medium) is indicated.

Results

Heparin activates FGFR-4 on transfected fibroblasts and myoblasts

In the course of characterizing the ligand specificities of FGFR-1 and FGFR-4 in transfected cells, which utilized soluble heparin as a potentiator of FGF activities (Burgess and Maciag, 1989; Clements *et al.*, 1993), we were surprised to discover that heparin stimulates tyrosine phosphorylation of FGFR-4 in the absence of exogenous FGF administration. As shown in the top panel of Figure 1, a 5 min challenge of FGFR-4-transfected NIH 3T3 fibroblasts with 1, 3 or 10 μg/ml heparin (20 000 average mol. wt) stimulates FGFR-4 tyrosine phosphorylation to an extent comparable with that of heparin plus saturating levels (200 ng/ml) of basic FGF. Heparin also induces phosphorylation of FGFR-4 on transfected L6 myoblasts, which express very low levels of endogenous FGF receptors (Mohammadi *et al.*, 1992; Peters *et al.*, 1992; Wang *et al.*, 1994), although a minimum of 5 μg/ml heparin is required for substantial stimulation (Figure 1, middle panel). By contrast, a kinase-defective mutant of FGFR-4 termed FR4/K500A (see schematic in Figure 2) expressed in L6 cells can bind FGFs, but does not undergo phosphorylation in response to FGFs or heparin (data not shown). Heparin therefore acts directly on wild type FGFR-4 to mediate receptor autophosphorylation.

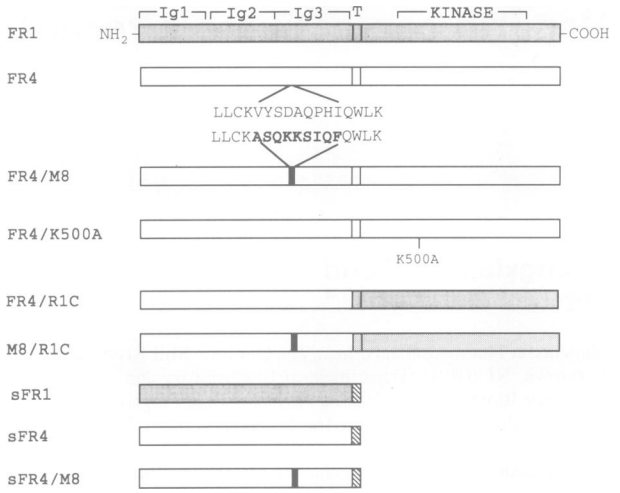

Fig. 2. Schematic representation of engineered FGF receptors. Sequence elements from FGFR-1 (shaded box), FGFR-4 (open box), CD-4 (dark box), and c-myc (hashed box) are indicated, as are the amino acid substitutions in FR4/M8 and FR4/K500A. Regions of FGF receptors encoding kinase domain, transmembrane domain (T) and extracellular immunoglobulin domains (Ig1, Ig2 and Ig3) are indicated above.

The stimulation of FGFR-4 by heparin is more dramatic in L6 cells cultured for several days in the presence of chlorate. Chlorate acts as an inhibitor of HSPG sulfation and, thereby, renders phosphorylation of FGF receptors by FGFs absolutely dependent upon exogenous heparin (Rapraeger *et al.*, 1991). As shown in the bottom panel of Figure 1, chlorate treatment abolishes the basal phosphorylation of FGFR-4 seen on untreated cells and renders the receptor insensitive to activation by FGF without heparin. In chlorate-treated cells, heparin is a potent FGF-independent activator of FGFR-4, mediating detectable receptor autophosphorylation at 30 ng/ml. Hence, the degree of cell surface sulfation determines the sensitivity of FGFR-4 to FGF-independent stimulation by heparin.

Heparin and functionally related sulfated polysaccharides interact with the ectodomain of FGFR-4 but not of FGFR-1

We have tested other glycosaminoglycans and anionic polymers for their ability to mimic the ability of heparin to induce FGFR-4 autophosphorylation on chlorate-treated L6 cells. Hyaluronic acid, heparan monosulfate, keratan sulfate, chondroitin sulfates and polyglutamate all failed to promote FGFR-4 autophosphorylation significantly (Figure 3). Two other sulfated polysaccharides, fucoidan (a marine alga derivative; Springer *et al.*, 1957) and synthetically sulfated dextran, were effective stimulators of FGFR-4 (Figure 3), paralleling the ability of these polysaccharides to mimic the anticoagulant and antiviral activities of heparin (Springer *et al.*, 1957; Ito *et al.*, 1987; Ueno and Kuno, 1987).

As shown in Figure 4, heparin, fucoidan and dextran sulfate were all incapable of inducing tyrosine phosphorylation of FGFR-1 in the absence of FGFs on either L6 or 3T3 cells. By contrast, FR4/R1C, a chimeric

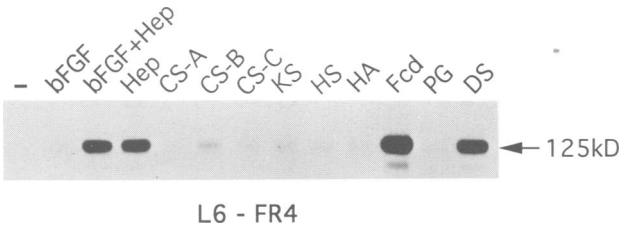

Fig. 3. Activation of FGFR-4 by heparin and functionally related polysaccharides. L6 cells expressing FGFR-4 were maintained in chlorate medium, then serum-starved and challenged with 10 μg/ml polysaccharides or polyglutamate with or without 100 ng/ml bFGF for 5 min. FGF receptors were immunoprecipitated with rabbit FGFR-4 antibodies, and receptor phosphorylation detected with 4G10 anti-phosphotyrosine after gel electrophoresis and membrane transfer. Hep, heparin; CS-A, -B, -C, chondroitin sulfates A, B, C; KS, keratan sulfate; HS, heparan monosulfate; HA, hyaluronic acid; Fcd, fucoidan; DS, dextran sulfate; PG, polyglutamate; bFGF, basic FGF.

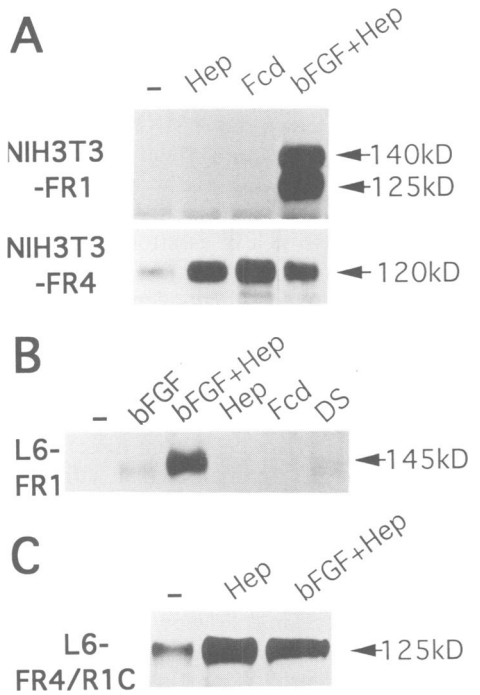

Fig. 4. Receptors with FGFR-4 and FGFR-1 ectodomains differ in susceptibility to activation by sulfate polysaccharides. (**A**) NIH 3T3 cells transfected with FGFR-1 (FR1) or FGFR-4 (FR4) expression vectors were serum-starved for 2 h and challenged for 5 min with 5 μg/ml heparin, 5 μg/ml fucoidan or 5 μg/ml heparin plus 100 ng/ml bFGF. Receptor phosphorylation was assayed following immunoprecipitation with FR1- or FR4-specific antibodies as described in in Figure 1. The sizes of FGFR-4 (120 kDa) and of transfected and endogenously expressed FGFR-1 (145 and 125 kDa, respectively) are indicated. (B and C) L6 cells expressing FGFR-1 (**B**) or FR4/R1C (**C**) maintained in the presence of chlorate were serum-starved and challenged with heparin, fucoidan, or dextran sulfate with or without bFGF, and receptor phosphorylation was assessed following immunoprecipitation with FR1-specific antibodies. Hep, heparin; Fcd, fucoidan; DS, dextran sulfate.

receptor bearing the ectodomain of FGFR-4 fused to the transmembrane and cytoplasmic domains of FGFR-1 (Wang *et al.*, 1994; see Figure 2 schematic), could be stimulated by these polysaccharides (Figure 4, and data below). Hence, the ectodomain of FGFR-4 dictates the ability of the receptor to respond to heparin and its analogs.

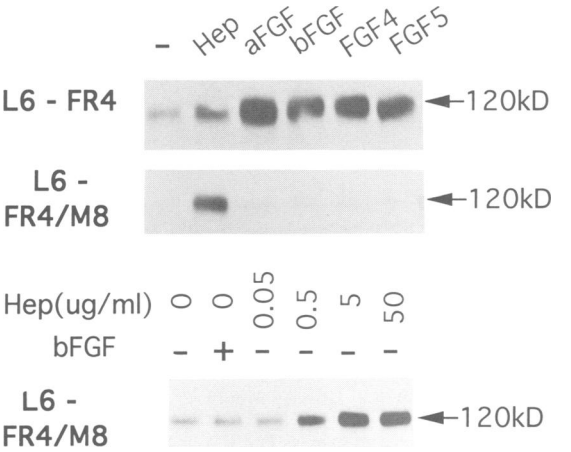

Fig. 5. An ectodomain mutation in FGFR-4 impairs activation by FGFs but not by heparin. (Upper panel) L6-FR4 and L6-FR4/M8 cells were serum-starved, then challenged with 20 μg/ml heparin (Hep) or FGFs for 5 min. Receptor phosphotyrosine was detected as described in Figure 1. aFGF, 200 ng/ml; bFGF, 200 ng/ml; FGF4, 200 ng/ml; FGF5, 1 μg/ml. (Lower panel) L6-FR4/M8 cells were serum-starved for 2 h, then challenged for 5 min with different concentrations of heparin with or without 100 ng/ml basic FGF, and lysates were assayed for receptor phosphotyrosine.

A central question raised by these observations is whether heparin directly stimulates FGFR-4 or, alternatively, whether heparin serves as a cofactor for endogenously expressed FGFs. The activity of heparin towards FGFR-4 and not towards FGFR-1 is one of several lines of evidence arguing against the participation of endogenous growth factors. The affinity of FGFR-1 towards each of six tested FGFs was at least equal to that of FGFR-4 (Vainikka *et al.*, 1992), yet FGFR-1 is refractory to FGF-independent heparin activation.

Heparin can activate a mutant form of FGFR-4 broadly impaired in FGF recognition

We have engineered a mutant form of FGFR-4, termed FR4/M8, in which a region encoding nine amino acid residues in the N-terminal portion of the Ig3 domain were replaced by a DNA segment encoding the corresponding residues in the Ig1 domain of human CD4 cell surface protein (Maddon *et al.*, 1985; Figure 2). FR4/M8 was stably expressed in L6 cells and was compared with wild type FGFR-4 for its ability to be activated by FGFs or heparin. As shown in Figure 5, acidic FGF (200 ng/ml), basic FGF (200 ng/ml), FGF-4 (200 ng/ml) and FGF-5 (1 μg/ml) can all activate FGFR-4, but not FR4/M8, on transfected L6 cells. By contrast, heparin remains an effective inducer of FR4/M8 phosphorylation on either untreated (Figure 5, lower panel) or chlorate-treated (data not shown) L6 cells. These data provide further evidence that heparin stimulation of FGFR-4 is not mediated through endogenously expressed FGFs.

Heparin stimulation of FGFR-4 and FR4/R1C induces activation of ERK protein kinases

To determine whether heparin-induced receptor autophosphorylation is truly indicative of receptor activation, we tested whether a characteristic biochemical response following FGF receptor activation, the phosphorylation of ERK serine/threonine protein kinases, is triggered by

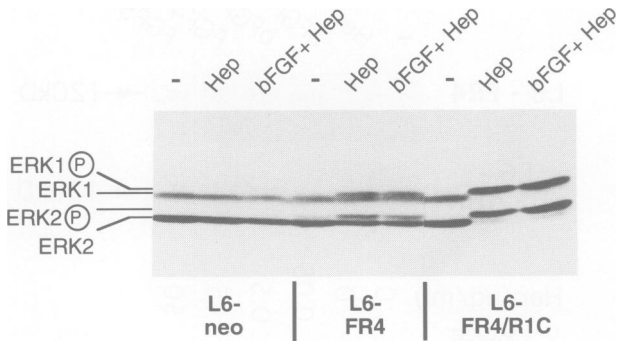

Fig. 6. Phosphorylation of ERK kinases in L6 cells following heparin stimulation of FGFR-4 or FR4/R1C. L6 cells lacking FGF receptors (L6-neo) or transfected to express FGFR-4 or FR4/R1C were serum-starved, and then challenged for 5 min with medium containing 5 μg/ml heparin (Hep), 5 μg/ml heparin plus 200 ng/ml basic FGF (bFGF + Hep) or without supplements (–). Lysates were assayed for electrophoretic mobility shifts of ERK kinases, indicative of ERK phosphorylation (ERK1-P and ERK2-P), as detected by Western blotting using anti-ERK antibodies.

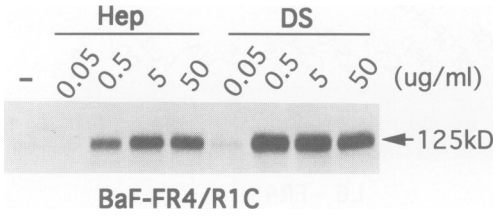

Fig. 7. Phosphorylation of FR4/R1C in BaF3 cells by heparin and dextran sulfate. BaF3 cells expressing FR4/R1C chimeric receptor were starved in IL3-free medium for 3 h, then challenged with different concentrations of heparin or dextran sulfate for 5 min. Tyrosine phosphorylation of the receptor was assayed by receptor immunoprecipitation, gel electrophoresis, filter transfer and probing with 4G10 anti-phosphotyrosine.

heparin. The ERK kinases are weakly activated by FGFR-4 stimulation and more strongly activated by FGFR-1 stimulation (Wang *et al.*, 1994). Transfected L6 cells expressing FGFR-4, FR4/R1C or a receptor-negative control (L6-neo) were treated with chlorate, stimulated for 5 min with basic FGF plus heparin or heparin alone, and cell lysates were analyzed by Western blotting with anti-ERK antibodies to detect the electrophoretic mobility shifts accompanying ERK1 and ERK2 phosphorylation. As shown in Figure 6, heparin with or without FGF did not induce ERK activation in control L6-neo cells. By contrast, weak ERK activation in L6-FR4 cells was achieved by heparin in the presence or absence of FGF, while strong ERK activation was achieved by heparin treatment of L6-FR4/R1C cells, due to the presence of the more potent FGFR-1-derived cytodomain in the chimeric receptor. Additionally, treatment of L6-FR4/R1C cells with heparin, alone or together with FGF, induced tyrosine phosphorylation of phospholipase Cγ and an 80 kDa protein (data not shown). Hence, heparin activates receptors with FGFR-4 ectodomains to achieve rapid characteristic biochemical responses.

Proliferation of FR4/R1C-transfected lymphoid cells in response to sulfated polysaccharides

IL-3 dependent BaF3 pro-B cells expressing FGFR-1 or FR4/R1C proliferate in response to FGFs in the absence of IL-3 (Ornitz and Leder, 1992; Wang *et al.*, 1994), making these cells ideal for testing whether polysaccharide-induced receptor activation can mediate a sustained biological response. A clone of BaF3 cells expressing FR4/R1C was first assayed for receptor tyrosine phosphorylation following stimulation with sulfated polysaccharides. As shown in Figure 7, both heparin and dextran sulfate were potent activators of FR4/R1C, inducing maximal receptor phosphorylation at ≥500 ng/ml polysaccharide.

Three independent clones of BaF3 cells expressing FR4/R1C were then tested for their ability to proliferate without IL-3 in response to heparin (1 μg/ml) alone or together with acidic FGF. Two of these clones were derived under conditions which promoted high levels of receptor expression (~100 000 receptors/cell; see Materials and methods), while the third clone, derived previously (Wang *et al.*, 1994), expressed <15 000 receptors/cell. As shown in Figure 8A, all three clones were able to proliferate in response to heparin, although growth was only 34–40% of that achieved in the presence of FGF. A dose–response analysis was conducted in BaF-pMXFR4R1C-clone 1 for heparin, fucoidan and dextran sulfate: all three substances were mitogenic for these cells and worked best at concentrations of 0.3–3 μg/ml, with 3 μg/ml dextran sulfate promoting cell growth nearly as strong as that achieved upon FGF stimulation (Figure 8B).

To rule out a contribution of endogenous FGF expression to polysaccharide stimulation of cells expressing an FGFR-4 ectodomain, BaF3 cells were transfected with either FGFR-1 or M8/R1C, a chimera containing the cytoplasmic domain of FGFR-1 and the ectodomain of FR4/M8 (Figure 2). When cells were directly selected in medium containing 20 μg/ml fucoidan without FGFs or IL3, the FGFR-1 transfectants showed no survival, while the M8/R1C transfection yielded 12 colonies from 5×10^6 plated cells (data not shown), an efficiency of colony formation comparable with that achieved with heparin plus acidic FGF selection following FGFR-1 or FR4/R1C transfection (Wang *et al.*, 1994). One such BaF3 clone expressing M8/R1C proliferated in response to heparin, fucoidan or dextran sulfate in a manner indistinguishable from BaF-FR4/R1C cells (Figure 8C), while BaF-FGFR-1 cells (Wang *et al.*, 1994) were absolutely dependent upon FGF for proliferation (Figure 8D).

The FGFR-4 ectodomain has strong affinity for heparin

FGFR-1 shows weak affinity for heparin which is mediated by a region of the receptor essential for FGF binding and activation (Kan *et al.*, 1993). This and other data suggest that the obligate FGF–heparin–FGFR-1 heteromeric complex is assembled in part through contacts between heparin and receptor. For FGFR-4 to be activated by heparin in the absence of FGF, we predicted that this receptor has a far stronger factor-independent affinity for heparin. To test this prediction, vectors were constructed to drive the expression of soluble receptors bearing the ectodomains of FGFR-1, FGFR-4 or FR4/M8 fused to a short segment of the c-myc protein (see Figure 2), allowing receptor detection with anti-myc 9E10 monoclonal antibody (Evan *et al.*, 1985). Soluble receptors in conditioned media from

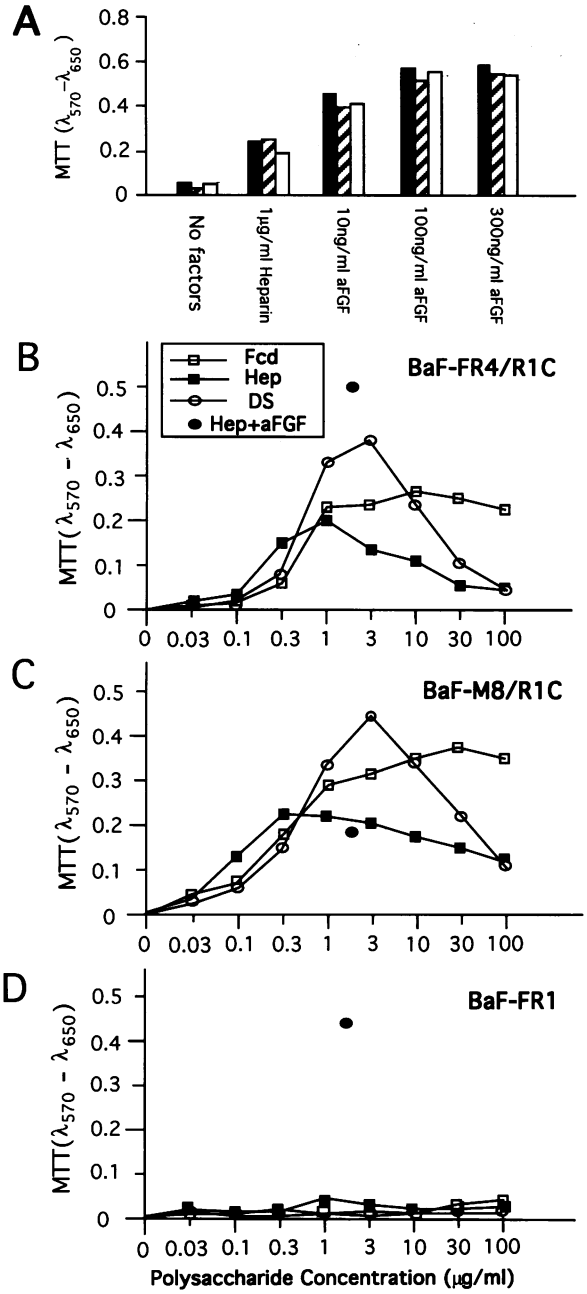

Fig. 8. Polysaccharide-mediated proliferation of FR4/R1C- or M8/R1C-transfected BaF3 cells. (**A**) Three independent clones of BaF3 cells expressing FR4/R1C (pMX-FR4/R1C-clone 1, open bars; pMX-FR4/R1C-clone 2, striped bars; pvcos-FR4/R1C, solid bars) were plated into microwells (10^4 cells/well) in medium without IL-3 and tested for proliferation in response to heparin (1 µg/ml) alone or together with 10, 100 or 300 ng/ml acidic FGF. Viable cells were quantitated after 62 h by MTT uptake and conversion. BaF3 cells expressing FR4/R1C (pMXFR4/R1C-clone 1; **B**), M8/R1C (**C**), or FGFR-1 (**D**) were cultured in IL-3-free medium containing different concentrations of heparin (Hep), fucoidan (Fcd) or dextran sulfate (DS), or containing 2 µg/ml heparin plus 100 ng/ml acidic FGF. After 62 h viable cells were quantitated by MTT uptake and conversion.

transiently transfected COS cells were assayed for their ability to bind to heparin–Sepharose at various sodium ion concentrations in the absence or presence of acidic FGF. Resin-bound receptors were eluted by boiling in SDS and detected following gel electrophoresis and blotting to PVDF membranes with 9E10 monoclonal antibody.

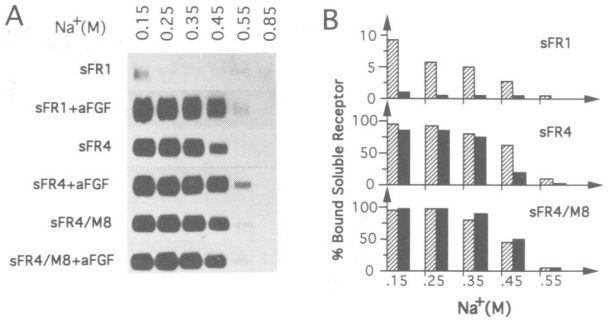

Fig. 9. Binding of soluble FGF receptors to immobilized heparin in the presence or absence of FGF. Soluble FGFR-1 (sFR1), FGFR-4 (sFR4) and FR4/M8 (sFR4/M8) bear the ectodomains of the receptors fused to a c-myc epitope tag. (**A**) Equivalent aliquots of soluble receptors (~75 ng) were mixed with 20 µl heparin–Sepharose resin for 2 h at 4°C in media containing different concentrations of sodium chloride and in the presence or absence of 150 ng acidic FGF. Bound receptors were eluted by boiling in buffer containing 2% SDS, subjected to polyacrylamide gel electrophoresis, transferred to PVDF membrane, and probed with anti-myc 9E10 mouse monoclonal antibody, followed by detection with peroxidase-conjugated anti-mouse IgG and ECL. Autoradiograms of 1 min exposure (sFR4 and sFR4/M8) and 10 min exposure (sFR1) are shown. (**B**) Filters were reprobed with 9E10 and ^{125}I-labeled anti-mouse IgG, and the radioactivity in each band was quantitated with a Fuji BAS 2000 imaging system and plotted as the percentage of input receptor bound.

As shown in Figure 9, soluble FGFR-4 (sFR4) is quantitatively bound to heparin–Sepharose at sodium ion concentrations up to 350 mM, while the presence of acidic FGF affords sFR4 a somewhat stronger heparin affinity. As anticipated from earlier receptor activation experiments, the soluble mutant FGFR-4 (sFR4/M8) also displayed strong affinity for heparin which could not be further stabilized by the presence of FGF (Figure 9). By contrast, in this assay system, soluble FGFR-1 (sFR1) showed almost no FGF-independent interaction with heparin resin, while acidic FGF allowed a portion of the receptors to bind heparin at sodium ion concentrations up to 450 mM (Figure 9). These data show that the ability of heparin to activate FGFR-4 and not FGFR-1 correlates with and is presumably determined by the greater affinity of heparin for FGFR-4 than for FGFR-1, and furthermore show that an Ig3 motif required for FGFR-4 interaction with FGF is not required for the interaction of the receptor with heparin.

Discussion

We have shown that heparin is an FGF-independent activating ligand for FGF receptor-4 on cultured cells. Heparin or functionally related polysaccharides can achieve levels of receptor autophosphorylation and downstream substrate activation indistinguishable from those achieved by FGF. These polysaccharides can also induce a sustained biological response in the absence of FGF, as shown by their ability to stimulate growth of lymphoid cells expressing the FR4/R1C chimeric receptor. It should be noted, however, that heparin alone is less mitogenic towards these reporter cells than is heparin together with FGF. The efficiency of heparin in short-term receptor activation and signaling and its inefficiency for sustained receptor-mediated responses could result from the relative instability of activated heparin–receptor complexes

compared with activated FGF–receptor complexes, or from inappropriate intracellular trafficking of receptors activated by heparin.

A fundamental question raised by our findings is whether heparin activates FGFR-4 in intact animals. Heparin fulfills the requirements of specificity, sensitivity and regulated secretion expected for a ligand which can act *in vivo*. While this glycosaminoglycan can stimulate FGFR-4, it is ineffective towards FGFR-1, or towards other receptor tyrosine kinases, such as TrkB and RET (M.Goldfarb, unpublished data). Furthermore, heparin activates FGFR-4 on cells lacking surface heparan proteoglycan weakly at 30 ng/ml and maximally at 500 ng/ml. This concentration range is comparable with or lower than those required for the other documented activities of heparin, including its ability to serve as an FGF co-factor for stimulation of other FGF receptors (Yayon *et al.*, 1991) and its inhibitory activities on the blood coagulation system (Jeske and Fareed, 1993). Our findings raise the possibility that endogenous heparin secreted upon mast cell activation or exogenous heparin administered as an anticoagulant may serve as a signaling ligand for cells expressing FGFR-4 in adult tissues. It is noteworthy that tissues infiltrated by mast cells include the lung, which is a prominent expression site for FGFR-4 (Partanen *et al.*, 1991).

Analysis of the structures of heparin and heparan sulfate proteoglycans provide a possible understanding for how heparin activates FGFR-4 and how cell surface sulfation antagonizes this activation, while still allowing for FGF-mediated receptor stimulation. If a short segment of a high molecular weight, densely sulfated heparin chain constitutes a binding site for FGFR-4, analogous to the short oligosaccharide motifs governing other heparin–heparin-binding protein interactions (Jackson *et al.*, 1991), then heparin is multivalent and thereby capable of inducing receptor clustering and concomitant activation. By contrast, the heparan chains of cell surface HSPGs are less extensively modified and bear only short stretches of dense sulfation (Lindahl, 1989; Montgomery *et al.*, 1992). HSPGs may be predominantly monovalent in their interactions with FGFR-4 and, when present at high concentration on the cell surface, competitively inhibit heparin-mediated receptor activation. Hence, the abundance of cell surface HSPG could dictate the sensitivity of FGFR-4 towards heparin in the absence of FGF.

The Ig2 and Ig3 domains of FGF receptors are required for FGF binding, while the Ig1 domain plays little or no role in FGF recognition (Crumley *et al.*, 1991; Miki *et al.*, 1991; Werner *et al.*, 1992; Yayon *et al.*, 1992; Cheon *et al.*, 1994). The Ig2 domain of FGFR-1 also bears a region which can bind heparin and appears necessary for FGF binding to its receptor (Kan *et al.*, 1993). Our failure to detect significant binding of soluble FGFR-1 ectodomain to heparin–Sepharose suggests that this interaction is of relatively low affinity. Does the corresponding Ig2 region of FGFR-4 account for its stronger interaction with heparin, or do other regions on this receptor mediate heparin binding and activation? Inspection of the FGFR-4 ectodomain sequence reveals that both the Ig1 and Ig2 domains contain multiple potential heparin-binding motifs not found in FGFR-1, characterized by clustered basic residues with interspersed hydropathic residues (Cardin and Weintraub, 1989; Jackson, 1991). The properties of chimeric receptors bearing portions of FGFR-1 and FGFR-4 ectodomains will be useful in mapping the domains on FGFR-4 which enable heparin to function as an activating ligand.

Materials and methods

Reagents

High mol. wt porcine heparin (20 000 average mol. wt), heparin oligosaccharides (4000–6000 average mol. wt), heparan monosulfate, keratan sulfate, chondroitan sulfates A, B and C, fucoidan, dextran sulfate and polyglutamic acid were purchased from Sigma Chemical. Murine IL-3 and human basic FGF, acidic FGF and FGF-4 were from R&D Systems. Human FGF-5 was prepared as described (Clements *et al.*, 1993). Rabbit polyclonal antibodies to a C-terminal peptide of murine FGFR-4 were described previously (Wang *et al.*, 1994). Antibodies to the FGFR-1 C-terminus were purchased from Santa Cruz Biotechnology, antibodies against phospholipase Cγ and ERKs were from Upstate Biotechnology, Inc., as was 4G10 anti-phosphotyrosine mouse monoclonal antibody. 9E10 anti-myc-epitope monoclonal antibody (Evan *et al.*, 1985) was provided by D.Morrissey, Regeneron Pharmaceuticals. Peroxidase-conjugated anti-mouse immunoglobulins and anti-rabbit immunoglobulins were from CALTAG and Sigma Chemical, respectively. ECL reagents were purchased from Amersham. ^{125}I-labeled anti-mouse immunoglobulins were from ICN Biochemicals. Heparin–Sepharose was purchased from LKB Pharmacia.

Receptor DNA constructs

To generate the FGFR-4 expression vector pMX-FR4, full-length murine FGFR-4 cDNA (FR4.2; Wang *et al.*, 1994) was cloned as an *Eco*RI fragment into pMX, which contains an SV40 replication origin and prokaryotic replication and selection components of pCMX (Davis *et al.*, 1991) and a polylinker placed between two Moloney murine leukemia virus (M-MLV) long terminal repeat elements (D.J.Glass, unpublished data). pMX-FR4/R1C expresses a chimeric receptor bearing the ectodomain of murine FGFR-4 fused to the transmembrane and cytoplasmic domains of murine FGFR-1 (Wang *et al.*, 1994). The pMO-FR1 vector expresses murine FGFR-1 from a M-MLV promoter (Yayon *et al.*, 1991).

pMX-FR4/M8 expresses a mutant receptor in which amino acids 291–299 (VYSDAQPHI) in the Ig3 domain of FR4 were replaced by amino acids 43–51 (ASQKKSIQF) in the corresponding region of the Ig1 domain of human CD4 (Maddon *et al.*, 1985). The mutant was constructed by replacing the *Eco*N1 fragment of FR4 (nucleotides 258–1413 of coding sequence; Stark *et al.*, 1991) with two PCR fragments generated using FR4 cDNA template: (i) a 0.5 kbp *Eco*NI–*Cla*I fragment using sense-strand oligonucleotide PF (nt 250–267; 5′-GCTTCCTTCCTGAGGAT) and antisense oligo MT8 (nt 797–780; 5′-ATATAGATCGATTTCTTCTGCGATCCTTGCAGAGTAGCTCCA) and (ii) a 0.6 kbp *Cla*I–*Eco*NI fragment using sense oligo MF8 (nt 835–852; 5′-ATATAGATCGATTCAGTTTCAGTGGCTGAAACACGT) and antisense oligo PT (nt 1418–1401; 5′-CCCTCACCCAGGGGCTT); the MT8 and MF8 primers bear the CD4 codons and *Cla*I recognition sites (underlined). pMX-M8/R1C was derived from the pMX-FR4/R1C chimera expression vector by replacing the *Eco*RI–*Eco*R5 FR4 ectodomain fragment with the corresponding fragment encoding the mutant FR4/M8 ectodomain.

pMX-FR4/K500A expresses a mutant FGFR-4 in which Lys500, a residue common to all protein tyrosine kinases (Hanks *et al.*, 1988) is replaced by alanine. FR4/K500A was constructed by replacing an *Msc*I–*Nco*I fragment of FGFR-4 (nt 1285–2540) with *Msc*I–*Spe*I and *Spe*I–*Nco*I PCR-derived FGFR-4 fragments. The primer K500A-sense (5′-ATATAGACTAGTACCGTGGCTGTG**GCA**ATGCTGAAAGACAATGCC), bearing the mutant alanine codon (bold, nt 1498–1500) and silent mutations creating an *Spe*I site (underlined, nt 1480–1485), and downstream primer was used to generate the 1.0 kbp *Spe*I–*Nco*I PCR fragment, while the K500A-antisense primer (5′-ATATAGACTAGTTTGGTCGGGCCGGGAGG), bearing silent mutations to create the *Spe*I site (underlined, nt 1485–1480) and upstream primer was used to generate the *Msc*I–*Spe*I 0.2 kbp PCR fragment.

Vectors for expressing c-myc epitope-tagged soluble receptor ectodomains (pCMX-sFR1, pCMX-sFR4, pCMX-sFR4/M8) were constructed using the pCMX-ECD vector, which bears *Asc*I and *Srf*I recognition sites 5′ to a *Srf*I–*Not*I myc epitope (PGGEQKLISEEDL) cassette in pCMX (D.Valenzuela, unpublished data). PCR of FGFR-1, FGFR-4 and FR4/M8 ectodomains using 5′ sense oligonucleotides bearing an *Asc*I recognition site and 3′ antisense oligonucleotides bearing

a *Srf*I site generated reaction products which were inserted into pCMX-ECD. PCR oligonucleotides used were (*Asc*I and *Srf*I sites are underlined, initiator methionine codon is in bold-face): sFR1: 5′-ATATAGGGCGCGCCGGGA**ATG**TGGGCTGGAA and 5′-ATATAGGCCCGGGCCCTCCAGGTAGAGCGGTGAGGTC; sFR4 and sFR4/M8: 5′-ATATAGGGCGCGCCA**ATG**TGGCTGCTCTTGG and 5′-ATATAGGCCCGGGCCATCTGTGTATCTGGCCTCA. Proteins encoded by all expression vectors are shown schematically in Figure 2.

Stable DNA transfection and cell selection

Rat L6 myoblasts and murine NIH 3T3 fibroblasts were transfected by the calcium phosphate method (Wigler *et al.*, 1979) using receptor expression plasmids (2 μg), drug selection plasmids (1 μg) and human placental DNA carrier (25 μg). BaF3 lymphoid cells were maintained in RPMI medium containing 10% fetal calf serum and 1 ng/ml murine IL-3, and were transfected by electroporation with linearized and ligase-concatamerized plasmids as previously described (Wang *et al.*, 1994). pLTRneo (Zhan *et al.*, 1987) was used for cell selection with 400 μg/ml G418, and pSV2His (Hartman and Mulligan, 1988) was used for selection with 1.5 mg/ml histidinol.

NIH 3T3 cells transfected with pLTRneo and either pMX-FR4 or pMO-FR1 were selected for G418 resistance, and pools of resistant cells were utilized in receptor activation experiments. L6 cells were transfected with pLTRneo, and a G418-resistant clone (L6-neo) were shown to lack detectable FGF receptors by radiolabeled acidic FGF binding, as previously described (Wang *et al.*, 1994). L6-neo cells were transfected with FGF receptor expression vectors and pSV2His, and histidinol-resistant clones were screened for receptor expression by immunoprecipitation and Western blotting with receptor-specific antibodies (see below).

BaF3 cells transfected with *Mlu*I-linearized and ligated pMO-FR1 or *Sfi*I-linearized and ligated pMX-FR4/R1C were selected with 10 μg/ml heparin and 50 ng/ml acidic FGF without IL-3 following plating in 96 microwell dishes at 5×10^4 cells/well. BaF3 cells transfected with linearized and ligated pMX-M8/R1C or pMO-FR1 were plated in microwells and selected with 20 μg/ml fucoidan without FGF or IL-3.

Cell stimulation and assays for protein phosphorylation

L6-derived cell lines were assayed for induction of receptor phosphorylation under three different culture conditions. (i) Cells were trypsinized and plated at confluent density in medium containing 10% fetal calf serum, and refed with serum-free medium 16–18 h later. After 2 h serum starvation, high mol. wt heparin (20 000 average mol. wt) and/or basic FGF was added for 5 min, followed by cell lysis. (ii) For the data shown in the upper panel to Figure 5, cells were maintained in serum-containing medium for 16–18 h, as above, then refed with serum-free medium containing 400 ng/ml low mol. wt heparin (4000–6000 average mol. wt) for 2 h before challenging for 5 min with FGFs or 20 μg/ml high mol. wt heparin. The low mol. wt heparin serves as a potentiator of acidic FGF and FGF-5 activities (Burgess and Maciag, 1989; Clements *et al.*, 1993) and does not mediate FGF-independent receptor activation on chlorate-untreated cells. We have found that despite up to 1000-fold differences among acidic FGF, basic FGF, FGF-4 and FGF-5 in 4°C equilibrium binding affinities for FGFR-4 (Vainikka *et al.*, 1992; Wang *et al.*, 1994), these FGFs differ only 20-fold in potency as activators of FGFR-4 phosphorylation at 37°C (G.Gao, unpublished data). (iii) To eliminate cell-surface heparan sulfate proteoglycan, cells were cultured in chlorate medium [Dulbecco's modified Eagle's medium containing 30 mM sodium chlorate, with reduced sodium chloride (80 mM) and cysteine (50 μM)] and 10% dialyzed fetal calf serum for 48 h, then trypsinized and replated in the same medium for 24 h before 2 h starvation in serum-free chlorate medium, followed by FGF and/or polysaccharide challenge for 5 min.

NIH 3T3-derived transfectant pools were cultured for 48 h in serum-containing medium before 2 h serum starvation and challenge with heparin with or without FGF. BaF3-derived cell lines were washed once and incubated in RPMI without IL-3 for 3 h before 5 min challenge with polysaccharides.

Cell lysates obtained using 1% NP-40 were assayed for receptor tyrosine phosphorylation, phospholipase Cγ phosphorylation, ERK electrophoretic mobility shift and 80 kDa protein tyrosine phosphorylation as previously described (Wang *et al.*, 1994). All Western blots originally probed for receptor tyrosine phosphorylation were reprobed with anti-receptor antibody to confirm the presence of equivalent amounts of receptor protein in each immunoprecipitate (data not shown).

BaF3 proliferation assay

BaF3-transfected clones derived with concatamerized receptor expression plasmids and previously derived BaF3 FR4/R1C and FGFR-1 clones expressing fewer receptors per cell (Wang *et al.*, 1994) were assayed for proliferation by washing and plating in 96-cluster microwells at 10^4 cells/well in RPMI containing 10% fetal calf serum with or without FGF and polysaccharides. After 62 h viable cells were monitored by the dimethylthiazolyl–dephenyltetrazolium bromide (MTT) uptake and reduction assay (Promega).

Production of soluble FGF receptors and heparin binding assay

COS7 cells transfected with soluble receptor expression vectors were cultured in serum-free medium for 3 days, and conditioned media were harvested as sources of soluble receptors. Aliquots of conditioned media were subjected to SDS–PAGE and Western blotting on PVDF membrane, using 9E10 anti-myc monoclonal antibody followed by peroxidase conjugated anti-mouse Ig and ECL detection to determine the relative concentrations of receptor preparations. By silver stain detection of receptors in a parallel gel, we estimated receptor concentrations to be ~100 ng/ml.

Equivalent aliquots of soluble receptors (~75 ng) were added to 20 μl heparin–Sepharose with or without 150 ng acidic FGF in DME supplemented with different concentrations of NaCl in a final volume of 1.2 ml. Suspended resins were mixed for 6 h at 4°C, then centrifuged, washed three times with ice-cold phosphate-buffered saline and bound receptors were eluted by boiling in 125 mM Tris pH 7–100 mM β-mercaptoethanol–2% SDS. Eluted receptors as well as aliquots of total receptors were detected by Western blotting as described above, and filters were stripped and reprobed with 9E10 and [^{125}I]anti-mouse Ig, to allow relative quantitation of receptors using a Fuji imaging plate and Fuji BAS 2000 system.

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