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Induction of Urokinase-type Plasminogen Activator by Fibroblast Growth Factor (FGF)-2 Is Dependent on Expression of FGF Receptors and Does Not Require Activation of Phospholipase Cγ1*

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The roles of heparan sulfate proteoglycans and tyrosine kinase fibroblast growth factor (FGF) receptors in mediating the induction of plasminogen activator (PA) by FGF-2 were investigated using L6 myoblast cells that normally do not express detectable FGF receptors. PA was induced by FGF-2 in a dose-dependent manner in L6 cells expressing transfected FGF receptor-1 but not in nontransfected cells or cells transfected with the vector alone. The PA produced in these cells was characterized as urokinase-type PA (uPA). Thus, expression of a tyrosine kinase FGF receptor was required for induction of uPA. Internalization of FGF through heparan sulfates does not seem to be involved in this response as soluble heparin and suramin at concentrations which inhibited FGF-2 binding to heparan sulfates but not receptors did not affect the induction of uPA by FGF-2. Mutant receptors in which the tyrosine kinase was inactivated were not able to respond to FGF-2. In contrast, mutation of the site of phospholipase Cγ (PLCγ) binding in the receptor, which causes loss of PLCγ activation, had no effect on uPA induction by FGF-2. These results suggest that PLCγ activation is not required for induction of uPA by FGF-2.

Basic fibroblast growth factor (FGF-2) is a potent mitogen for a variety of mesenchymal cell types (1, 2). In addition to its mitogenic activity, FGF-2 also stimulates cell motility, induces neurite outgrowth in neuroectodermal cells, and regulates the expression of a variety of proteins. One of the best studied of the nonmitogenic effects of FGF-2 is its regulation of the proteolytic enzyme plasminogen activator (PA). The ability to regulate PA levels is thought to be central to the role of FGF-2 as an angiogenesis factor (3). Increased PA activity contributes to the ability of angiogenic endothelial cells to invade and grow into surrounding tissues. Addition of FGF-2 to cultured endothelial cells results in a dramatic increase in production of urokinase-type PA (uPA), uPA receptors, and collagenases (4–7). uPA catalyzes the conversion of the ubiquitous, plasma-derived zymogen plasminogen to active plasmin, which, in turn, converts the procollagenases to active collagenases. Thus, FGF-2 action results in the generation of a constellation of proteolytic activities that are active against most components of basement membranes and the extracellular matrix. This increased proteolytic activity of endothelial cells is correlated with an increased ability to invade basement membranes, and the invasive properties of endothelial cells can be inhibited with specific inhibitors of these enzymes (8).

The signaling pathway used in the induction of uPA by FGF-2 is unknown. Four related tyrosine kinase receptors for FGFs have been identified. At least three of these receptors can occur in multiple alternatively spliced forms (9). The IIIc isoforms of FGF receptor-1 and FGF receptor-2 are high affinity receptors for FGF-2, but FGF-2 can also bind with lower affinity to other members of the family. Tyrosine kinase activity is stimulated upon binding of FGF-2 to the extracellular domain of these receptors, leading to autophosphorylation on tyrosine residues (10), as well as tyrosine phosphorylation of other cytoplasmic target-proteins (11). One of the downstream signaling pathways from the activated receptor involves binding of phospholipase Cγ (PLCγ) to a phosphorylated tyrosine at residue 766 in the receptor (10, 12). The bound PLCγ is phosphorylated and activated. In addition to the tyrosine kinase receptors, FGF-2 also binds to a cysteine-rich receptor which has no tyrosine kinase domain (13). The role of this molecule in signal transduction is not clear. Finally FGF-2 binds with lower affinity to heparan sulfate proteoglycans on the cell surface (14). The heparan sulfates can modify the interaction of FGF-2 with the tyrosine kinase receptor. In addition, FGF-2 can be internalized directly through its interaction with heparan sulfates (15–18).

Recent investigations have suggested that some responses to FGF-2 can occur in the absence of tyrosine kinase receptors (19, 20). In addition, some site-directed mutations in FGF-2 that have no affect on its mitogenic properties abolish its ability to induce PA, suggesting that an additional signal beyond that necessary for mitogenesis is required for induction of PA (21). We have investigated whether induction of PA requires a tyrosine kinase FGF receptor and whether both mitogenesis and induction of PA can be conferred by transfection of a single receptor, the IIIc isoform of FGF receptor-1, into L1 myoblast cells, which are devoid of endogenous FGF receptors.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human FGF-2 was a gift from Synergen, Inc. (Boulder, CO). Porcine intestinal mucosa heparin, grade II, was purchased from Sigma. Suramin was obtained from Mobay Chemical Corp. (New York, NY). Na+.пал* was purchased from DuPont NEN. A BCA protein assay kit was purchased from Pierce.

Cell Cultures and Transfections—L6 myoblasts cells transfected with the cDNA encoding the human three immunoglobulin-like domain form of FGF receptor-1 (WT-7 and WT-4 clones) and several mutated forms of FGF receptors (Y766F, Y766F/Y776F, K514A, CD58, and Y653F/Y664F) were described previously (22–25). The L6 parental myoblast

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§ The abbreviations used are: FGF, fibroblast growth factor; PA, plasminogen activator; uPA, urokinase-type PA; tPA, tissue-type PA; PLCγ, phospholipase Cγ; PBS, phosphate-buffered saline; MAP, microtubule-associated protein; PDGF, platelet-derived growth factor.

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cells demonstrated levels of high affinity binding that were only barely detectable above background and were less than 5% of the high affinity binding present in the transfected cells. The transfected clones used in this study express approximately 20,000 to 50,000 receptors/cell as determined by analysis of their ability to bind 125I-FGF-2. Cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

125I-Labeled FGF-2—Recombinant human FGF-2 was labeled with 125I using Iodogen (Pierce) as described previously (14). The radiola- beled FGF-2 was separated from unincorporated 125I by chromatogra- phy on Sephadex G 25 (Pharmacia Biotech Inc.), using an elution buffer containing 150 mM NaCl, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin. The specific activity of various preparations ranged between 3 \times 10^4 and 1 \times 10^5 cpm/μg.

Binding Assays—Studies of the binding of FGF-2 to L6 myoblast cells were performed as described previously (14, 26). Briefly, cells were washed twice with cold PBS, then incubated in serum-free medium containing 0.15% gelatin, 25 mM HEPES, pH 7.4, and 10 ng/ml 125I-FGF-2 and other ingredients (unlabeled FGF-2, heparin, and suramin) for 2 h at 4 °C on a shaker. At the end of the incubation time, cells were washed twice with PBS, and 125I-FGF-2 bound to low affinity binding sites was released with two washes with 2 mM NaCl in 20 mM HEPES, pH 7.4. 125I-FGF-2 bound to the receptor was released with two washes with 2 mM NaCl in 20 mM sodium acetate, pH 4.0.

FGF-2 Internalization—Myoblast cells were plated at 500,000 cells/35-mm dish in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum with or without 30 mM chlorate. After 24 h, cells were washed twice with cold PBS and incubated in fresh medium containing 0.15% gelatin and 10 ng/ml 125I-FGF-2 with or without 10 μg/ml heparin. Heparin was added to a final concentration of 30 μg/ml to the medium of the cultures that had been preincubated in chlorate. After 0.5, 1, or 2 h at 37 °C, the cells were washed twice with 2 M NaCl in 20 mM sodium acetate, pH 4.0, to remove FGF-2 bound to heparan sulfates and receptors on the cell surface (26) and then twice with PBS. Cells were extracted in 0.5% Triton X-100, and the amount of radioactivity in the extract was determined. To control for FGF-2 nonspecifically associated with the cell layer, parallel cultures incubated at 4 °C were washed and extracted at the same time points. The amount of radioactivity associ- ated with cells incubated at 4 °C was subtracted from the amount of radioactivity associated with cells incubated at 37 °C.

Plasminogen Activator Assay—Myoblast cells were plated in 24-well plates at 50,000 cells/well. After 24 h, cells were washed twice with PBS and incubated in fresh medium containing 0.5% calf serum for 12 h. The medium was replaced with medium containing 0.5% serum and varying concentrations of FGF-2 with or without other additions (heparin, suramin, and sodium acetate). After incubation in this medium at 37 °C for 12 h, the cells were washed twice in PBS and extracted with 0.5 ml of 0.5% Triton X-100 in PBS. Aliquots of the samples were assayed on fibrin plates as described previously (27). Data are expressed as milliPloug units/μg of protein.

Zymography—Myoblast cell lysates were prepared by extracting cells with 0.5% Triton X-100 in 0.1 M Tris, pH 8.1. The protein concentra- tion in the lysate was determined with the BCA protein assay reagent. Equal amounts of protein from each lysate were run in an SDS-8% polyacrylamide gel under nonreducing conditions. The gel was soaked in 2.5% Triton X-100 for 1 h to remove the SDS. The polyacryla- mide gel was laid on top of a freshly made milk/agar indicator gel and incubated at 37 °C in a humid atmosphere. The milk/agar indicator gel consisted of 2% (w/v) non-fat dry milk, 2.5% agarose, and 40 μg/ml purified human plasminogen in PBS. Periodically, the milk/agar gel was examined for lysis zones. When lysis zones were observed, the gel was photographed using Polaroid 55 film.

RESULTS

FGF-2 Induces PA Activity Only in Myoblast Cells Expressing FGF Receptor-1—The ability of FGF receptor-1 to mediate induction of PA in response to FGF-2 was evaluated in L6 myoblast cells. In these cells, PA activity is not detectable in response to FGF-2. After transfection with a cDNA encoding the isoform of human FGF receptor-1 with three immunoglobulin-like domains, clones of L6 cells expressing 20,000 to 50,000 high affinity FGF receptors per cell were selected (22). Nontransfected L6 myoblast cells expressing FGF receptor-1 were in- cubated at 37 °C with varying concentrations of FGF-2, and cell extracts were assayed for PA activity after 12 h. FGF-2 increased PA activity in L6 cells expressing FGF receptor-1 in a dose-dependent manner reaching a plateau with 30 ng/ml FGF-2 (Fig. 1). At maximal stimulation, PA activity was increased 7-fold over the basal level of the enzyme. Half-maximal stimulation was obtained with approximately 5 ng/ml FGF-2, similar to the concentration required to stimulate PA in endothelial cells (4). A similar increase in PA activity was measured in the conditioned medium of the cells (data not shown). In contrast, PA activity remained unchanged in nontransfected L6 cells regardless of the FGF-2 concentration. These results show that FGF-2 induces PA production only in L6 cells expressing the FGF receptor.

The PA Induced by FGF-2 in L6 Cells Expressing FGF Receptor-1 Is uPA—Since both uPA and tissue-type PA (tPA) can convert plasminogen to plasmin, the activity measured in our assay might represent either enzyme. To examine whether the FGF-mediated changes in PA activity are due to uPA or tPA, we performed zymographic assays that separate PAs on the basis of their molecular weights. Zymography of cell extracts of nontransfected L6 cells and L6 cells expressing FGF receptor-1 showed a single zone of lysis corresponding to an M, of 50,000, which is approximately the M, of murine uPA (Fig. 2). Exposure of the cells to 10 ng/ml FGF-2 increased the zone of lysis in L6 cells expressing FGF receptor-1 but not in the nontrans- fected L6 cells (Fig. 2). These results show that FGF-2 regulates uPA activity in L6 myoblasts through an interaction with the FGF receptor.

Induction of PA Activity by FGF-2 Is Not Affected by the Presence of Soluble Heparin or by Treatment of the Cells with Chlorate—FGF-2 can be internalized by cells through both receptor-mediated and heparan sulfate-mediated processes (15–18). The FGF-2 internalized through heparan sulfate-me- diated processes has been proposed to have a role in some responses to FGF-2 (18, 19). To verify that these two internalization processes take place in L6 cells, L6 cells expressing FGF receptor-1 and nontransfected L6 cells were incubated with 10 ng/ml FGF-2 in the presence or absence of 30 mM chlorate, which inhibits the sulfation of heparan sulfates (28) and abol-
chlorate treatment of cells expressing FGF receptor-1 had no effect on basal level of PA activity in these cells (Fig. 4). In addition, chlorate treatment of nontransfected cells did not affect the induction of PA activity by FGF-2 (Fig. 3B). The combination of chlorate treatment and addition of heparin decreased the amount of FGF internalized by about 20% after 2 h. Thus, internalization of FGF-2 by nontransfected L6 cells is inhibited by soluble heparin and chlorate treatment, whereas internalization of FGF-2 by L6 cells expressing FGF receptors is insensitive to these treatments.

To determine if FGF-2 internalized through heparan sulfates has a role in the induction of uPA, the ability of FGF-2 to stimulate uPA production in the presence of soluble heparin was assessed. As shown above, 10 μg/ml soluble heparin blocks internalization of FGF-2 through the heparan sulfate-mediated mechanism but does not block binding to the receptor. L6 cells expressing FGF receptor-1 and nontransfected L6 cells were incubated with 10 ng/ml FGF-2 in the presence or absence of 10 μg/ml heparin, and PA activity was measured 12 h later. In nontransfected cells, addition of FGF-2 had no effect on PA activity either in the absence or presence of heparin. In contrast, FGF-2 increased PA activity 3.5-fold over control values in cells expressing FGF receptor-1 (Fig. 4). Addition of soluble heparin had no effect either on basal PA levels or on the induction of PA by FGF-2. Thus, heparan sulfate-mediated internalization of FGF-2 is not involved in the induction of uPA.

To determine if cell-associated heparan sulfates are involved in the induction of PA activity by FGF-2, chlorate was used to inhibit sulfation of the heparan sulfates. As shown above, treatment of cells with chlorate inhibited internalization of FGF-2 through the heparan sulfate-mediated pathway. However, chlorate treatment of nontransfected cells did not affect the basal level of PA activity in these cells (Fig. 4). In addition, chlorate treatment of cells expressing FGF receptor-1 had no effect either on basal PA levels or on the induction of PA by FGF-2 in these cells (Fig. 4). Addition of soluble heparin also had no effect on basal PA levels or on the induction of PA by...
FGF-2 in the chlorate-treated cells (Fig. 4). Thus, cell-associated heparan sulfates do not appear to be involved in the induction of uPA.

Induction of PA Is Inhibited by Suramin—The polysulfonated polyaromatic compound suramin interferes with the interactions of a number of ligands, including FGF-2, with specific receptors (30, 31). The ability of suramin to inhibit induction of PA by FGF-2 was examined. Preliminary experiments were performed to evaluate the effect of suramin on the binding of FGF-2 to L6 cells expressing FGF receptor-1. When these cells were incubated with 10 ng/ml 125I-FGF-2 and varying suramin concentrations, binding of 125I-FGF-2 to both heparan sulfates and receptors on the cells was inhibited (Fig. 5A). Binding of 125I-FGF-2 to heparan sulfates was more sensitive to suramin than binding to receptors. Half-maximal competition for binding to heparan sulfates was obtained with 20 μM suramin, whereas half-maximal inhibition of binding to receptors was obtained with 300 μM. Complete inhibition of 125I-FGF-2 binding to both types of binding sites occurred with 1 mM suramin.

Cells incubated for 12 h at 37 °C with 10 ng/ml FGF-2 and varying concentrations of suramin were assayed for PA activity. With suramin concentrations lower than 20 μM, induction of PA by FGF-2 was unaffected (Fig. 5B). Induction of PA by FGF-2 was inhibited with 1 mM suramin, a concentration that inhibited binding to both heparan sulfates and receptors. At intermediate concentrations, 30 and 300 μM, induction of PA by FGF-2 was slightly increased. Thus, concentrations of suramin that blocked binding to heparan sulfates but still permitted binding to receptors did not inhibit FGF-2 stimulation of PA activity. These results demonstrate that suramin is able to inhibit the PA-inducing capacity of FGF-2 only at concentrations that were effective in preventing binding to its tyrosine kinase receptor.

PA Induction Is Independent of PLCγ Activation—The ability of mutated FGF receptors to stimulate PA activity in response to FGF-2 was evaluated. L6 cells expressing a receptor in which the tyrosine kinase activity was eliminated by substitution of an alanine for a lysine at position 514 in the kinase domain (K514A) did not produce increased PA in response to FGF-2 (Fig. 6). Similarly, no alteration of PA production in response to FGF-2 was observed in L6 cells expressing FGF receptor-1-containing mutations in two tyrosine residues in the kinase domain (Y653F/Y654F). Phosphorylation of these two tyrosine residues has been shown to be essential for kinase activity of the receptor (25). Together, these results confirm that kinase signaling through the receptor is required for PA induction. In contrast, a mutant in which tyrosine 766, the major autophosphorylation site and the binding site of PLCγ (10), was replaced with a phenylalanine responded to FGF-2 with increased PA production (Fig. 6). Similarly, a mutant in which both tyrosine 766 and neighboring tyrosine 776 were replaced by phenylalanines also responded to FGF-2 with increased PA production. Moreover, deletion of the C-terminal 58 amino acids of the receptor containing these two tyrosines did not prevent FGF-2 induction of PA activity. These three latter mutations have been shown previously to abolish activation of PLCγ and phosphoinositide hydrolysis in these cells, but to mediate mitogenic signaling (22, 23). All L6 cell clones expressing mutated receptors produced less PA in response to FGF-2 than the clone expressing wild-type receptors. This difference in response may be related to the numbers of receptors expressed by these cells.
A second clone of L6 cells expressing fewer wild-type receptors responded to FGF-2 to the same extent as the clones expressing mutant receptors (data not shown). These results suggest that 1) induction of PA by FGF-2 requires a functional FGF receptor capable of autophosphorylation, 2) PLCγ activation and phosphatidylinositol hydrolysis are not essential for PA induction, and 3) tyrosines 776 and 766 and the 58 amino acids at the carboxyl terminus of the receptor containing these two tyrosines are not involved in PA induction.

**DISCUSSION**

The results presented here demonstrate that an active tyrosine kinase FGF receptor is required for induction of uPA activity in response to FGF-2. A similar finding was recently reported by Rusnati et al. (32). Apparently internalization of FGF-2 through its interaction with heparan sulfates is not involved in this response as 1) heparan concentrations that block internalization of FGF-2 through the heparan sulfate-mediated pathway did not inhibit the induction of uPA by FGF-2 in cells expressing FGF receptors, 2) inhibition of heparan sulfate-mediated internalization by chlorate treatment had no effect on induction of uPA by FGF-2, and 3) suramin, at concentrations that completely block binding to heparan sulfates, did not block induction of PA by FGF-2. Furthermore, it has previously been shown using these same cells that introduction of FGF receptor-1 established a mitogenic response to FGF-1 (22). We have confirmed that DNA synthesis is also stimulated by FGF-2 in L6 cells expressing FGF receptor-1 (data not shown). Together, these results demonstrate that both a mitogenic response and induction of PA can be mediated by a single isoform of FGF receptor-1. Differences in sensitivity of these two responses to various reagents (32) may be due to differences in post-receptor pathways of signal transduction.

In the experiments presented here, treatment of L6 cells with chlorate inhibited binding of FGF-2 to heparan sulfates (data not shown) and inhibited FGF-2 internalization through the heparan sulfate-mediated pathway, but had no effect on the induction of uPA by FGF-2 in cells expressing FGF receptor-1. This result is consistent with our previous finding that heparan sulfates or heparin are not required for the binding of FGF-2 to its receptor (33) and with the recent report that heparan sulfates are not required for FGF-2 activity in Balb/c 3T3 cells (34). Although heparin or heparan sulfates are not required for binding of FGF-2 to its receptor, they alter the affinity of the FGF-2-receptor interaction so that FGF-2 binds to its receptor at lower concentrations in the presence of heparin than in the absence of heparin (33). The amount of FGF-2 used in the experiments presented here was sufficient to saturate FGF receptor-1 both in the presence and absence of heparin (33). Thus, under these conditions, the absence of heparan sulfates should have no effect on FGF-2 interactions with FGF receptor-1.

PA can be induced in endothelial cells by both FGF-2 and phorbol esters (4, 27). Both FGF-2 and phorbol esters increase PA levels to the same extent at their maximum effective concentrations, and their effects are not additive. Addition of FGF-2 to suboptimal concentrations of phorbol ester can completely eliminate without adverse effect on the induction of PA by FGF-2, suggesting that the Ras pathway is the predominant signaling pathway used in FGF-2 induction of uPA. It is interesting to note that almost the entire C terminus of FGF receptor-1 beyond the tyrosine kinase domain can be eliminated without adverse effect on the induction of PA by FGF-2. This finding demonstrates that the signaling molecules required for induction of uPA interact with sequences in the juxtamembrane region, the kinase insert, or the kinase domain itself. Additional tyrosines in these regions that are phospho-acylated upon activation of FGF receptor-1 have recently been identified (25). FGF activation of its receptor has been shown to activate the grb-2/sos, Ras, Raf-1, MAP kinase pathway. However, the signaling molecules other than PLCγ that interact directly with FGF receptor-1 have not yet been identified. Further characterization of the signaling elements required for uPA induction must await the identification of the molecules that directly interact with the FGF receptor.

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