Embryoglycan ectodomains regulate biological activity of FGF-2 to embryonic stem cells

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Accepted 20 July; published on WWW 9 September 1998

SUMMARY

Basic fibroblast growth factor (FGF-2) functions as a natural inducer of mesoderm, regulator of cell differentiation and autocrine modulator of cell growth and transformation. The FGF-2 signals are transduced through receptors with intrinsic protein tyrosine kinase activity. However, receptor binding and activation is governed by extracellular matrix, cell surface or soluble proteoglycans. This paper focuses on the role of proteoglycans synthesized by embryonic cells, embryoglycans, in FGF-2 signaling via FGF receptor-1 (FGFR-1).

We found that embryoglycan ectodomain Lewis X, analog of developmentally regulated embryonic cell surface epitope TEC 1, promotes oligomerization of FGF-2 in the cell free chemical crosslinking. In vitro assays show that a large molar excess of extracellular Lewis X does not inhibit binding of FGF-2 to embryonic stem (ES) cells, but prevents the mitogenic effect of FGF-2. Western blot analysis of ES cells revealed the presence of abundant 52 kDa and trace amounts of 67 and 125 kDa isoforms of FGFR-1. However, none of these isoforms undergo any detectable changes in tyrosine phosphorylation under the conditions that modulate the mitogenic effect of FGF-2. Rather, a primary substrate of all receptor tyrosine kinases, phospholipase Cγ (PLCγ), is activated by both FGF-2 and Lewis X. The combination, FGF-2 plus Lewis X, leads to weak inhibition, when compared with the effects of FGF-2 and Lewis X, respectively. In accordance, the level of phosphorylation of non-receptor tyrosine kinase c-Src is reduced in a reversed pattern to PLCγ. Furthermore, in this particular cell type we show the presence of activated forms of extracellular signal-related kinase (ERK) in all nontreated and treated cells.

These findings demonstrate that embryoglycan ectodomains may act as negative regulators of FGF-2-induced ES cell proliferation, most likely through the FGFR-1-independent signaling pathway.

Key words: Embryoglycan, Lewis X, FGF-2, FGFR-1, Mouse ES cell

INTRODUCTION

FGF-2 can bind to two different classes of receptors, with high affinity to transmembrane signaling receptors endowed with tyrosine kinase activities and with much lower affinity to cell surface or extracellular matrix heparan sulfate proteoglycans. Both classes of receptors cooperate to generate a cellular response (Klagsbrun and Baird, 1991).

To date, four known members of the signaling FGFR family have a related structure that is comprised of three extracellular ligand-binding domains, a single transmembrane region and a cytoplasmic tyrosine kinase domain with a short carboxy-terminal tail (Green et al., 1996). Activation of receptor tyrosine kinases results in binding to intracellular substrates containing Src homology 2 domains such as PLCγ. In the following signaling pathway, activated FGFR interacts with several other substrates including ERK 1, ERK 2 and c-Src (Kanai et al., 1997).

Proteoglycans, including their embryo-specific form embryoglycans (mostly polylactosaminoglycans), are enormously complex macromolecules that each contain a core protein with covalently bound glycosaminoglycans (GAG) side chains (Wight et al., 1991). GAGs are linear disaccharide repeats, either highly sulfated, acidic and negatively charged or less sulfated or nonsulfated and rather neutral. This has been shown particularly for high-molecular mass embryoglycans (Kimber et al., 1993). Embryoglycans carry a number of developmentally regulated carbohydrate epitopes strongly expressed and shared in the embryonic ectoderm of early embryos, embryonic stem cells and embryonal carcinoma (EC) cells (Muramatsu, 1984, 1992). One of them, trisaccharide Gal(β1→4)-[Fuc(α1→3)]GlcNAc (Lewis X, TEC 1), has been determined to mediate cell adhesion in preimplantation embryos and aggregation of EC cells (Kojima et al., 1994; Boubelík et al., 1996). This function stems from the proposed cell-cell interaction between the galactosyltransferase and N-acetylglucosamine residues involved in polylactosaminoglycans (Muramatsu, 1992). A similar mechanism can also provide an explanation for the developmental downregulation of Lewis X expression, since it
was shown that the transcriptional rate for the α1,3-galactosyltransferase gene is increased in mouse teratocarcinoma F9 cells treated with the differentiation agent all-trans-retinoic acid and this elevated activity causes masking of Lewis X epitopes by terminal α-galactosyl residues (Cho et al., 1996). We have recently proposed that embryonic Lewis X acts as recognition molecules for FGF-2 and plays an active role in the formation of ligand-receptor complexes (Dvořák et al., 1997).

It has been shown that the key event in transmembrane signaling by receptors with tyrosine kinase activity, including FGFs, is ligand-induced dimerization (Heldin, 1995). First, FGF-2 can interact with either free or cell surface- and extracellular matrix-bound proteoglycans. Then, growth factor molecules are oligomerized through sugar residues and this event facilitates the binding of complexes by FGFs and subsequent receptor dimerization and activation (Yayon et al., 1991; Ornitz et al., 1992, 1995). Although heparin as well as heparan sulfate proteoglycan is highly sulfated, small nonsulfated oligosaccharides can also activate the FGF signaling pathway (Ornitz et al., 1995). The recent model suggests that by binding of FGF to the abundant membrane proteoglycans and their free oligosaccharide fragments, complexes will exhibit rapid lateral mobility and this effect of ‘reduced dimensionality’ for FGF molecules will allow more frequent encounters with the main signaling receptors (Schlessinger et al., 1995). Backwards, it is possible that a large molar excess of free oligosaccharides will occupy all binding sites on the growth factor molecule and this can restrict the effect of FGF in receptor dimerization and transmembrane signaling, as it was suggested for syndecan-1 ectodomain (Mali et al., 1993).

We report here that embryoglycan ectodomain can serve as a regulator of the effect of FGF-2 on embryonic cells. The elevated expression and shedding of specific oligosaccharide structures during early stages of mouse development and in certain types of embryonic cells may, in fact, inhibit the effect of FGF-2. We speculate that such specific membrane interactions modulating the downstream signaling may have time and space restricted functional significance to maintain the nondifferentiated state of ES cells.

MATERIALS AND METHODS

Cells and reagents

The germline-transmissible C3H/He-derived ES cell line (established in the Division of Experimental Animal Research, RIKEN, Tsukuba, Japan) was used for all experiments. Recombinant human FGF-2 was obtained from Sigma (St Louis, MO) and biologically active bovine FGF-2 was purchased from Boehringer (Mannheim, FRG). Heparin (Mr 16,000) and heparan sulfate (Mr 7,5,000) were from Sigma. Trisaccharide Lewis X was obtained from Oxford GlycoSystems (Abingdon, UK).

Chemical crosslinking of FGF-2

FGF-2 (375 nM) was mixed with either 78 nM to 20 μM of Lewis X, 78 nM to 10 μM of heparan sulfate or 78 nM to 5 μM of heparin. All reactions were carried out in a volume of 40 μl at room temperature for 40 minutes. After incubation, the covalent crosslinker disuccinimidyl suberate (DSS; Pierce, Rockford, IL) was added to achieve a final concentration of 0.19 mM. This reaction mixture was incubated for 20 minutes at room temperature and then quenched by 50 mM Tris-HCl, pH 7.5, and 100 mM glycine for the next 15 minutes. The crosslinked products were mixed 1:1 with 2× Laemmli sample buffer, electrophoresed on an 8-16% SDS-PAGE gel and immunoblotted with mouse monoclonal anti-FGF-2 antibody (Sigma). This was followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated antibody (Sigma). The FGF-2 bands were visualized by the chemiluminescence detection system (ECL; Amersham, UK) and quantified by scanning densitometry.

Receptor binding assays and competition experiments

ES cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/BRL, Gaithersburg, MD) containing 20% fetal calf serum, 2 mM glutamine, 0.1 mM non-essential amino acids, 0.05 mM mercaptoethanol, 100 i.u./ml penicillin, 0.1 mg/ml streptomycin and 1×10^5 units/ml leukemia inhibitory factor (LIF). Mitomycin C-treated mouse embryonic fibroblasts were used as a feeder layer. For the receptor binding assay, cells were seeded on gelatin-coated glass coverslips in 4-well dishes (Nunc; Roskilde, Denmark) in medium without LIF and feeder layer and cultured for 4 days. The cells were then rinsed and incubated for 10 minutes at room temperature in DMEM, 15 mM Hepes and 0.5% BSA (DMEM/H/B) containing 0.09 to 3 mM (0.05 to 1.6 μg/ml) Lewis X, 0.07 to 0.53 mM (0.5 to 4 μg/ml) heparan sulfate, or 0.06 to 0.5 μM (1 to 8 μg/ml) heparin. Control cells were maintained in DMEM/H/B. Biotinylated FGF-2 was then added to each well at a final concentration of 17 nM (0.3 μg/ml) and cells were incubated for 2 hours on ice. Cells were then rinsed 3 times with ice-cold DMEM/H/B, fixed in ethanol/acetic acid (1:19; 95% EtOH, 1% acetic acid) for 30 minutes on ice, slowly rehydrated and quenched with 5% goat serum (NGS) and 1% BSA in phosphate buffered saline, pH 7.2 (PBS; Ca^{2+}-Mg^{2+}-free) for 1 hour at room temperature. This was followed by an overnight incubation at 4°C with mouse monoclonal anti-biotin IgG (Sigma) diluted in PBS/NGS/BSA. A control without primary antibody was also included. After washing with PBS containing 0.05% Tween-20, secondary goat anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (Sigma) was applied for 2 hours at room temperature. Then cells were counterstained with propidium iodide (10 μl/ml; Sigma), repeatedly rinsed and mounted in Mowiol with n-propyl gallate.

Fluorescence signal analysis was performed using the Nikon Diaphot 300 microscope equipped with Argus-50 image processor and color CCD camera C 2400 (Hamamatsu Photonics).

Proliferation assay

ES cells were seeded at an initial density of 2,000 cells/well in 96-well tissue culture plates without feeder layer and were serum-starved (DMEM supplemented with 5% fetal calf serum) for 24 hours. Then cells were washed with PBS and grown for the next 24, 48 and 72 hours in DMEM/5% serum containing various combinations of FGF-2 and oligosaccharides to be tested. The low serum media supplemented with appropriate growth factor and oligosaccharide were changed daily. Following each period of cultivation, 10% of cell well tissue culture plates were serum-starved in DMEM/5% serum and harvested into Laemmli sample buffer. Samples were subjected to 10% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham) and blotted with polyclonal anti-FGFR-1 antibody directed against the carboxy terminus of the receptor (anti-Flg; Santa Cruz Biotechnology, CA). For comparison, ES cells grown without feeder layer and LIF for 8 days and EC cells (P19) were subjected to the same analysis.

For metabolic labeling, ES cells were cultured without feeder layer and LIF for 4 days and then incubated with [35S]methionine (300 μCi/ml) for 4 hours. After washing, the cells were solubilized in cold lysis buffer (50 mM Hepes-NaOH pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA) and metabolic labeling of cells

ES cells were cultured as for receptor binding assays. Then cells were washed with PBS and harvested into Laemmli sample buffer. Samples were subjected to 10% SDS-PAGE gel and immunoblotted with mouse monoclonal anti-FGF-2 antibody (Sigma). This was followed by incubation with anti-mouse IgG horseradish peroxidase-conjugated antibody (Sigma). The FGF-2 bands were visualized by the chemiluminescence detection system (ECL; Amersham, UK) and quantified by scanning densitometry.
further characterize the biological significance of Lewis X in repeating ectodomain of large embryoglycans. In order to oligosaccharide Lewis X was chosen as the representative process we performed chemical crosslinking studies in which tyrosine kinase activity was required for transphosphorylation of all receptors with intrinsic common mechanism of ligand-induced oligomerization is transmembrane signaling receptors with higher activity than simultaneously to several FGF-2 molecules that associate to Lewis X induces oligomerization of FGF-2 molecules.

**RESULTS**

**Protein tyrosine phosphorylation**

For the detection of tyrosine phosphorylation of the FGFR-1, ES cells were grown without feeder layer and LIF for 3 days. Then, subconfluent cell cultures were serum-starved for an additional 18 hours and stimulated with FGF-2 or FGF-2 plus Lewis X for 5 and 10 minutes at 37°C. Cells were then washed twice with PBS and solubilized in cold lysis buffer as described above. Cell lysates were centrifuged, protein amounts were equalized, precleared with Protein G beads and immunoprecipitated with polyclonal anti-FGFR-1 antibody directed against the carboxy terminus of the receptor (anti-Flg; Santa Cruz Biotechnology, CA) for 2 hours at 4°C. Then, the immune complexes were collected by Protein G agarose beads (Sigma), washed with cold lysis buffer and mixed 1:1 with 2x Laemmli sample buffer. Proteins were then resolved by 10% SDS-PAGE and immunoblotted with biotinylated mouse anti-phosphotyrosine antibody (Sigma, clone PT-66). Phosphotyrosine detection was done using horseradish peroxidase streptavidin (Vector Laboratories, Burlinghame, CA) and visualized by enhanced chemiluminescence (ECL Plus, Amersham).

In order to compare the level of immunoprecipitated FGFR-1 in differently treated ES cells, the Immobilon P membrane (Millipore, Bedford, MA) was stripped in 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM mercaptoethanol (Spivak-Kroizman et al., 1994), washed and reimmunoblotted with anti-FGFR-1 antibody.

For the detection of tyrosine phosphorylation of PLCγ and c-Src, ES cells were subjected to various stimulations, lysed, and clarified lyses were immunoprecipitated with anti-phosphotyrosine antibody (Santa Cruz Biotechnology, clone PY-20), resolved by 10% SDS-PAGE, transferred to membrane and probed with anti-PLCγ or anti-c-Src antibodies (Santa Cruz Biotechnology).

For the examination of MAP kinases activation, treated cells were directly harvested into Laemmli sample buffer, separated by 10.5% shift SDS-PAGE, transferred to membrane and blotted with anti-ERK antibody (Santa Cruz Biotechnology). The activation/phosphorylation state of ERK 1 and ERK 2 was assessed by examining the electrophoretic mobility shifts and compared to the mobility shift of mouse oocytes during meiotic maturation (Verlhac et al., 1994).

**Lewis X does not compete with FGF-2 for binding to receptor**

Based upon the structural requirements essential for the formation of ternary complexes composed of FGR, FGF-2, and surface proteoglycan, we have designed a competition experiment with free Lewis X, heparin or heparan sulfate and biosynthetically labeled FGF-2 using ES cells. For this purpose, control ES cells were maintained 3 and 4 days in culture without feeder layer and LIF and then probed by immunofluorescence for the expression of dominant FGF-2-binding receptors, FGFR-1 and FGFR-2. FGFR-1 was highly expressed whereas FGFR-2 was not detectable (data not shown). Our prediction was that the presence of free oligosaccharides at a large molar excess over FGF-2 could antagonize the binding of growth factor to cognate receptor either by steric inhibition or by saturating all binding sites on FGF-2 molecules that make FGF-2 unavailable for further interaction with surface embryoglycans and tyrosine kinase receptors.

Heparan sulfate and heparin inhibited the binding of FGF-2 to surface receptors (Fig. 2C and D) in a dose-dependent fashion (Table 1), with exogenous heparin having significantly stronger inhibitory activity than heparan sulfate. Surprisingly, as demonstrated in Fig. 2A and B, trisaccharide Lewis X has no effect on FGF-2 binding to surface receptor. Thus, it is apparent that the ability of free oligosaccharides to inhibit the binding of FGF-2 to ES cells correlates with size.

**Extracellular Lewis X causes inhibition of FGF-2-induced ES cell proliferation**

We show here that Lewis X oligomerizes FGF-2 while having no effect on FGF-2 binding to ES cells. Therefore, we also performed a proliferation experiment in which both Lewis X and FGF-2 were added to culture medium at different concentrations.

As demonstrated in Fig. 3, a clear mitogenic response to 10 ng/ml FGF-2 was observed. Lewis X alone at a concentration of 10 ng/ml had no effect on ES cell proliferation, while a higher concentration, 100 ng/ml, induced the mitogenic response at 72 hours of the treatment. Interestingly, the combination of FGF-
2 and Lewis X at two different concentration ratios produced a strong inhibition of mitogenic response.

Thus, although extracellular Lewis X at concentrations up to 1.6 \( \mu \text{g/ml} \) does not inhibit the binding of FGF-2 to receptors, subsequent mitogenic response is already blocked at much lower concentrations of about 10 ng/ml. In addition, the mitogenic effect of Lewis X alone at a concentration of 100 ng/ml suggests that some interactions, other than binding to FGF-2, may contribute to the biological activity of embryoglycan-derived oligosaccharides.

**FGF-2/Lewis X fail to induce autophosphorylation of FGFR-1 but activate PLC\(\gamma\) and reduce phosphorylation of c-Src**

A central question raised by proliferation experiments is whether the mitogenic response of ES cells to FGF-2 and inhibition of this response by extracellular Lewis X correlates with the alteration of FGFR signaling pathway. Therefore we compared the tyrosine phosphorylation levels of FGFR-1 and several intracellular substrates in ES cells treated as in the proliferation experiment reported above.

First, to gain insight into the complexity of possible phosphorylated FGFR-1 isoforms in C3H/He-derived ES cells we either immunoprecipitated \[^{35}\text{S}\]methionine labeled cell lysates by anti-FGFR-1 antibody (Fig. 4A) or directly subjected cell lysates to western analysis (Fig. 4B). ES cell extracts contained anti-FGFR-1 antibody immunoreactive proteins that migrated with a molecular mass of approximately 52 kDa, 67 kDa, and 125 kDa. An additional 78 kDa band appears in \[^{35}\text{S}\]methionine labeled cell lysates. The 52 kDa isoform is predominant in C3H/He-derived ES cells as revealed by western blotting. We assume that this band represents the truncated form of the 125 kDa receptor. This is not true for EC cells, in which the relative amounts of the high molecular mass isoform is much higher (Fig. 4C).

Immunoprecipitation of FGFR-1 followed by tyrosine phosphorylation analysis showed that all three forms, 52 kDa, 67 kDa and 125 kDa, detected by western blotting are.

**Fig. 1.** FGF-2 crosslinking in the presence of Lewis X (A), heparan sulfate (B), and heparin (C). Dimer (32 kDa) and trimer (50 kDa) band intensities are plotted above each lane. No formation of trimers is seen in control experiments (D). The molar ratios of FGF-2 to Lewis X, heparan sulfate, and heparin are the same in all experiments and are shown in E. M, monomer; D, dimer; T, trimer.
Table 1. Competition experiments*

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<th>Molar concentrations (nM)</th>
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*Competition for FGF receptor binding was carried out in the presence of 17 nM biotinylated FGF-2.
†Data for each group were calculated from total ES cell area of 11.6 to 23 mm² and from two independent experiments.

phosphorylated. However, biotinylated anti-phosphotyrosine antibody failed to detect the 78 kDa band that was revealed by immunoprecipitation in lysates of [35S]methionine labeled cells. Importantly, no significant differences in the ability to elicit tyrosine phosphorylation of FGFR-1 between the differentially treated ES cells were detected. As shown in Fig. 5, each of the four treatments results in a very similar pattern of FGFR-1 tyrosine phosphorylation that is no different from that in control nontreated ES cells.

Further studies were carried out to determine the activation of the potential downstream substrates (Fig. 6). After stimulation, cell lysates were immunoprecipitated with anti-phosphotyrosine antibody and immunoblotted with anti-PLCγ or anti-c-Src antibodies. The treatment with FGF-2 resulted in phosphorylation of PLCγ. Moreover, Lewis X stimulates PLCγ in the absence of FGF-2, while the combination of FGF-2 and Lewis X has a weak inhibitory effect. In contrast, non-receptor tyrosine kinase c-Src shows a reversed pattern of phosphorylation. c-Src in ES cells is constitutively phosphorylated and the level of phosphorylation is strongly reduced after FGF-2 stimulation. Similarly, Lewis X (100 ng/ml) causes the reduction in phosphorylation of c-Src. We also tried to determine the activation of MAP kinase isoforms ERK 1 and ERK 2. To address this point, the extracts from ES cells treated with FGF-2/Lewis X were immunoblotted with anti-ERK 1/2 antibody. Electrophoretic mobility shifts revealed that ERK 1 as well as ERK 2 exist in both forms, nonphosphorylated and phosphorylated, independently on the treatment. The prominent shift in the mobility of MAPKs of nonmatured

Fig. 2. Representative images of control FGF-2/biotin (0.3 μg/ml = 17 nM) binding to ES cells (A), and competition binding of FGF-2 to ES cells in the presence of 1.6 μg/ml (3 μM) Lewis X (B), 2 μg/ml (= 0.27 μM) heparan sulfate (C), and 4 μg/ml (= 0.25 μM) heparin (D), respectively. Bar, 20 μm.

Fig. 3. The effect of FGF-2, Lewis X and the combination of both on the proliferation of C3H/He-derived ES cells. Cells were cultured for 24, 48, and 72 hours in medium containing indicated concentrations of FGF-2 and Lewis X and proliferation was measured by WST-1-based colorimetric assay. The differences between each culture conditions after 72 hours were statistically significant (t-test, P<0.01). The mean values were derived from at least 30 wells and from three separate experiments. Standard error values are indicated.
(germinal vesicle, GV) and matured (metaphase II – M II) mouse oocytes served as a marker.

Thus, while FGFR-1 tyrosine kinase domain-dependent downstream cascade is dominant for many of the cell types, an alternative pathway for FGF-2 signaling may exist in embryonic stem cells. Phosphorylation of PLC\(_g\) and reduced phosphorylation of c-Src in response to FGF-2 strongly suggest that another member of the FGF receptor family is involved in the FGF-2-induced signaling.

**DISCUSSION**

In early embryonic cells, embryoglycans are abundantly present (Kimber et al., 1993) and due to the proteolytic cleavage of the protein core, their saccharide ectodomains could be released from the cell membrane (Ishihara et al., 1993; Schlessinger et al., 1995; Subramanian et al., 1997). Thus, our first concern was whether the embryoglycan-derived oligosaccharides can interact with FGF-2 in a cell free system. Nonsulfated trisaccharide Lewis X (TEC 1 epitope) was used as a soluble low affinity receptor, since it was shown that small di- and trisaccharides also induce FGF-2 self-association (Ornitz et al., 1995; Herr et al., 1997) and native embryoglycan-specific ectodomains are mostly nonsulfated and thus neutral (Kimber et al., 1993). We show here that Lewis X promotes FGF-2 association which leads to the formation of oligomers. In this context, it should be noted that our original FGF-2 samples contained monomeric as well as dimeric species. Thus, the formation of trimers (monomer/dimer or three monomers) is much more frequent than tetramers (dimer/dimer, dimer + two monomers or four monomers) due to the original monomer/dimer ratio in FGF-2 samples. Second, the stability of tetramers is generally lower than trimers due to the size of the complex. However, in the case of Lewis X the absence of tetramers is likely to have resulted from the length of the trisaccharide unit. The fact that Lewis X can inhibit oligomerization of FGF-2 at a larger excess, but in a similar manner as heparin or heparan sulfate suggests its biological role during development.

FGFR-1 and -2 have been reported to play essential roles during early embryogenesis. In ES cells, FGFR-1 mRNA is expressed constitutively, while the expression of FGFR-2 mRNA increases upon differentiation (Mummery et al., 1993; McDonald and Heath, 1994) and their biologically relevant ligand, FGF-2, is implicated in patterning of mesodermal and neural lineages (Smith, 1993; Cornell and Kimelman, 1994). However, very little is known about the action of FGF low affinity receptors on embryonic cells. In addition, in this...
particular cell type the FGF-2-induced signaling pathway has not yet been clarified. Thus, we asked whether free embryoglycan ectodomain, in addition to surface bound embryoglycans, may change the receptor (either low or high affinity) binding of FGF-2, FGF-2-induced mitogenesis, and downstream signaling. There are several lines of evidence for such presumption. It was shown that heparin can act as a growth factor-independent activating ligand for FGFR-4 but not for FGFR-1 (Gao and Goldfarb, 1995). It was also demonstrated that both free heparin and heparan sulfate can reconstitute a low affinity binding of FGF-2 in heparan sulfate-deficient CHO cells (Yayon et al., 1991). However, in Swiss 3T3 fibroblasts treated with chlorate, excess of heparin or heparin fragments competes for high affinity binding and reduces receptor phosphorylation, while mitogenic effects are not diminished (Kruftk et al., 1996). In nontreated adrenocortical endothelial cells, free heparin-derived hexa- and octasaccharides were shown to selectively inhibit the interaction between FGF-2 and surface low affinity receptors and FGF-2-induced mitogenic effect, while larger decasaccharides had an opposite effect (Ishihara et al., 1993, 1994).

In a defined in vitro system utilizing embryonic stem cells, we show that while heparan sulfate and heparin can dramatically alter FGF-2 binding to ES cells, Lewis X is ineffective. Despite this, the binding of Lewis X to exogenous FGF-2 has biological significance, hence Lewis X can efficiently inhibit the mitogenic effect of FGF-2 to ES cells. Our results in ES cells differ from those that report that exogenous heparin inhibits the binding of FGF-2 to low affinity receptors but does not inhibit the FGF-2 binding to high affinity receptors (Ishihara et al., 1993). We believe that these seemingly controversial data may suggest that regulation of two hierarchies of FGF-2 activity, receptor binding and mitogenic signaling, is cell type-specific. It is likely that in the case of heparin and heparan sulfate our observation is related to the hypothesis that increased shedding of proteoglycan ectodomain into culture medium in NIH 3T3 cells overexpressing syndecan-1 could make FGF-2 unavailable also for the high affinity receptors (Mali et al., 1993). Moreover, in our experiments Lewis X at higher concentrations has some mitogenic effect alone, probably by the same mechanism as was reported for heparin and FGFR-4 (Gao and Goldfarb, 1995). Altogether, these experiments demonstrate that there is a fine equilibrium between the size of exogenous, potentially shedded ectodomains and biological features of the cell such as growth factor binding and mitogenic response.

The experiments described above raised the fundamental question of how the receptor tyrosine kinase and the potential downstream substrates are modified when Lewis X: (A) associates with FGF-2, (B) does not compete with binding of FGF-2 to ES cells, and (C) inhibits mitogenic activity of FGF-2. Surprisingly, we were not able to detect any ligand-dependent changes in tyrosine phosphorylation of FGFR-1, with FGFR-1 being significantly phosphorylated regardless of the treatment. Anti-phosphotyrosine western analysis of immunoprecipitates made using anti-FGFR-1 antibody revealed three bands which clearly correspond to those detected by the same anti-FGFR-1 antibody in direct western blots. Since the anti-FGFR-1 antibody recognizes the epitope that maps to the C-terminal amino acids 808-822 (outside the TK domains), we hypothesize that predominant 52 kDa and also less abundant 67 kDa isoforms represent FGFR-1 truncated in extracellular ligand-binding or tyrosine kinase domains. Barely detectable 125 kDa protein probably represents the full length form of FGFR-1 (Safran et al., 1990). Significantly, several active or inactive FGFR-1 isoforms of low molecular mass were described (Hill et al., 1995; Maher, 1995; Tanahashi et al., 1996), some of them associated with the nuclear fraction (Kilkenny and Hill, 1996). The inability to detect the significant differences in phosphorylation of FGFR-1 could be due to either high expression of truncated low molecular mass receptors or FGFR-1-independent FGF-2 signaling in ES cells. The later seems to be true, because we have recently shown that FGFR-1-deficient ES cells (Deng et al., 1994) have normal basal growth and proliferate in response to FGF-2 (unpublished results). This is in clear contrast to the data reported for mouse fibroblasts and human melanoma cells lacking the intracellular kinase domain (Yayon et al., 1997). Also, ES cells resemble rapidly proliferating ectodermal cells of approximately 6 to 8 day old mouse embryos, at the time of mesoderm induction (Lawson et al., 1991) when the ES cells are highly competent to accept a variety of extracellular signals. Thus, the inability to detect possibly minute phosphorylation changes upon the treatment with the free ligand may still be simply due to already elevated phosphorylation caused by other undefined stimulatory signals.

We show that activated forms of extracellular signal-related kinases ERK 1 and ERK 2 are present in proliferating ES cells and they are not influenced by FGF-2. The presence of activated forms of ERKs has been shown in animal hemispheres during the mesoderm induction in Xenopus (Gotoh et al., 1995). Therefore, the activation of ERKs in ES cells may mirror their commitment toward the differentiation into mesoderm. In contrast, PLCγ and c-Src are regulated upon FGF-2/Lewis X in the specific manner that is basically consistent with proliferation response to various combinations of FGF-2 and Lewis X. It is interesting that significant phosphorylation of PLCγ in ES cells correlates with the FGF-2/Lewis X-induced proliferation, since it was reported that in rat myoblasts PLCγ is not required for mitogenic response mediated by FGF (Peters et al., 1992; Shaoul et al., 1995) or mesoderm induction (Muslin et al., 1994). On the other hand, PLCγ serves as a primary substrate of tyrosine kinases of several growth factor receptors (Muslin et al., 1994). c-Src, a cytoplasmic membrane-associated non-receptor tyrosine kinase, has been shown to be differentially regulated in signal transduction mediated by FGFs. For example, in lung capillary endothelial cells and mouse fibroblasts, FGF-2 stimulation leads to increased phosphorylation. In contrast, in porcine aortic endothelial cells and lung fibroblasts from Chinese hamster, treatment with FGF-2 causes reduced phosphorylation (Landgren et al., 1995). Our observation of downregulated c-Src phosphorylation after FGF-2/Lewis X stimulation of ES cells may be relevant to the finding of Kanai et al. (1997) that activated FGFR-3 results in reduced phosphorylation of c-Src.

Together, it therefore appears that an alternative or parallel pathway to FGFR-1-related one is utilized by FGF-2 for regulation of downstream signaling and proliferation of ES cells. Further experiments, specifically those using FGFR-deficient ES cell lines, are needed to clarify this mechanism.

We thank Dr E. M. Thompson and Dr Y. Hirabayashi for critical comments. This work was supported by funds from the Ministry of Education, Youth and Sports of the Czech Republic (VS96115) and from the Grant Agency of the Czech Republic (524/96/K162 and 312/97/0393).
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