A activation of an Enhancer on the Syndecan-1 Gene Is Restricted to Fibroblast Growth Factor Family Members in Mesenchymal Cells

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Fibroblast growth factors (FGFs) induce a variety of biological effects on different cell types. They activate a number of genes, including immediate-early genes, as the transcription factors Fos and Jun, which are also common targets for other tyrosine kinase receptor-activating growth factors. Here we describe a secondary far-upstream enhancer on the syndecan-1 gene that is activated only by members of the FGF family in NIH 3T3 cells, not by other receptor tyrosine kinase-activating growth factors (e.g., epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, or serum). This FGF-inducible response element (FiRE) consists of a 170-bp array of five DNA motifs which bind two FGF-inducible Fos-Jun heterodimers, one inducible AP-2-related protein, a constitutively expressed upstream stimulatory factor, and one constitutive 46-kDa transcription factor. Mutational analysis showed that both AP-1 binding motifs are required, but not sufficient, for FiRE activation. Moreover, agents such as 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, or forskolin, which are known to activate AP-1 complexes and AP-1-driven promoters, fail to activate FiRE. However, FiRE can be activated by the tyrosine kinase phosphatase inhibitor orthovanadate. Taken together, this data implies a differential activation of growth factor-initiated signaling on AP-1-driven regulatory elements.

Fibroblast growth factors (FGFs) are a family of heparin-binding peptides that currently include nine members. FGFs are known to induce the transcription of a number of genes, including transcription factors, components of the cytoskeleton, and ribosomal genes. Basic fibroblast growth factor (FGF-2), the best-characterized member of this family, is synthesized by and acts on various cell types and tissues. For example, in vitro it is a strong mitogen for cells of mesodermal origin, can modulate cell motility and differentiation, and is a potent angiogenic factor. In vivo, it potentiates neovascularization and stimulates proliferation of most of the cell types involved in wound healing, including keratinocytes, fibroblasts, and vascular and capillary endothelial cells (2, 3, 6, 29). It plays a crucial role in fetal development, in which it seems to possess various activities. Several studies have implicated FGFs as the prime candidates for the limb bud apical ectodermal ridge (AER) growth signal. FGF-2 is detectable in chick limb bud, and replacing the AER with FGF-2 restores limb development (13). FGF-2 can also induce additional limb formation in chick embryos, as placing FGF-2 beads in the embryos results in formation of complete ectopic limbs (7). Other members of the FGF family, FGF-4 and FGF-8, have also been implicated in the AER growth signal and have been shown to retain the outgrowth of the limb (10, 25, 36).

FGFs act through a family of transmembrane tyrosine kinase receptors (FGFRs) (23). Heparin or heparan sulfate proteoglycans participate in the regulation of FGF action. Several mechanisms, for both negative and positive regulation for FGF action by proteoglycans, have been postulated (48). An integral membrane heparan sulfate proteoglycan, syndecan-1 (46), can simultaneously bind FGF-2 and extracellular matrix molecules, and this complex is able to promote DNA synthesis in 3T3 cells (44). However, it is known that different heparin sequences can either activate or inhibit FGF-2 function (14) and that the composition and length of the syndecan side chains vary in a cell- and tissue-dependent manner (40, 43, 45). Negative regulation of FGF action by syndecan-1, which might be due to the glycaminoglycan side chain modification or a different stoichiometric ratio of FGF and coreceptor, has also been reported elsewhere (4). FiRE can be activated by the tyrosine kinase phosphatase inhibitor orthovanadate. The expression of syndecan-1 follows morphogenetic rather than histological tissue boundaries (4). It is expressed at the four-cell stage (51), but during later development, it is expressed mainly by epithelia and only transiently by several condensing mesenchymes, including tooth (53), kidney (56), and developing limb mesenchyme (49). FGF-2 is also detected in limb bud mesenchyme (47), similar to FGF-4 (35) and FGF-8 (16). Furthermore, syndecan-1 is colocalized with FGF-3 in developing tooth mesenchyme (60) and with the heparin-binding growth factor-like molecule, midkine, in developing skin (33). Syndecan-1 expression is also induced up to 20-fold in keratinocytes during wound healing (11), suggesting that these growth factors might be involved in the regulation of syndecan-1 expression.

Several growth factors, including FGFs, can elicit immediate-early responses after their receptor tyrosine kinase (RTK) activation. Well-characterized examples are epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), which induce, via the mitogen-associated protein (MAP) kinase pathway transcription factors Fos and Jun, the serum response factor and ternary complex factor (17). The cyclic AMP (cAMP) response element (CRE), bound by the CRE-
binding protein homodimer or as heterodimers in association with members of the ATF family, is also under the influence of growth factors. EGF and PDGF, but not FGFs, are also able to induce activation of signal transducer and activator of transcription (STAT) transcription factors which act on Sis-inducible element (SIE) or interferon-stimulated response element (18, 24). While able to induce the same signaling molecules, like MAP kinases and subsequently several early genes such as AP-1, it remains less well understood how differential transcriptional activation elicited by different growth factors on AP-1-driven promoters is obtained.

Our previous data has indicated that simultaneous exposure of cultured 3T3 cells to FGF-2 and transforming growth factor β (TGF-β) enhances syndecan-1 expression (12), indicating that members of the FGF family are involved in the regulation of syndecan expression. Whole-cell extracts were prepared by freezing the cells after harvesting and were subsequently resuspended in a 400 mM sodium salt buffer and ultracentrifuged (Sorval R-C M-120) for 5 min at 50,000 rpm, and the supernatant was used for gel shift analysis, with approximately 6 μg of protein extract for each reaction.

Cell proliferation assays were made by incubating cells for 4 to 6 h with 0.25 μCi of [32P]iodo-deoxyuridine (5’-32P)Jur; A mersham, washed several times with phosphate-buffered saline, and solubilized in 1 M NaOH. Radioactivity was measured by a gamma counter (Wallac).

For gel mobility shift assays, double-stranded oligonucleotides were end labeled with [γ-32P]ATP (ICN Biomedicals) by T4 polynucleotide kinase (Promega). Corresponding to footprint regions (see Fig. 3), oligonucleotides (top strand) were 5'-dGCGAACCACCACACGTGAGACCT3' (motif 1), 5’-TTGGCAACCACCGAGGAGGATG-3' (motif 2), 5’AGTGGTCAGGGTGACTCT-3' (motif 3), and 5’-AGGAGGTGAGCCATGCGACC-3' (motif 4), and 5’-CTGGGCTATTGAGACTCTGG-3' (motif 5). In a 12-μl reaction mixture, 2 μg of nuclear extracts was incubated with labeled oligonucleotidest, 2 μg of poly(dI-dC), and 2 μl reaction buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 2 mM EDTA, and 10% glycerol) for 15 min at room temperature. Nonlabeled competitor oligonucleotides were used at 50× molar excess. The complexes were analyzed by electrophoresis in a 4.5% polyacrylamide gel. For supershifts, 1 μl of specific antibody (Santa Cruz) was added to the reaction 15 min before the labeled oligonucleotide.

For UV cross-linking experiments, gel mobility assays were run as described above. The gel, after being run, was exposed to 245-nm UV light (3.600/25 cm2) in a Stratagene UV cross-linker. The gel was exposed for several hours, and specific bands were cut from the gel, eluted overnight at +4°C, precipitated with ethanol, resuspended in Laemmli buffer, denatured at +95°C for 5 min, and run on a sodium dodecyl sulfate (SDS)-10% polyacrylamide gel together with a 1°C-labeled molecular weight marker. The gel was stained with Coomassie Brilliant Blue R-250 and destained in 10% acetic acid.

For nuclear extracts, 3T3 NIH cells were plated on 16-cm dishes and treated with or without FGF-2 for 2 to 8 h. Nuclear proteins were extracted by a modification described by Lee et al. (27). Protein concentrations were measured by the Bradford reaction, and approximately 3 μg of extract was used for each reaction. Whole-cell extracts were prepared by freezing the cells after harvesting and were subsequently resuspended in a 400 mM sodium salt buffer and ultracentrifuged (Sorval R-C M-120) for 5 min at 50,000 rpm, and the supernatant was used for gel shift analysis, with approximately 6 μg of protein extract for each reaction.

RESULTS

Syndecan-1 gene is activated by FGF-2 in 3T3 cells. Syndecan-1 expression is usually very low in mesenchymal cells compared to that in epithelial cells. Yet, many mesenchymal genes can transiently induce syndecan-1 expression (56, 57). Our earlier work has indicated that syndecan-1 expression is upregulated in 3T3 cells after 24 h of simultaneous FGF-2 and TGF-β exposure (12). However, our previous data suggested that transiently FGF-2 alone could also activate the syndecan-1 gene. To demonstrate this, 3T3 cells were exposed to FGF-2 at 10 ng/ml in growth factor-depleted conditions and the syndecan mRNAs were quantified at various time points. As shown in Fig. 4, the mRNA levels were increased severalfold already at 4 h after FGF-2 treatment. This induction reverted to low levels within the next 8 to 24 h, however. Furthermore, a nuclear run-on experiment revealed that this upregulation was transcriptional. In nuclei isolated 4 h after FGF exposure, the level of transcription of the syndecan-1 gene was elevated, as
were the levels of c-Jun and Nur used as positive controls. The transcription of syndecan-1 was no longer detectable after 24 h (Fig. 1B), in agreement with the results of Northern hybridization.

In 3T3 cells, a novel FGF-2-specific, far-upstream element is responsible for enhanced syndecan-1 expression. In order to find transcriptional elements responsible for syndecan-1 expression following FGF-2 exposure, the 5'-9 region of the gene was cloned and sequenced 12 kb upstream from the translation initiation site. Fragments of the 5'-9 region ranging in size from 1.1 to 4.5 kb were fused with the proximal promoter (−1.1 kb upstream from the translation initiation site) of the syndecan-1 gene (19, 58) and inserted into a CAT reporter plasmid. These constructs (see Fig. 2A) were assessed by transiently transfecting them into 3T3 NIH cells, which were then treated with or without FGF-2. The most distal 2.2-kb part of the syndecan-1 gene produced a 7- to 10-fold enhancement when the reporter construct was expressed in FGF-2-treated cells (Fig. 2A). All other fragments, as well as the 1.1-kb proximal promoter clone alone (pCATProm), revealed no response to FGF-2 (Fig. 2A). All other fragments, as well as the 1.1-kb proximal promoter clone alone (pCATProm), revealed no response to FGF-2 (Fig. 2A). The 2.2-kb FGF-responsive region was cut into halves (pXSp1 and pXSp2), and the derived pXSp1 was further cut into shorter fragments (Fig. 2B). These constructs were assessed for FGF-2-induced enhancer activity as described above. A 280-bp element, termed FiRE, was found to mediate the full FGF-2 response, regardless of its orientation within the plasmid (pFiRErev).

FiRE binds FGF-2-inducible and noninducible nuclear factors. DNAse I footprinting was performed with the end-labeled FiRE fragment and nuclear extracts derived from FGF-2-treated or nontreated 3T3 NIH cells in order to find DNA-protein interactions along the enhancer sequence. Five protein binding sites, ranging from 14 to 38 bp in length, close to each other and covering a total of 170 bp, were revealed (Fig. 3A). Binding of nuclear proteins by motifs 1 and 2 was evident both in FGF-2-stimulated and nonstimulated 3T3 cells, but motifs 3, 4, and 5 clearly indicated binding of FGF-2-dependent nuclear factors (Fig. 3A).

Gel retardation assays were performed with double-stranded oligonucleotides corresponding to all five motifs. A SP1 consensus oligonucleotide was routinely used as a control to check the functionality of the nuclear extracts. Binding of one or more protein complexes to each motif was observed. To reveal specific binding, each oligonucleotide was competed with a 50-fold excess of specific and nonspecific oligonucleotides. The results from these experiments indicated that all motifs shifted at least one specific band in the gel retardation assay. The motifs 1 (Fig. 4A) and 2 (Fig. 4B) revealed no FGF-2-inducible nuclear factors, as observed earlier in the footprint experiment. Instead, motifs 3 (Fig. 4C), 4 (Fig. 4D), and 5 (Fig. 4E) all showed binding of at least one specific nuclear protein induced by FGF-2. To further support the finding that motifs 1 and 2 are occupied by constitutively expressed nuclear proteins but motifs 3, 4, and 5 are occupied
only after FGF exposure of 3T3 cells, five independent gel shift assays were performed and the specific bands (indicated by a line in Fig. 4) were scanned. These results are shown in Fig. 4F and clearly indicate a 6- to 10-fold induction for the nuclear factors binding to motifs 3 to 5 but only a 1.2- and a 1.6-fold induction for motifs 1 and 2, respectively.

Characterization of FiRE-binding nuclear proteins. The nucleotide sequence of FiRE (Fig. 3B) was compared to the known sequences of different transcription factor binding elements stored in the Transcription Factor Database. Only motif 2 revealed a known transcription factor binding consensus site, an E box. Motif 4 contained an AP-1-like consensus binding site with one mismatch, and motif 5 contained two AP-1-like consensus sites. Motifs 1 and 3 were not found to contain any known transcription factor sequences. Therefore, a large array (including AP-1, AP-2, AP-3, Ets, GATA, SIE, CRE, MEF-1, MEF-2, Max-Myc, SP-1, NF-κB, and C/EBP) of commercial consensus oligonucleotides were competed with each binding site in order to reveal possible protein binding outside the established consensus sites. As expected, a Max-Myc consensus oligonucleotide was able to abolish the binding to motif 2 (Fig. 5D). For motifs 4 and 5, AP-1 consensus oligonucleotides also competed the binding (Fig. 5A and B). Surprisingly, binding on motif 3 was abolished by AP-2 oligonucleotide although there is no AP-2 consensus site present in motif 3 (Fig. 5C). Motif 1 could not be competed by any of the consensus oligonucleotides tested (data not shown). These competition assays suggested that motifs 4 and 5 may bind an AP-1 complex, motif 3 may bind an AP-2 complex, motif 2 may bind a helix-loop-helix factor, and motif 1 may bind an unknown nuclear factor.

Based on the competition experiment results above, specific antibodies were tested by adding them to the gel retardation reaction. As indicated in Fig. 5, Jun and Fos antibodies were able to remove the specific binding on motifs 4 (Fig. 5A) and 5 (Fig. 5B) and also to produce supershifts. Anti-USF or anti-ATF-3 antibodies analyzed at the same time had no effect. As an AP-2 consensus oligonucleotide was able to abolish the
binding by motif 3, an AP-2 antibody was tested with it. This, however, had no effect (Fig. 5C). The AP-2 antibody was also tested with labeled AP-2 oligonucleotide by using FGF-2-induced 3T3 cell nuclear extracts, and it was shown to be functional, as it produced a supershift (data not shown). This data suggests that the protein bound by motif 3, which is able to bind an AP-2 consensus site but is not AP-2, is perhaps an AP-2-related transcription factor and may bear homology within the DNA binding domain. This protein was named FIN-1 for FGF-inducible nuclear factor. For motif 2, neither Max nor Myc antibodies had any effect. However, another basic helix-loop-helix protein, USF, which is known to be constitutively expressed in 3T3 cells (32), was shown to occupy motif 2, as USF antibody removed the specific band (Fig. 5D) while not influencing the binding of other motifs.

As the proteins binding to motifs 1 and 3 remained unknown, we performed a UV cross-linking experiment to reveal their approximate molecular weights. Gel retardation was run as for Fig. 4, and the specific bands were cut, eluted, and run on SDS-PAGE gels. The oligonucleotide mass was subtracted from the estimated molecular weights revealed by the gel analysis. This method indicated one reproducible 46-kDa band for motif 1 and two bands of 78 and 50 kDa for motif 3 (Fig. 5C and E). Interestingly, AP-2 is known to be a 50-kDa protein. Figure 5F summarizes the structural model of FiRE, which includes constitutive and FGF-inducible transcription factors, in an active form.

**Newly synthesized AP-1s are required but not sufficient for FiRE activation.** To determine whether all the binding sites are required for enhancer activation, deletion mutants for each motif were generated by PCR (see Materials and Methods). For DelM1 and DelM5, motifs 1 and 5 were totally deleted. For DelM2, the E box (CACC TG) on motif 2 was changed to a KpnI recognition site (GGTACC). For DelM3, a central part of the motif (TCAGGT) was replaced by a SpeI site (AATCACTAGTGATG). For DelM4, the AP-1 site (GGTACCCTAGTGATG) was replaced by a Spel site (AATCACTAGTGATG). Transfections and CAT assays were performed as for Fig. 2. Except for the binding domain for USF (DelM2), deletion of each motif (DelM1 and DelM3 to M5) dramatically decreased the activation of FiRE by FGF-2.
least two Fos-Jun complexes are required and that alone they are not sufficient to activate FGF-induced transcription.

To study whether the activation of the different inducible components of FiRE is due to direct posttranslational modifications or whether they are newly synthesized, simultaneous cycloheximide and FGF treatment and subsequent protein extraction were performed. As shown in Fig. 7A, the FGF-induced binding of AP-1s to motifs 4 and 5, as well as the binding of FIN-1 to motif 3, was abolished when translation was blocked by cycloheximide. This suggests that all the inducible proteins involved in FiRE require de novo protein synthesis and, furthermore, that FiRE represents a secondary response element in FGF-initiated signaling. This is further demonstrated by the fact that cycloheximide also blocks the effects of FGF on syndecan-1 mRNA, a Northern analysis was performed, and the blot was probed with syndecan-1 and subsequently with a probe recognizing the ribosome 28S as a loading control (B).

FiRE shows selectivity for FGFs in 3T3 cells. To examine the responsiveness of FiRE to other growth factors and serum, the FiRE-CAT construct was transfected into 3T3 NIH cells treated with FGF-1, FGF-2, FGF-4, FGF-7 (KGF), PDGF/BB, EGF, IGF-I, TGF-β, gamma interferon, and 5% FCS. Although all of the growth factors known to act on 3T3 cells stimulated 3T3 proliferation, as assayed by 5-125IIdU incorporation after a 24-h growth factor treatment (Fig. 8B), FGFs were the only growth factors to clearly increase the reporter gene activity (Fig. 8A). Interestingly, FGF-1 and FGF-4 had less effect than FGF-2 (Fig. 8A). FGF-7 is known to act only on epithelial cells and had no effect. Although serum is known to contain several growth factors, 5% FCS also gave no response. PDGF, IGF, and EGF clearly enhanced cell proliferation but...
had no effect on the enhancer activity. Thus the stimulation of different tyrosine kinase receptors does not lead to activation of FiRE, nor does the proliferation alone correlate with the activation of FiRE.

As the original construct contained over 1 kb of syndecan-1 proximal promoter, we wanted to rule out the possible suppressive action of this basal promoter on the function of the other growth factors. Therefore, nearly all of the promoter was deleted, leaving only 98 bp of the proximal promoter (p-271CAT) which included only the putative TATA box without any upstream regulatory elements (58). As shown in Fig. 8C, the removal of the syndecan promoter had no effect on the pattern of growth factor-induced FiRE activation, indicating that the growth factor specificity is not regulated by the proximal promoter.

Agents activating AP-1 are not sufficient for FiRE activation. Since FiRE binds AP-1 transcription factors, whose activation is well characterized, we tested chemicals known to cause AP-1-dependent gene activation. These included the protein kinase C (PKC) activator 12-O-tetradecanoylphorbol-13-acetate (TPA), the protein phosphatase-1 and -2 inhibitors okadaic acid (OA) (10 nmol) and calyculin A (10 nmol), the CaMP activator forskolin (5 μmol), and the tyrosine kinase phosphatase inhibitor orthovanadate (100 μmol) were used.

**FIG. 9.** TPA or other activators of AP-1 do not activate FiRE. Different agents, known to induce Fos-Jun complexes and activate AP-1-driven promoters, were tested for FiRE activation in a CAT assay with the minimal promoter FiRE (p271FiRE) stably transfected 3T3 NIH cells. The PKC activator TPA (10-nmol final concentration), the protein phosphatase-1 and -2 inhibitors okadaic acid (OA) (10 nmol) and calyculin A (10 nmol), the cAMP activator forskolin (5 μmol), and the tyrosine kinase phosphatase inhibitor orthovanadate (100 μmol) were used.

**DISCUSSION**

In this paper, we have characterized a response element for members of the FGF family, factors which are known to influence a variety of biological systems. This far-upstream FiRE employs a rather complex composition of different nuclear factors. It consists of an array of five binding motifs, bound by several different transcription factors, and presents a novel possibility for FGFs to induce differential gene activation. Combining growth factor specificity and ability to upregulate an FGF-binding cell surface molecule, syndecan-1, the model also presents a mechanism by which members of the FGF family could autoregulate their own function.

A novel FiRE. The 280-bp FiRE described in this paper is located 11.6 kb upstream from the translation start site of the syndecan-1 gene and shows at least a 10-fold activation in FGF-2-treated 3T3 cells. In mesenchymal cells, this activation seems to be restricted to FGFs and shows no response to serum or other growth factors tested.

FiRE consists of several FGF-inducible and noninducible nuclear factors in an organized array (Fig. 5F). Components of AP-1, Fos and Jun, are rapidly activated by numerous extracellular stimuli. Their activation is brought about by either direct gene activation or posttranslational modifications (24).

In several studies, FGFs have been shown to activate c-Fos. FGF-2 is also known to induce the expression of c-Fos and c-jun in 3T3 cells (22). Our results show that FGF treatment results in transcriptionally active Fos-Jun heterodimers and that FiRE is bound by at least two AP-1 complexes, both of which seem to be required for FGF induction. Removal of the binding site of one AP-1 (motif 4 or motif 5) dramatically reduces the level of the FGF effect, as does the removal of the binding site for FIN-1. This indicates that FiRE requires concatenation of at least three FGF-inducible transcription factors on the same array of DNA. However, this seems not to be sufficient, since removing motif 1 also nearly abolishes the FGF response. This suggests that, besides gathering inducible factors together, the element demands interactions between inducible and constitutive DNA-binding proteins. USF is a ubiquitously expressed homodimeric transcription factor (15) constantly expressed in 3T3 cells (32). It is not known to be under the influence of any growth factor. In FiRE, USF is not activated by FGF, but it is involved in the complex with inducible components. Replacement of the E box does not, however, reduce the FGF effect on FiRE. This might be due to replacement of USF with another constantly active transcription factor which might be able to interact with the inducible components. Alternatively, protein-protein interactions might hold USF in the FiRE complex despite the removal of its optional DNA binding target.

Besides AP-1 and USF, FiRE involves another noninducible component as well as a putatively novel FGF-inducible AP-2-like transcription factor. AP-2 is a cell-specific 50-kDa transcription factor expressed by several tissues at high levels (26). Multiple forms of AP-2 are generated by alternative splicing (31), and also one AP-2-related transcription factor, AP-2b, has been cloned (34). FIN-1, the FGF-inducible nuclear factor that binds motif 3 and could be competed with an AP-2 consensus oligonucleotide but was not recognized by AP-2 antibody, might also be a member of a larger AP-2 family. It is known that AP-2 can form heterodimers without DNA binding and that AP-2 dimerization is required for binding (61). This implies that the larger of the two bands seen in our cross-linking analysis might represent a dimer form of FIN-1 (Fig. 5). Our current effort is targeted to the cloning of this factor.

The interactions between far-upstream regulatory gene elements and basal transcriptional complexes are currently not fully understood. However, looping of DNA and physical interactions between these two separate elements are supposed to occur. The exchange of the syndecan-1 promoter with the simian virus 40 promoter did not inactivate the enhancer, and neither did removal of most of the proximal promoter, suggesting that the FiRE-type element does not require any specific upstream activators on proximal promoter and, further-
more, that a similar element might be able to activate other genes besides syndecan-1.

FGFs, EGF, PDGF, and IGF signal by binding to their own cell surface tyrosine kinase receptors (RTKs). FGFs, as well as the other growth factors, activate the MEK-MAP kinase pathway downstream from the receptor activation via ras and raf (5, 18, 29, 37, 55), but other signal transduction pathways also might contribute to the FGF signaling (29). Several endpoints for growth factor signaling on gene elements have been described elsewhere. These include (i) EGF and PDGF activation of the serum response element (SRE), which is bound by a ternary complex factor-serum response factor complex; (ii) activation of the CRE, which besides CRE-binding protein is also bound by AP-1 and ATF family members; (iii) response elements for signal transducer and activator of transcription factors (interferon-stimulated response element and SiE), which are activated through J anus kinases by EGF and PDGF as well as by various cytokines like interleukons; and (iv) activation of NF-κB by TGF-α (18, 24). FGFs are also reported to activate the SRE (39). CRE, together with forskolin, activates the CRE (52) and subsequent transcription of cellular genes. Together with these examples, FiRE, which does not contain either SRE or CRE, illustrates the multiple ways in which FGFs can induce transcription.

All the RTK-activating growth factors can induce cell proliferation and activate Fos and Jun transcription factors in 3T3 cells. They still have different biological effects and can induce partly different subsets of secondary target genes. As the cycloheximide inhibition of the FGF response reveals, FiRE is a secondary response element in FGF-induced signaling. Furthermore, based on the mutational analysis and the treatment with several AP-1-activating agents, it is clear that, while AP-1 activation is mandatory for FiRE activation, it is not sufficient for it. This element can also distinguish the action of different RTK-stimulating growth factors upon an AP-1-driven DNA element, since only FGFs, not other AP-1-inducing growth factors or chemicals, can activate it. The mechanisms underlying this specificity still remain to be studied. Several possibilities exist as to why FGFs are able, but other growth factors and serum fail, to activate FiRE. The unresponsiveness of FiRE to serum could be explained by the low concentration of FGFs in it, as the most abundant growth factor in serum is thought to be PDGF. The activation of RTKs other than FGFRs can result in differential activation of a diverse subset of cytoplasmic kinases and, subsequently, also different transcription factors such as members of the Fos and Jun families. This again could result in a switch of one family member to another that binds to FiRE while lacking the inductive capacity. Equally, it is also possible that the other growth factors cannot induce the FIN-1 modification transcription factor while they are still able to activate AP-1s. One possible mechanism would be different posttranslational modification, such as phosphorylation or dephosphorylation on the inducible (e.g., Fos or FIN-1) or constitutive (e.g., USF) proteins. For example, c-Jun is known first to require dephosphorylation to bind on DNA and then to require further phosphorylation in order to activate transcription (for a review, see the work of Karin (24)). Finally, signaling through RTKs other than FGFR could induce inhibitory transcription factors that bind to FiRE but lack the capacity to activate it. For example, the high PDGF content in serum might activate signaling pathways downstream of the FiRE. Therefore, FiRE, as an end point for signaling, can distinguish various RTK-activated cascades and may be a very useful tool for future studies elucidating biological differences such as the differential activation of these cascades.

**Physiology of FiRE.** FGF-2 is produced by fibroblasts and is also found in association with extracellular matrix and basement membranes, where it can be released by proteolytic activity. FGF-2 enhances the accumulation and proliferation of fibroblasts, keratinocytes, endothelial cells, and macrophages. In animal models, it induces neovascularization, cell migration, and granulation tissue formation, and during development, it seems to possess a vast number of different functions, including induction of mesenchyme. In adult tissues, syndecan-1 is expressed mainly in epithelia, but during induction of mesenchyme, syndecan appears in condensing and proliferating mesenchyme (53, 56). Syndecan can be colocalized to the target tissues of many members of the FGF family, for example, the mesenchyme underlying ectoderm in limb bud (49), which is a target for FGF-2 (13), FGF-4 (36), and FGF-8 (10). FGF-8 can also be colocalized with syndecan-1 in limb bud (16). In tooth development, syndecan is colocalized with FGF-3 and is also detected in the mesenchyme and epithelium, which are putative target tissues for FGF-4 (54). This raises the possibility that FiRE is the regulatory element and the end point for FGF-initiated signaling of syndecan upregulation in mesenchyme.

Heparin is shown to be required for FGF oligomerization and subsequent FGFR dimerization and signal transduction (50). Heparin is in the form of heparan sulfate proteoglycans at the cell surface, and it is not yet fully understood how proteoglycans can participate in the regulation of FGF. Syndecan-1 can bind simultaneously FGF-2 and an extracellular matrix molecule, and this complex can promote FGF-induced cell proliferation (44). However, there is also evidence that syndecan-1 expression could be inhibitory for FGF action. Syndecan-1 isolated from lung fibroblasts has been shown to inhibit FGF binding to FGFR (1). Furthermore, the overexpression of syndecan-1 on the cell surface of 3T3 NIH cells by transfection abolishes the proliferative response of FGF-2 (28). Several possibilities can explain this type of inhibition, including an unfavorable stoichiometric ratio of FGF and syndecan-1 that does not support ternary complex formation. Alternatively, an altered heparan sulfate structure may generate antagonistic activity and subsequent release of growth factor from FGFR. Whatever the mechanism, this inhibition provides an interesting inhibitory loop for FGF action. Cells with a low cell surface proteoglycan content can activate the FGF signaling pathway resulting in the activation of FiRE. This results in the enhancement of syndecan-1 expression, which subsequently could block further FGF action. This type of restriction of FGF action would be extremely useful during development, for example, as FGFs could otherwise cause inappropriate proliferation of mesenchymal cells in time and space. This principle could be applied also to other physiological and pathophysiological conditions in which proliferation of mesenchyme or fibroblasts by growth factors occurs.

Cancerous cells are also known to be able to activate AP-1 complex, and it is known that c-Fos is required for malignant tumor progression (42). However, in many cases, syndecan-1 is shown to disappear when cells transform and become invasive (20, 21). It is therefore tempting to speculate that, if FiRE is needed for high-level expression of syndecan-1, then perhaps the activation of the non-AP-1 transcription factors, such as FIN-1 and USF, of FiRE may be disturbed in malignant cells while Fos and Jun dimers remain active, resulting in decreased syndecan-1 levels.
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