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The activation and composition of FiRE (an FGF-inducible response element) differ in a cell type- and growth factor-specific manner

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The expression of the heparan sulfate proteoglycan, syndecan-1, is induced both in keratinocytes and in fibroblasts during development and tissue regeneration. Here we report that in keratinocytes the syndecan-1 gene was stimulated by EGF but not by FGF-2. In fibroblasts it was stimulated by FGF-2 but not by EGF. Likewise, the recently discovered FGF-inducible response element (FiRE) on the gene of syndecan-1 was stimulated by FGF-2 in fibroblasts and by EGF in keratinocytes, but not *vice versa*. The FiRE has two binding sites for an activator protein-1 (AP-1), one for an FGF-inducible nuclear factor (FIN-1) and one for an upstream stimulatory factor-1 (USF-1). The growth factor-stimulated binding of these transcription factors, as well as their requirement for FiRE activation, varied between the two cell types. First, although AP-1s were required for activation of FiRE in both cell types, the binding of AP-1 to FiRE was increased by growth factor-stimulation only in fibroblasts and not in keratinocytes. Secondly, FiRE did not bind FIN-1 nor needed the FIN-1 binding site for EGF-stimulated activation in keratinocytes, in contrast to the FGF-stimulated activation of FiRE in fibroblasts. Thirdly, EGF, which did not activate FiRE in fibroblasts, failed to activate FIN-1 in these cells. Finally, an USF-1 binding site that was necessary for activation of FiRE in keratinocytes was not needed in fibroblasts. These data suggest mechanisms by which members of the EGF- and FGF-families can differentially stimulate transcription through AP-1 regulated elements in a cell type-specific manner.

Keywords: AP-1; EGF; FGF; FIN-1; KGF; Syndecan-1; transcription

Introduction

Members of the epidermal growth factor (EGF) and the fibroblast growth factor (FGF) families share many biological functions and frequently activate same signal transduction pathways in their target cells. However, the genes that these growth factors upregulate in a given cell type are not always identical. Both EGF and the basic fibroblast growth factor, FGF-2, act on epithelial cells and fibroblasts stimulating their migration and cell proliferation (Basilico and Moscatelli, 1992; Bennet and Schultz, 1993; Tsuboi *et al*., 1993). There are also common pathways, such as the extracellular regulated kinase (ERK) pathway of MAP kinases, which transduce signals upon activation of both EGF and FGF receptors (Karin *et al*., 1997). Furthermore, EGF and FGF-2 are able to promote regeneration processes, such as wound healing (Andree *et al*., 1994; Brown *et al*., 1989; Davidson and Broadley, 1991). It is remarkable that although sharing all these activities, the gene expression pattern the growth factors induce in one cell type is only partially overlapping. This has been shown, for example, in neural cells where EGF and FGF can differentially upregulate the expression of several proteins (Loret *et al*., 1989). Similarly, in fibroblasts FGF, but not EGF, upregulates the vinculin gene (Ben-Ze'ev *et al*., 1990).

The induced expression of genes is mainly achieved by activating transcription through the activation of transcription factors. The transcription factors of the Fos – family (c-Fos, FosB, Fra-1, Fra-2) and the Jun-family (c-Jun, JunB, JunD) form hetero- and homodimers called activator protein-1 (AP-1). AP-1 dimers bind to DNA at consensus sequences such as the classical TPA-responsive element (TRE) and the cAMP-responsive element (CRE) to activate transcription, but the binding site can vary depending on the composition of the AP-1 dimer. Furthermore, some other transcription factors adjacent to AP-1 are supposed to have an ability of modulating the transactivation induced by AP-1. A wide variety of genes are known to have an AP-1 site in their promoter. The Fos- and Jun-family members are mostly associated with proliferation and survival of cells and are induced by several mitogenic stimuli, including growth factors (Hill and Treisman, 1995; Karin *et al*., 1997). EGF and FGF-2 are known to induce the AP-1 transcription factor family members in numerous cells. EGF and FGF-2 also upregulate several AP-1 driven promoters (Felts *et al*., 1995; Han *et al*., 1992; Pestell *et al*., 1995; Tan *et al*., 1994). However, the signaling induced by EGF and FGF can not be identical. This can be seen in situations where in a single cell type one growth factor elicits expression of a gene, while the other one fails to do it. Furthermore, if this expression is dependent on an AP-1 regulated promoter, it is obvious that these growth factors can not equally regulate the transactivation capacity of AP-1. The specificity of growth factors to differentially regulate transcription, also among the AP-1 driven promoters, is not well understood.

Syndecan-1 is one example of a protein that is induced in a cell type-specific manner. It is a heparan sulfate proteoglycan expressed in keratinocytes and

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other epithelia (Bernfield *et al*., 1992). The level of syndecan-1 expression in skin keratinocytes is upregulated during wound healing. Both mRNA and protein levels of syndecan-1 are induced up to 20-fold in migrating keratinocytes (Elenius *et al*., 1991; Gallo *et al*., 1996). Furthermore, during development, e.g. in tooth and kidney, syndecan-1 is mostly found in epithelia, but transiently at high levels in condensing mesenchyme as well (Vainio *et al*., 1989; Vainio and Thesleff, 1992). During both development and wound healing, the inducible expression of syndecan-1 has been postulated to be regulated by growth factors. Previously, syndecan-1 expression has been shown to be upregulated by FGF-2 in NIH3T3 fibroblasts (Elenius *et al*., 1992; Jaakkola *et al*., 1997). Therefore, we addressed the question whether syndecan-1 can be regulated by growth factors also in keratinocytes, and furthermore, which are the mechanisms underlying the inducible regulation of syndecan-1 both in keratinocytes and fibroblasts.

We have previously characterized the activation of an FGF-inducible response element (FiRE) in NIH3T3 fibroblasts. FiRE is a 280 bp gene fragment located at −10 kb upstream of syndecan-1 promoter (Jaakkola *et al*., 1997). In NIH3T3 cells FiRE was shown to be selectively induced by FGFs (mainly by FGF-2) but not by other growth factors, such as EGF, keratinocyte growth factor (KGF) or platelet-derived growth factor (PDGF). In NIH3T3 fibroblasts FiRE was shown to bind two inducible AP-1 complexes, an inducible AP-2 related 50 kD protein referred to an FGF-inducible nuclear factor (FIN-1), a constitutively expressed upstream stimulatory factor-1 (USF-1) (Gregor *et al*., 1990), and an uncharacterized 46 kD protein. Here we demonstrate that syndecan-1 expression is regulated by EGF in keratinocytes but not in fibroblasts and, that the differential regulation similarly applies to the FiRE in both cell lines. Furthermore, we show that FiRE uses different patterns of transcription factors in fibroblasts and keratinocytes and that the growth factors differ in their ability to induce transcription factors in these cells. Finally, we provide evidence which suggests that the main regulation of FiRE activity upon growth factor stimuli might happen at the level of transcription factor binding in fibroblasts but not in keratinocytes.

Results

Syndecan-1 mRNA is induced by EGF and KGF in keratinocytes and by FGF-2 in fibroblasts

We have previously shown that in NIH3T3 fibroblasts syndecan-1 mRNA is transiently upregulated by FGF-2 and that the upregulation is transcriptional (Jaakkola *et al*., 1997). Since syndecan-1 is strongly induced in keratinocytes *in vivo* (Elenius *et al*., 1991), we studied whether the syndecan-1 expression could be regulated by growth factors in these cells. We used a keratinocyte cell line MCA3D (Kulesz-Martin *et al*., 1983) and stimulated the cells with EGF, KGF or FGF-2, growth factors known to stimulate biological responses in keratinocytes. The MCA3D cells were grown in absence of serum for two days and treated with growth factors for 5 h. A Northern blot was performed followed by quantification of the syndecan-1 RNA levels by scanning and correlation to glyceraldehyde 3- phosphate dehydrogenase (GAPDH) loading control. As shown in Figure 1a, EGF and KGF, but not FGF-2, were able to increase the levels of syndecan-1 mRNA in MCA3D cells. In contrast, the syndecan-1 mRNA levels were increased by FGF-2, but not by EGF, in NIH3T3 cells (Figure 1b). Thus, syndecan-1 expression could be differentially regulated by growth factors in a cell type-specific manner.

FiRE is induced by EGF and KGF in keratinocytes but by FGF-2 in fibroblasts

We have recently described in fibroblasts a 280 bp far upstream FGF-2 inducible response element (FiRE), on the gene of syndecan-1 (Jaakkola *et al*., 1997). To investigate whether FiRE could be responsible for the growth factor induced expression of syndecan-1 also in keratinocytes, a plasmid containing the FiRE element together with 1.1 kb of syndecan-1 proximal promoter (Vihinen *et al*., 1993) in front of a chloramphenicol acetyltransferase (CAT) gene (Figure 2d), was transiently transfected into MCA3D cells. The transfected cells were grown without serum for two days and treated with different growth factors overnight. CAT enzyme activity was subsequently determined. FGF-2 did not activate FiRE in MCA3D keratinocytes (Figure 2a), in contrast to NIH3T3 fibroblasts (Figure 2c). However, KGF and EGF, which had no effect on NIH3T3 cells, were potent activators of FiRE in MCA3D cells (Figure 2a). Furthermore, serum and PDGF failed to activate FiRE in both cell lines.

To demonstrate that FiRE was responsible for the growth factor induced reporter gene activation, the syndecan-1 proximal promoter fragment (pSynPr), without FiRE, was tested in the CAT reporter gene assay. As shown in Figure 2b, the pSynPr fragment did not respond to any of the tested growth factors in keratinocytes. The data demonstrated that FiRE activity correlates strictly to induced syndecan mRNA levels in both MCA3D and NIH3T3 cells, suggesting that FiRE mediates the growth factor induced syndecan-1 upregulation in both cell lines. Furthermore, it suggested that this gene element is growth factor-selective in a cell type-specific manner.

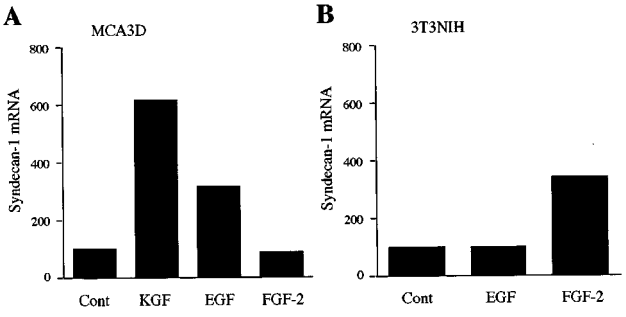

Figure 1 Syndecan-1 is activated by different growth factors depending on the cell type. MCA3D keratinocytes (**a**) and NIH3T3 fibroblasts (**b**) cells were grown to 70% confluence, serum starved for two days and treated with indicated growth factors for 5 h. RNA was isolated, Northern blot performed and probed with syndecan-1 and GAPDH as a loading control. Gels were scanned and syndecan-1 was correlated to GAPDH

To control the biological activity of the growth factors but also to study the relationship between growth factor induced cell proliferation and FiRE activation, we measured the incorporation of 5-[^{125}I]Iodo-2′-deoxyuridine (IdU) into the DNA of MCA3D and NIH3T3 cells treated with growth factors (data not shown). EGF, KGF and FGF-2 were able to induce cell proliferation in MCA3D. In NIH3T3 cells EGF elicited equal DNA synthesis compared to FGF-2. As expected, KGF had no effect on the IdU incorporation in NIH3T3 cells. This implied that the activation of FiRE did not correlate to the ability of these cells to proliferate in response to growth factors.

Growth factor-stimulation increases the binding of nuclear proteins to FiRE in fibroblasts but not in keratinocytes

The FiRE element has been shown in NIH3T3 cells to consist of five nuclear factor binding sites (motifs 1–5) in an array covering 170 bp (Figure 2d). In these cells the motif 1 was occupied by an 46 kD unknown protein, the motif 2 by an USF-1, the motif 3 by a 50 kD nuclear factor (termed FIN-1 for FGF-inducible nuclear factor), and motifs 4 and 5 by Fos-Jun (AP-1) complexes. The USF-1 and 46 kD transcription factors were found to bind DNA constitutively regardless of the growth factor stimulation, in contrast to the AP-1s and the FIN-1 protein which were induced by FGF-2 stimulation in NIH3T3 cells (Jaakkola *et al*., 1997).

We first tested the 170 bp fragment of FiRE in a reporter gene (CAT) assay and found this to give 15-fold induction with EGF treatment in MCA3D cells (data not shown). Thereafter, binding affinity of all the five motifs was investigated, by using whole cell or nuclear extracts derived from non-treated (control) and KGF or EGF treated MCA3D cells, in a gel shift assay (Figure 3). Motifs 1,2,4 and 5 were found to bind two protein complexes each. Their migration corresponded to that found previously from NIH3T3 derived extracts (Jaakkola *et al*., 1997). The arrows in Figure 3 point to the previously found specific bands in NIH3T3 cells. No marked enhancement of protein binding to motifs 1 or 2, compared to control, was seen with extracts from KGF or EGF treated MCA3D cells. Similarly, the growth factor-treatment (KGF or EGF) in MCA3D cells did not enhance or enhanced only slightly the nuclear protein binding to motifs 4 and 5, in contrast to the growth factor-treatment in NIH3T3 cells. The duration of the growth factor stimuli (ranging from 1–18 h) did not have effect on the protein binding. Equally to EGF and KGF, FGF-2 could not increase the binding of proteins to the motif 2 or 4 in MCA3D cells (not shown).

Since EGF fails to activate FiRE in NIH3T3 cells, we investigated whether in NIH3T3 cells EGF and FGF-2 could differentially regulate binding of the nuclear proteins to FiRE. Protein extracts from EGF- and FGF-2-stimulated NIH3T3 cells were prepared and tested with these motifs in a gel shift experiment. Similarly to the results obtained previously from FGF-2 stimulated cells (Jaakkola *et al*., 1997) specific proteins bound to motifs 1 (Figure 3a) and 2 (Figure

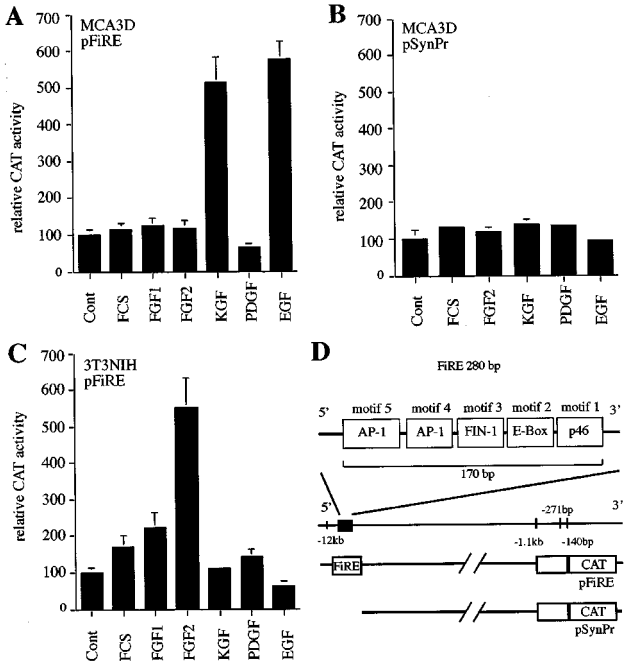

Figure 2 FiRE is activated by different growth factors depending on the cell type. MCA3D keratinocytes (**a**) and NIH3T3 fibroblasts (**c**) were transiently transfected with FiRE-syndecan proximal promoter- CAT (pFiRE) plasmid (**a** and **c**) or syndecan proximal promoter- CAT (pSynPr) plasmid (**b**). Cells were serum starved and treated overnight with indicated growth factors or 5% fetal calf serum (FCS) followed by determination of CAT activity. CAT activity is shown as percentile increasement of non-treated cells (Cont). The means and standard deviations of three independent experiments are shown. (**d**) Schematic model of the 280 bp FiRE element and the reporter gene plasmids used in this paper. FiRE has five transcription factor binding sites (motifs 1–5) in an array covering 170 bp. The numbers (−12 kb to −140 bp) indicate the location relative to the syndecan-1 translation site

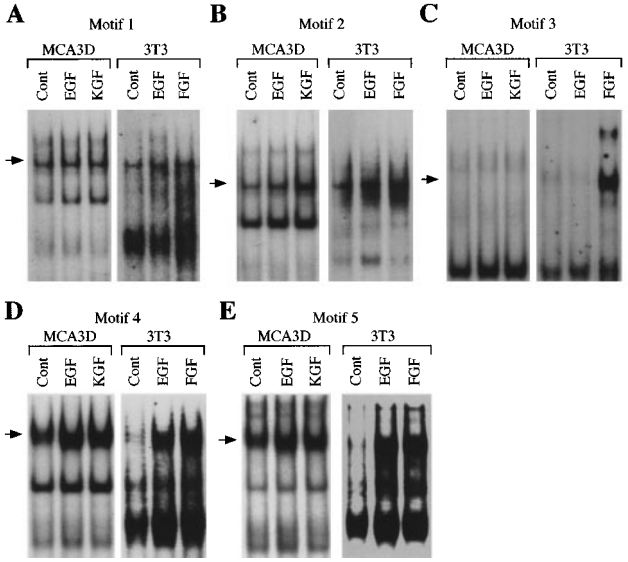

Figure 3 Analysis of nuclear protein binding to the five motifs of FiRE in keratinocytes and fibroblasts. Gel retardation analysis for each motif (1–5) was performed to reveal protein complex formation with nuclear extracts. Extracts were derived from untreated (Cont) MCA3D cells or MCA3D cells treated for 90 min with EGF or KGF, and from untreated (Cont) NIH3T3 cells or NIH3T3 cells treated with EGF or FGF-2. Specific binding, found previously in NIH3T3 cells, is indicated by an arrow

3b) were unaffected by EGF-stimulation while binding of nuclear proteins to the motifs 4 (Figure 3d) and 5 (Figure 3e) was significantly increased by EGF treatment.

EGF fails to induce binding of FIN-1 in both fibroblasts and keratinocytes

We have previously characterized an AP-2-like 50 kD nuclear protein (FIN-1) bound to the motif 3 of FiRE. It was strongly induced by FGF-2 treatment in NIH3T3 cells and the binding site was shown to be critical for activation of FiRE (Jaakkola *et al*., 1997). Therefore, we studied if the growth factors were able to induce FIN-1 in keratinocytes. Protein extracts and gel shift experiments were performed as described. As shown in Figure 3c, the binding of FIN-1 was totally absent in keratinocytes. Binding of this protein was not seen in gel shift assays performed with either nuclear or whole cell extracts derived from control, KGF or EGF treated MCA3D keratinocytes (Figure 3c) and did not depend on the duration of the growth factor stimuli. Equally to EGF and KGF, FGF-2 could not increase the protein binding to the motif 3 in MCA3D cells. Furthermore, we studied if EGF, which did not activate FiRE, could induce the binding of the FIN-1 protein in fibroblasts. Interestingly, EGF-stimulation failed to induce the binding of FIN-1 to motif 3. Equal results were obtained by using either nuclear or whole cell extracts. In contrast to EGF-stimulation, FGF-2-stimulation produced a strong band representing binding of FIN-1 to motif 3 (Figure 3c).

FiRE binds AP-1s and USF-1 in EGF-stimulated keratinocytes and fibroblasts

To study if the proteins bound to motifs 2 (an E-Box), 4 and 5 (AP-1 like sites) in EGF-stimulated MCA3D and NIH3T3 cells were the same as found previously in NIH3T3 cells upon FGF-2 stimulus, supershift experiments by using specific antibodies against transcription factors, were performed (Figure 4). The protein binding to motif 2 was removed by antibody to USF-1 in both EGF-treated keratinocytes and fibroblasts (Figure 4a), while the antibodies against Jun-family proteins (αJUN) or another E-Box binding transcription factor, Max (αMAX), had no effect. In contrast, the antibody to USF-1 had no effect on the protein binding to motifs 4 (Figure 4b) and 5 (Figure 4c) in either cell line. The antibodies recognizing Jun and Fos-family members (αJUN and αFOS, respectively), however, abolished binding to these motifs, and moreover, produced supershifts on motif 4 (Figure 4b, horizontal line). Equal results were obtained by using nuclear extracts derived from either KGF- or non-treated keratinocytes (data not shown).

Taken together the results demonstrated that equally to EGF and FGF-treated NIH3T3 cells, in EGF-treated MCA3D cells FiRE binds USF-1 and AP-1 complexes. However, in keratinocytes the growth factors could not increase the binding of AP-1 complexes, in contrast to the fibroblasts. Furthermore, the results demonstrated that the binding of the putatively novel FIN-1 is totally absent in keratinocytes, and also in fibroblasts stimulated by EGF, while strongly induced in fibroblasts by FGF-2, suggesting that it might have an important function in activating FiRE in fibroblasts.

FiRE utilizes differentially the USF-1 and FIN-1 binding sites in keratinocytes and fibroblasts

To investigate if all five protein binding sites of FiRE are needed for its growth factor induced activity, each of these motifs were separately mutated in the pFiRE plasmid (see Materials and methods) and stably transfected into MCA3D and NIH3T3 cells. The G418 resistant cells were pooled after which the EGF induced FiRE activity in MCA3D cells and the FGF-2 induced activity in NIH3T3 cells was assayed as described above. Deletion of an AP-1 site (either motif 4 or motif 5) resulted in a dramatic reduction or a total abolishment of CAT activity in both cell lines (Figure 5). In contrast, mutation of the binding site for FIN-1 (motif 3) decreased only slightly FiRE activity in MCA3D cells whereas in NIH3T3 cells the reduction was marked. Mutation of the USF-1 binding site (motif 2), resulted in clear reduction of EGF-induced FiRE activity in keratinocytes, but had no marked effect on FGF-2-induction of FiRE in fibroblasts. This indicated that in both NIH3T3 and MCA3D cells AP-1s are crucial for the function of FiRE. However, although in NIH3T3 cells the USF-1 seems not to be required, it is likely to be essential in

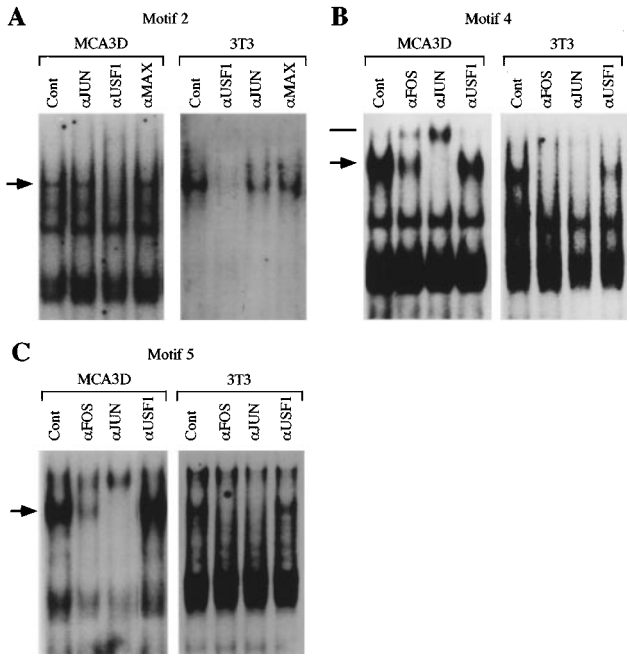

Figure 4 FiRE binds USF-1 and two AP-1 complexes in both EGF-treated MCA3D and NIH3T3 cells. Gel retardation analysis, with nuclear extracts derived from EGF treated MCA3D and 3T3 cells, was performed in the presence of indicated antibodies. (**a**) Antibody against USF-1 (αUSF1) (see Materials and methods) abolished the specific band on motif 2, but the antibody raised against Jun-family members (αJUN) or antibody against the E-Box binding Max protein (αMAX) had no effect in either cell line. (**b**) Antibodies against the members of Fos and Jun families (αFOS and αJUN) removed the specific bands. The supershift band is indicated by a horizontal line on motif 4. Antibody against USF-1, used as a negative control, had no effect. (**c**) Equally to motif 4, antibodies against both Fos and Jun, but not USF-1, abolished the specific binding to motif 5 in both cell types

keratinocytes. Finally, the FIN-1 which is found only in FGF-2-stimulated fibroblasts and also required in fibroblasts, is not mandatory to fully activate FiRE in keratinocytes.

EGF does not alter the AP-1 complex formation of FiRE in keratinocytes

The finding that FGF-2 but not EGF can induce binding of FIN-1 to motif 3 and binding of AP-1 to motifs 4 and 5 and that these motifs are required for FiRE activity in fibroblasts, together with the fact that the FGF-2 induced binding of FIN-1 and AP-1s depends on *de novo* protein synthesis (Jaakkola *et al*., 1997), suggests that the activation of FiRE is regulated at the level of DNA-binding in fibroblasts. In contrast, the growth factors did not increase DNA-binding of any of the nuclear proteins to FiRE in keratinocytes. AP-1s were, however, necessary for activation of FiRE also in keratinocytes. Therefore, we investigated if FiRE binds different AP-1 family members in non-treated and growth factor treated cells. It is known that a single AP-1 complex can be composed of dimers of at least four members of the Fos-family (c-Fos, FosB, Fra-1, Fra-2) and three members of the Jun-family (c-Jun, JunB, JunD). We performed a supershift experiment using extracts derived from non-treated and EGF-treated MCA3D cells, a labeled AP-1 (motif 4) oligonucleotide and specific commercial antibodies raised against each of the Fos- and Jun-family members. We found that an antibody raised against JunD produced a supershift band (arrow, Figure 6a), whereas antibodies to c-Jun and JunB did neither abolish nor raise the AP-1 complex. The same result was obtained from both EGF-treated and non-treated cells (Figure 6a). An antibody raised against c-Fos could also produce a supershift band and reduced the DNA-binding of the AP-1 complex (Figure 6b).

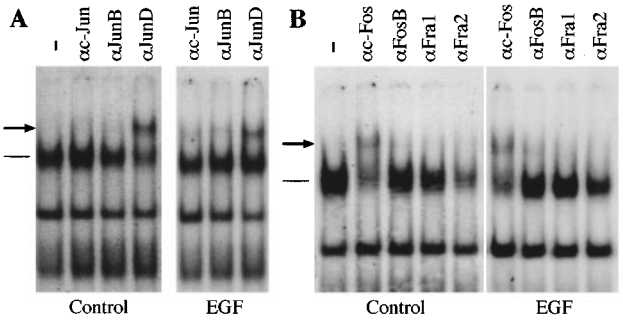

Figure 6 FiRE binds c-Fos/JunD complexes in EGF-treated and non-treated keratinocytes. Gel retardation analysis, with nuclear extracts derived from EGF-treated (EGF) and nontreated (control) MCA3D cells, was performed in the presence of indicated antibodies (see Materials and methods) raised against specific members of the Jun- (**a**) and Fos-family (**b**) proteins. JunD raised a supershift (arrow) for control and EGF-treated extracts. c-Fos similarly raised a supershift for both extracts and Fra-2 reduced the protein binding of the AP-1 complex (horizontal lines)

Antibodies to FosB and Fra-1 had no effect, but the antibody to Fra-2 reduced binding of the AP-1 complex to some extent (Figure 6b). Interestingly, similarly to the Jun-family members, no changes were seen for Fos-family members between non-treated and EGF-treated extracts. Same results were obtained by using the motif 5 oligonucleotide instead of motif 4, for both Fos- and Jun-family members. These results demonstrated that FiRE preferentially binds c-Fos/JunD heterodimers in keratinocytes, but that Fra-2 might also be involved. Most importantly, the results demonstrated that the growth factor treatment did not alter the composition of the AP-complex, and thus can not explain the difference in FiRE activation between growth factor-treated and non-treated keratinocytes.

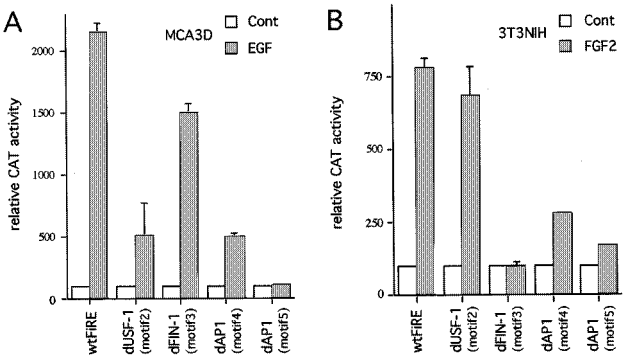

Figure 5 Deletion analysis of nuclear protein binding sites in keratinocytes and fibroblasts. To estimate the importance of each motif, deletion mutants for all the protein binding DNA regions were generated by PCR (see Materials and methods). For dAP-1 (motif 5) the entire motif was deleted and dAP-1 (motif 4) the central part was replaced by a *Spe*I site. For dUSF-1 the E-Box (CACCTG) on motif 2 was changed to *Kpn*I recognition site (GGTACC). For dFIN-1 a central part of the motif (TCAGGGT) was replaced by a *Spe*I site (AATC*ACTAGT*GA). Transfections and CAT assays were performed as in Figure 2. Means and ranges of two independent experiments are shown. Except for the binding domain for FIN-1, deletion of each motif dramatically decreased the activation of FiRE by EGF in MCA3D cells. In contrast, deletion of the USF-1 site in 3T3 cells had no effect, while binding site for FIN-1 was mandatory

Discussion

Syndecan-1 heparan sulfate proteoglycan is expressed in cell type- and development-specific pattern (Kim *et al*., 1994). In this study we have shown that syndecan-1 is induced by different growth factors in a cell type-dependent manner. Thereafter, we have shown that similarly to syndecan-1 mRNA, the growth factor inducible gene element (FiRE) is regulated by different growth factors depending on the cell type. We have demonstrated that FiRE is regulated by EGF, but not by FGF-2, in keratinocytes, while in fibroblasts it is regulated solely by FGF. We have showed that although the transcription factors of the AP-1-family, Fos and Jun, are required for the EGF-induction in keratinocytes and for the FGF-induction in fibroblasts, differences in utilization and induction of the FIN-1 nuclear protein occur between these two cell types. Differences in the utilization of USF-1 were also found in the two cell types. Furthermore, the growth factor-stimulation increased binding of transcription factors to FiRE in fibroblasts, but not in keratinocytes. Finally, we showed that the growth factor-stimulation did not alter the composition of the AP-1 complex in keratinocytes. Taken together the results suggest that the growth factor-specificity of FiRE is obtained by the

transcription factors adjacent to the AP-1 and that the differential regulation by EGF and FGF-2 happens at the level of regulating DNA-binding of transcription factors in fibroblasts, but not in keratinocytes. Furthermore, it suggests that the regulation of syndecan-1 by growth factors could be obtained by a single element. This would be advantageous, for example, in organ development. During tooth development syndecan-1 is tightly regulated in time and space at histological boundaries by the epithelial-mesenchymal interactions (Vainio *et al*., 1989), known to be governed by several growth factors. Although if there was overlapping expression of FGF-2 and EGF at the border of epithelium and mesenchyme, FiRE could target the expression of syndecan-1 strictly to epithelium by EGF at one time and switch it to mesenchyme by FGF-2 at another time which could explain the sequential expression of syndecan-1 during formation of tissues.

Growth factor-induced activation of FiRE is distinguished from the growth factor-induced mitogenic activity

Keratinocytes respond to EGF, FGF-2 and KGF, shown by our results in MCA3D cells, and previously by other studies in different cultured keratinocytes (Tsuboi *et al*., 1993). Also NIH3T3 fibroblasts were shown to respond to both EGF and FGF-2. Therefore, it is clear that the activation of FiRE, in different cells by different growth factors, does not simply follow the ability of the growth factors to induce cell proliferation. This suggests that the activation of EGFR and FGFR-1 have to differ in their ability to induce a subset of intracellular kinase cascades and subsequently transcription factors. Whether the amount of the cell surface growth factor receptors is equal in the two cell lines or whether the amount could have impact on the activation of FiRE has not been studied. However, even though the lower amount of receptor expression would result in proliferation of cells, but not in activation of FiRE, it would still imply that differential growth factor initiated signaling for cell proliferation and activation of AP-1 regulated genes is required. Therefore, by having the ability to distinguish between cell proliferation and AP-1 activity, FiRE could be useful for studying the possible difference in signaling.

FiRE uses different set of transcription factors depending on the cell type and growth factor stimulus

Besides Fos-Jun complexes, FiRE uses other transcription factors, namely USF-1 and the 50 kD FIN-1 nuclear factor. The usage of these proteins is likely to be responsible for the specificity of FiRE, since in general the AP-1 driven promoters are activated by a wide variety of growth factors (Karin, 1994) and also in the case of FiRE, the AP-1s can not explain the growth factor selectivity. USF-1 has been shown to be expressed both in keratinocytes (Ushikai *et al*., 1994) and fibroblasts (Jaakkola *et al*., 1997; Klucher and Spector, 1990), but FIN-1 binding activity was found only in fibroblasts. It seems that FiRE requires only four out of five nuclear protein complexes for maximal growth factor-induced activation. In NIH3T3 cells these include two AP-1 complexes and the FIN-1 protein but not USF-1. The requirement of FIN-1 is underlined by the fact that EGF, which fails to activate FiRE in fibroblasts, is also unable to activate FIN-1. In contrast, USF-1 might replace the FIN-1in MCA3D cells, which also require AP-1. Notably, other ubiquitous basic helix-loop-helix transcription factors, such as Myc-Max, did not bind to FiRE in either fibroblasts or keratinocytes. The differences in the FiRE composition suggest that the interactions with the basal transcriptionary mechanism, through TAFs, could also differ depending on the cell type.

The low level of induction of AP-1 complexes with either KGF or EGF, compared to the robust induction seen in NIH3T3 cells by FGF-2, might be due to at least two reasons. First, it is possible that small changes in the amount of transcription factor binding could result in large changes in the overall transcription rate. Secondly, in keratinocytes most transcriptional activation could be induced by phosphorylation of the JunD proteins, at sites not affecting the DNA-binding. This is actually suggested by the finding that none of the complexes bound to FiRE in keratinocytes is clearly upregulated by growth factors and furthermore, that the composition the AP-1 complexes is not affected by the growth factors. Therefore, it is likely that the growth factors, by inducing kinase activity, post-translationally modify the phosphorylation of JunD in keratinocytes resulting in enhanced capacity of transactivation, but not in enhanced capacity of DNA-binding. In fibroblasts the main effect of growth factors seems to be enhancing DNA-binding of transcription factors AP-1 and FIN-1. Actually, this was previously suggested by the finding that binding of both AP-1 and FIN-1 is dependent on *de novo* protein synthesis as it can be blocked by cycloheximide (Jaakkola *et al*., 1997). However, additional phosphorylation of the AP-1 complex also by FGF-2 in fibroblasts can not be ruled out. Interestingly, the MCA3D keratinocytes which do not differentiate upon calcium or growth factor treatment (Kulesz-Martin *et al*., 1983), did not show binding of other Jun-family members than JunD. JunD has been shown to be constitutively expressed in differentiating keratinocytes while e.g. JunB is upregulated (Gandarillas and Watt, 1995). Surprisingly, binding of c-Jun, which was absent in keratinocytes, was found in fibroblasts both for motifs 4 and 5 (not shown).

FiRE- a novel tool for analysis of growth factor-induced transcription

Our study raises several questions on the possible mechanisms of differential transcriptional regulation executed by the growth factors. Firstly, one needs to ask what are the differences in the intracellular signaling cascades induced by EGFR and FGFR. Secondly, how do these pathways lead to activation of different subset of transcription factors and are all the nuclear proteins involved in activation of FiRE induced by separate signaling cascades. Finally, how does the enhancer complex formation by different proteins lead to equal activation of the basal transcription machinery. These questions will make FiRE a useful tool for studying the mechanisms behind the specificity of growth factor induced signaling and transcriptional activation.

Materials and methods

Cell culture, plasmids and transfections

NIH3T3 mouse fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) to approx. 70% confluence. MCA3D cells were cultured in Ham's F-12 medium (Gibco BRL) supplemented with 10% FCS. For growth factor treatment FCS was replaced with 2% carboxy-methyl-Sepahadex eluted FCS (CMS) 2 days before adding growth factors. Growth factors were used at 10 ng/ml overnight. The human recombinant growth factors were purchased from PeproTech.

The plasmid constructs used in this study have been described in detail previously (Jaakkola *et al*., 1997). Briefly; to make the pFiRE construct *Pst*I–*Sty*I/blunt fragment from −10 kb of syndecan-1 promoter was subcloned into the *Pst*I–*Eco*RV or *Pst*I–*Sma*I sites of pBluescript vector and transferred to the *Xba*I–*Sph*I sites in pCATProm vector. pCATProm vector was constructed by exchanging the SV40 promoter of pCATpromoter vector (Promega) to 1.1 kb *Bgl*II–*Xho*I fragment of syndecan-1 promoter. Mutant plasmids were generated by PCR. For dAP-1 (motif 5) the motif 5 was totally deleted. For dUSF-1 the E-Box (CACCTG) on motif 2 was changed to *Kpn*I recognition site (GGTACC). For dFIN-1 a central part of the motif 3 (TCAGGGT) was replaced by a *Spe*I site (AATC*ACTAGT*-GA). For dAP-1 (motif 4) the AP-1 site on motif 4 (GGAG*TGAGCCA*TGCC) was replaced by *Spe*I site (AATC*ACTAGT*GATT).

For transient transfections NIH3T3 and MCA3D cells were plated at equal density on six-well plates (Falcon). Plasmid DNA was transfected into cells by calcium phosphate method (Chen and Okayama, 1987). A β-galactosidase expressing plasmid (pSV-β-galactosidase, Promega) was co-transfected with CAT constructs to monitor transfection efficiencies. Three parallel transfections were used in every assay. Directly after transfection growth factors were added, media changed the next day and cells were harvested at day two. CAT activities were measured by liquid scintillation and β-galactosidase activities spectrofotometrically at 420 nm (Vihinen *et al*., 1993). Stable transfections were made by transfecting simultaneously pMAMNeo plasmid and 10-fold molar excess of CAT reporter plasmids by calcium phosphate method and selecting cells with 750 μg/ml of G418. Several independent clones were pooled.

For Northern blot analysis cells were lysed in 4M guanine isothiosyanate and RNA was isolated by acid guanium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987), run on 1% agarose and transferred on Hybond-N$^+$ (Amersham) nylon membrane. Membrane was prehybridized as recommended by the manufacturer and hybridized with multi-prime (Promega) labeled partial cDNA of mouse syndecan-1 gene (PM-4). The membrane was washed as recommended by the manufacturer and rehybridized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Nuclear extracts and gel retardation assays

For nuclear extracts MCA3D and NIH3T3 cells were plated on 16 cm dishes, grown to 50–70% confluency, serum starved for 2 days and treated with or without growth factors for 4 h. Nuclear proteins were extracted by a modification described by Lee *et al*., (1988). Protein concentrations were measured by Bradford reaction. Whole cell extracts were prepared by freezing the cells after harvesting and pelleting. They were subsequently resuspended in a 400 mM sodium salt buffer and ultracentrifuged for 5 min at 50 000 r.p.m. and the supernatant was used for gel shift analysis, incubating approximately 6 μg of protein extract for each reaction.

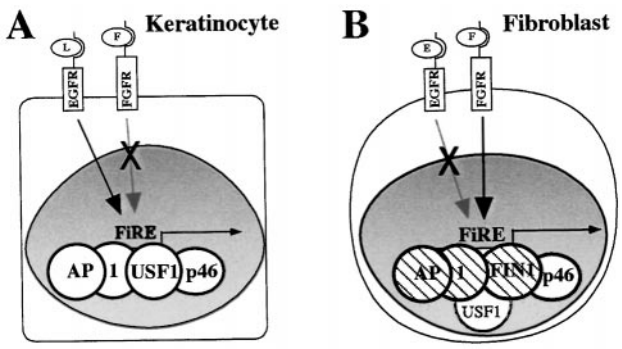

Figure 7 A model summarizing the transcription factors involved in the activation of FiRE in keratinocytes and fibroblasts. FiRE uses different transcription factors upon growth factor-stimulation in keratinocytes and fibroblasts. Hatched circles represent transcription factors induced by growth factors; FGF-2 in fibroblasts and EGF in keratinocytes. The empty circles represent constitutively bound transcription factors. The circle in faint gray (USF-1 in fibroblasts) represents a possibly non-mandatory transcription factor

For gel mobility shift assays double stranded oligonucleotides were end-labeled with γ-^{32}P-dATP (ICN Biomedicals) by T4 polynucleotide kinase (Promega). Oligonucleotides used (top strand) were 5′-dGCTGGCACAC CCACCGT-CAC GAGAGCTTCC-3′ (motif **1**), 5′-TTGGCACACC TGGGAGGATG-3′ (motif **2**), 5′-AGTGGTTCAG GGT-GACTCT-3′ (motif **3**) and 5′-AGGAGTGAGC CATGC-CACC-3′ (motif **4**) and 5′-CTGGGTCATT GATGACT-GTT GTGTGGGATA CCTG-3′ (motif **5**). In a 12 μl reaction 5 μg of nuclear extracts were incubated with labeled oligonucleotide, 2 μg of poly-d(I-C) and 2× reaction buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA and 10% glycerol) for 15 min at RT. The complexes were analysed by electrophoresis in 4.5% polyacrylamide gel. For supershifts 1 μl of specific antibody was added to the reaction 15 min before the labeled oligonucleotide. All the antibodies used were purchased from Santa Cruz Biotechnology Inc. Antibodies used in Figure 3 recognizing all Fos-family and Jun-family members were c-Fos (K-25)X and c-Jun/AP-1 (D)X, respectively. For USF-1 and Max proteins the USF-1 (C-20)X and Max(C-17)X antibodies were used. For specific Fos and Jun-family members the antibodies used in Figure 4 were; c-Fos (4)X, FosB (102)X, Fra-1 (N-17)X, Fra-2 (Q-20)X, c-Jun/AP-1 (N)X, JunB (N-17)X and JunD (329).

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