

Wound reepithelialization activates a growth factor-responsive enhancer in migrating keratinocytes

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ABSTRACT Wound reepithelialization and keratinocyte migration require strictly ordered gene expression, which is assumed to be initiated by locally released mitogens and exposure of the cells to different matrix components. The mechanisms triggering gene expression specifically during reepithelialization are poorly understood. The far upstream AP-1-driven, FGF-inducible response element (FiRE) of the syndecan-1 gene was activated during cutaneous wound healing in transgenic mice. FiRE was induced selectively in migrating but not in proliferating keratinocytes at the wound edge. The activation was initiated at the start of the cell migration, was persistent throughout the merging and stratification phases, and was terminated after completion of reepithelialization. Although FiRE has been found within the gene of syndecan-1, the proximal promoter of syndecan-1 was not required for activation of FiRE in the migrating keratinocytes. The wounding induced activation was inhibited by blocking cell surface growth factor receptors with suramin. However, the activation of FiRE in resting skin required simultaneous growth factor- and stress-induced signals, but could also be elicited by the phosphatase inhibitor, okadaic acid. The activation by both wounding and chemical stimuli was blocked by inhibiting extracellular regulated kinase and p38 MAP kinases, suggesting the involvement of at least two parallel signal transduction pathways in wounding induced gene activation. As FiRE shows specificity for migrating keratinocytes only, it can be a useful tool for future wound healing studies and for targeting genes to injured tissues.—Jaakkola, P., Kontusaari, S., Kauppi, T., Määttä, A., Jalkanen, M. *FASEB J.* 12, 959–969 (1998)

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WOUND REPAIR is a complex process that requires the ordered expression of several genes. Reepithelialization, requiring keratinocyte migration over the provisional matrix, is essential to rebuilding a protective layer after wounding. Reepithelialization starts after

a lag period of several hours and lasts until the keratinocytes have covered the dermis, stratified, and a new basement membrane has formed (1). It is not completely clear which cells lead the migration, but they seem to include basal and suprabasal keratinocytes in both the wound edge skin and hair follicle remnants (2). Several genes are specifically expressed during keratinocyte migration. These include $\alpha 5 \beta 1$, $\alpha \nu \beta 6$, and $\alpha \nu \beta 5$ integrins, and various proteases such as plasminogen and matrix metalloproteinases (2). In addition, many proteins such as growth factor receptors (3) and cell surface proteoglycans (4, 5) are up-regulated during these processes. Less knowledge is available about transcription factor induction, but evidence exists for wound edge expression of at least the activator protein 1 (AP-1)³ family members c-Jun and c-Fos (6, 7). However, the mechanisms triggering gene expression in mammals during keratinocyte migration are not well understood. Our knowledge is especially restricted about the mechanisms whereby specific genes are turned on and off when the resting keratinocytes become migratory at the wound edge.

While the peptide growth factors seem to play an essential role in the initiation and control of wound healing and reepithelialization by regulating the chemotaxis, proliferation, and migration of different cells (8), they are also major candidates for regulating

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³ Abbreviations: AP-1, activator protein 1; CAT, chloramphenicol acetyltransferase; CMS, carboxy-methyl-Sephadex eluted FCS; EGF, epidermal growth factor; ERK, extracellular regulated kinase; FGF-2, basic fibroblast growth factor; FiRE, FGF-inducible response element FCS, fetal calf serum; KGF, keratinocyte growth factor; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; PCNA, proliferating cell nuclear antigen; PKC, protein kinase C; PI-3 kinase, phosphatidylinositol 3-kinase; RTK, receptor tyrosine kinase; TGF- α , transforming growth factor alpha; X-Gal, 5-bromo-4-cholo-3-indolyl- β -D-galactopyranoside; SynPr, syndecan-1 proximal promoter; FiRE-TK, FiRE-thymidine kinase promoter.

wounding induced gene expression. Growth factors involved in the reepithelialization process include, at least, the epidermal growth factor (EGF) family members EGF, transforming growth factor alpha (TGF- α), and the fibroblast growth factor (FGF) family members FGF-2 and keratinocyte growth factor (KGF or FGF-7). EGFs are produced by several different cell types and act on epithelial cells, stimulating their migration and cell division (9). Likewise, FGF-2 and KGF, which are produced by fibroblasts, act on keratinocytes (10–12). EGF has been shown to enhance wound healing (13) as well as KGF (14) and FGF-2 (15). Besides growth factors, other extracellular signals, including disruption of cell–cell or cell–matrix contacts and the provisional matrix, might contribute to the initiation of migration, reepithelialization, and activation of gene expression (1, 2, 16).

To study the growth factor-initiated transcriptional regulation *in vivo*, we have made transgenic mice with a previously characterized far upstream FGF-inducible response element (FiRE) (17) in front of a β -galactosidase reporter gene. The FiRE element has two binding sites for Fos and Jun complexes, called AP-1—one for USF-1 and one for a putatively novel transcription factor, FIN-1. The AP-1 sites are mandatory for activation of FiRE. However, FiRE also requires the other transcription factors adjacent to AP-1s. In fibroblasts, FiRE is induced only by members of the FGF family but not by, for instance, platelet-derived growth factor or EGF (17). The FiRE has been found on the gene syndecan-1, which is a transmembrane heparan sulfate proteoglycan that is strictly regulated during development and tissue regeneration (18). It is known to be up-regulated in migrating and proliferating keratinocytes during cutaneous wound healing, and also in endothelial cells close to the wound site (4, 5). In this study, we show that FiRE is activated selectively in migrating keratinocytes during the reepithelialization phase of wound healing. We also show that the syndecan-1 proximal promoter (SynPr) is not necessary for wounding induced activation of FiRE. Furthermore, by developing a method to monitor FiRE activity in the presence of inhibitory chemicals in organ culture, we suggest that the induction of FiRE requires the involvement of at least two signaling pathways. As this activation is seen only in regenerating and not in resting skin, FiRE could prove to be a valuable tool for wound healing investigations and might have implications in modifying wound healing by targeting genes into wound sites.

MATERIALS AND METHODS

Plasmids, transfections, and generation of transgenic mice

The SynPr construct carries a XhoI–SphI (–115 to –2163 bp from the syndecan-1 translation initiation site) fragment

(19) in front of a reporter gene in the Lac-Z vector. The FiRE construct is the same, but has the 280 bp far upstream (from approx. –10 kb) of the FGF-inducible element (PstI–StyI), next to the SynPr fragment. For the FiRE-thymidine kinase promoter (FiRE-TK) construct, the entire SynPr was deleted by BamHI–BglII digestion from the FiRE plasmid. An BglII–BamHI fragment (+124 to +293) of thymidine kinase promoter derived from a pBLCAT2 cloning vector was inserted into this site. Constructs were released from vector sequences by NotI digestion, separated on agarose gels, electroeluted, and dissolved into the injection buffer. Transgenic mice were generated by microinjecting (C57Bl/6*DBA/2)F1 fertilized eggs, using standard methods (20). Founder animals were identified from tail DNA by polymerase chain reaction analysis (21), using Lac-Z specific oligonucleotides. After the initial experiments, the FiRE strain mice were regularly tested for β -galactosidase enzyme expression by performing a tail wound (22).

MCA3D cells were cultured in Ham's F-12 medium (Gibco Brl, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS). For growth factor treatment, FCS was removed 2 days before adding growth factors. The plasmid constructs used for MCA3D transfections have been described in detail (17). Briefly, the p-271FiRECAT construct contains the FiRE element and 98 bp proximal promoter fragment of syndecan-1 gene in front of chloramphenicol acetyltransferase (CAT) gene. Stable transfections were made by transfecting simultaneously pMAMNeo plasmid and 10-fold molar excess of CAT reporter plasmids by a calcium phosphate method and selecting cells with 750 μ g/ml of G418. Several independent clones were pooled.

Wounding experiments

For wound healing studies, 8- to 12-wk-old mice of both sexes were anesthetized by 2.5% avertin and the hair was cut; full thickness incisional wounds were made on the back or on the tails of the mice with a scalpel, and left unsutured and uncovered. The experimental procedures were approved by the ethics committee of the University of Turku. At specified time points, the mice were killed, the wounded parts were removed, fixed in 4% paraformaldehyde for 30 min, washed three times for 30 min, and stained overnight with 1 mg/ml of 5-bromo-4-cholo-3-indolyl- β -D-galactopyranoside (X-Gal) at RT (22).

Skin culture and analysis of FiRE activation

For organ culture studies, mice were killed and approximately 1 cm² of intact skin pieces, after hair removal, were removed with a scalpel. They were allowed to float in cell culture conditions overnight in DMEM supplemented with 10% FCS or 2% CMS (carboxy-methyl-Sephadex eluted FCS) in a 24-well plate. Growth factors and chemicals were added to the culture medium for the next 24 h, followed by fixation, β -galactosidase staining, and production of histological sections. Inhibitory chemicals were added 0.5 h before growth factors or okadaic acid. Growth factors were purchased from PeproTech (London, U.K.) and other compounds from CalbioChem, Nottingham, U.K. (PD 098059, suramin, genistein, bisindolylmaleimide) or Sigma, St. Louis, Mo. (anisomycin, wortmannin, rapamycin). SB 203580 was a kind gift from SmithKline Beecham (Philadelphia, Pa.).

To study the impact of the inhibitory chemicals on FiRE activity, mice were killed and their tails were cut into consecutive pieces of approximately 0.5 cm of length (10–12 pieces were obtained from a single tail). Each tail piece was placed separately in a single well of a 24-well plate (Falcon, Oxnard, Calif.) and maintained overnight in cell culture conditions

(DMEM supplemented with 10% FCS). The inhibitory chemicals (at concentrations indicated in the text and in Figs. 5 and 7) were added to the culture medium, with a lag period of 0.5 to 4 h after cutting the tail. The next day medium was removed, followed by fixation and X-Gal staining, as described above. The keratinocytes tend to migrate over the surface of the cut tail end. These cut ends were photographed with a microscope; the photographs were subsequently scanned (Agfa Arcusscan II) at 150 dots per inch and imported into an image analyzer system of the Microcomputer Image Device system (MCID, Imaging Research Inc.). FiRE activation was evaluated as the area of β -galactosidase staining (blue color, identified by the analyzing program) proportioned to the total surface of the cut tail end. In each experiment, β -galactosidase activity in the chemical-treated wound edges was compared to the nontreated (control) tail ends by assigning a value of 1.00 (100%) to the proportional area of the blue color in the control tail (Fig. 5D and Fig. 7A).

Immunohistochemistry

Tissues were fixed in 4% formalin, dehydrated in ascending concentrations of ethanol, and embedded in paraffin. Tail pieces were treated with 4% formic acid for 2–4 days before embedment. Microtome sections (5 μ M) were mounted on glass slides treated with poly-L-lysine. Standard techniques were used for hematoxylin-eosin staining. For immunohistochemistry, proliferating cell nuclear antigen (PCNA) (Novocastra) recognizing primary antibody and subsequent avidin-biotin-peroxidase complex techniques (Vectastain, Vector Laboratories, Burlingame, Calif.) were used.

RESULTS

FiRE is activated in reepithelializing keratinocytes of healing wound

To assess the *in vivo* function of FiRE, two separate transgenic mouse strains harboring a reporter gene (Lac-Z) ligated to the syndecan-1 promoter regions were produced: the first with 2.25 kb of the SynPr (23, 24) in front of the β -galactosidase reporter gene, and the second with an additional far upstream 280 bp enhancer element (FiRE) positioned immediately upstream of the SynPr-LacZ gene (Fig. 1A). Because FiRE was known to be growth factor dependent in fibroblasts and because several growth factors are known to participate in wound healing, we targeted our investigations to wounded skin, where syndecan-1 expression is also known to be up-regulated (4, 5). Linear wounds were made to the back and to the tail of mice; the skin and tail were subsequently removed and stained with X-Gal. Dermal cells did not show Lac-Z activity, whereas in all 12 mouse lines that were DNA positive with the FiRE construct, Lac-Z activity was found at the wound site keratinocytes (Fig. 1B; Fig. 2, Fig. 3), in contrast to the six DNA-positive SynPr mouse lines, which illustrated no staining (Fig. 1C). The intact skin and several other adult organs showed no consistent staining in either SynPr or FiRE strains. However, during embryonic development

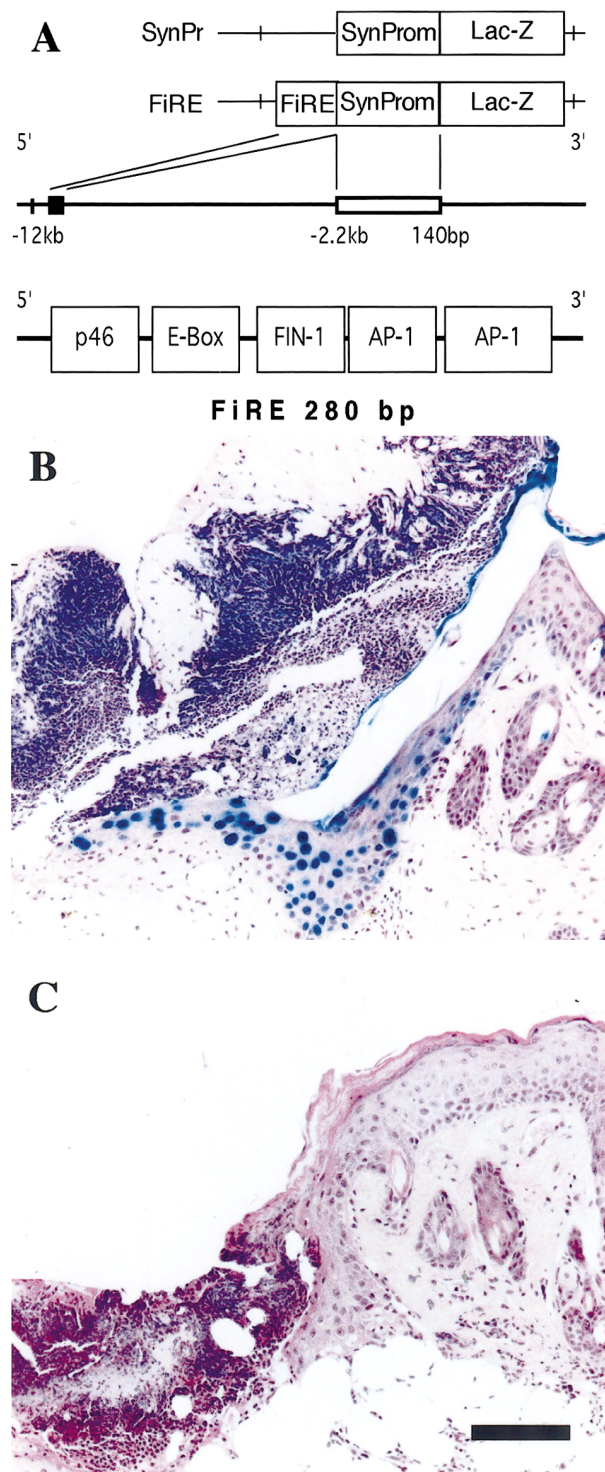


Figure 1. FiRE is activated during wound healing. A) Schematic model of the two transgenic constructs containing 2.25 kb of syndecan-1 basic promoter region (SynPr) in front of β -galactosidase gene, and an additional AP-1-driven far upstream 280 bp enhancer (FiRE). A detailed structure of nuclear factor binding sites (Jaakkola et al., ref 17) for FiRE is provided in the lower panel. B) At day 3 postwounding, FiRE activity is intense throughout the migrating keratinocyte layer. β -Galactosidase staining marking FiRE activity is shown in blue on the epithelial cell nuclei. C) Back wound of SynPr mouse at day 3 postwounding shows no Lac-Z activity. Bar, 100 μ m.

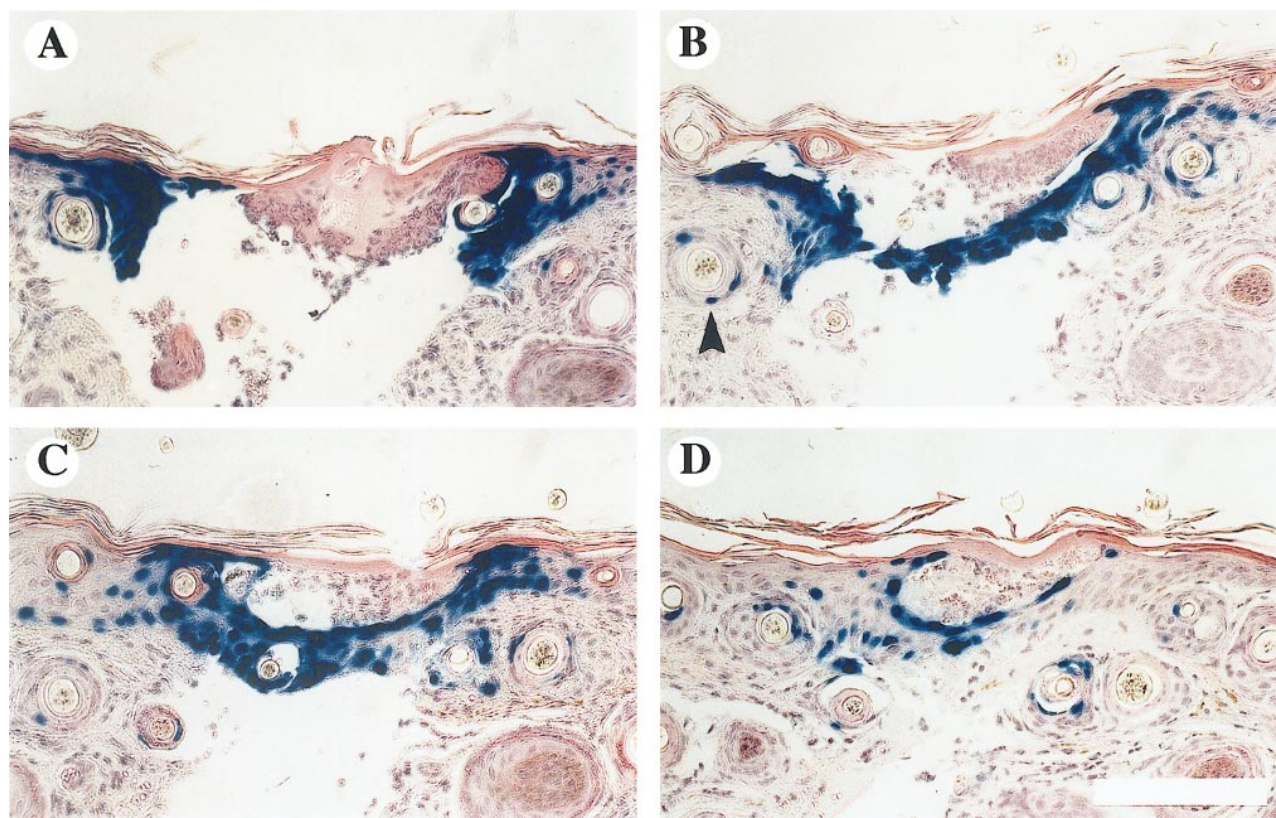


Figure 2. Activation of FiRE is restricted to the reepithelialization phase of wound healing. Consecutive sections of a single tail wound of a FiRE mouse. *A*) β -Galactosidase staining (blue) is intense throughout the reepithelialization phase including the initial stage of migration, the merging epithelium (*B*), and subsequent stratification (*C*). *D*) In fully epithelialized wound, FiRE activity fades away. The arrow in panel *B* points to one of the FiRE-positive hair follicle keratinocytes. Bar, 100 μ m.

both FiRE and SynPr strains showed nonspecific Lac-Z activity in neural tissue and limbs, demonstrating that the quiescence of the SynPr strain during wound healing was not due to its chromosomal inactivation. Furthermore, the 2.2 kb proximal promoter fragment has been shown to drive high transcriptional activity in cultured keratinocytes and fibroblasts (19).

Several time points spanning from 4 h up to 15 days were selected to study the timing of FiRE activation. During the early inflammatory phase (from 4 to 18 h), no Lac-Z activity was seen. However, at 20 to 24 h postwounding, β -galactosidase staining, indicating activation of FiRE, appeared at the wound edge. This correlates with the time at which keratinocyte migration is assumed to begin. The staining was most abundant at the third day (Fig. 1*B*) and started to decline at approximately day 7. To reveal the FiRE activation on different stages of wound healing, consecutive sections of a single tail wound were produced (Fig. 2). In this model, a cross section from the middle of the incision shows a situation where the keratinocyte migration is just starting, whereas sections from the ends of the incision present a reepithelialized keratinocyte sheet. Staining was first seen in the stratified epidermal sheet of the keratinocytes nearest to the incision site starting migration

downward (Fig. 2*A*). In the more advanced phase, FiRE activity was very strong at the leading edge of the migratory keratinocytes and in the merging epithelium (Fig. 2*B*). During subsequent stratification of epithelial cells, FiRE activation was still evident (Fig. 2*C*), but at the end of reepithelialization the staining started to decline, as demonstrated by the most distal section of the tail wound (Fig. 2*D*). The epidermis further from the incision site, as well as the dermal cells and the wound clot, remained negative for β -galactosidase activity throughout the repair process. During the later phases, when dermal integrity was being reestablished and the epidermis still maintained a hyperproliferative state, FiRE was not reactivated. In the hair follicle keratinocytes adjacent to the incision site, FiRE was also activated (Fig. 2*B*, arrow). These cells, as well as the wound edge keratinocytes, are supposed to be starting sources for keratinocyte migration.

FiRE is activated in migrating but not in dividing keratinocytes

The relationship between FiRE activity and cell proliferation was investigated by staining the FiRE wound sections with a PCNA antibody (25). During

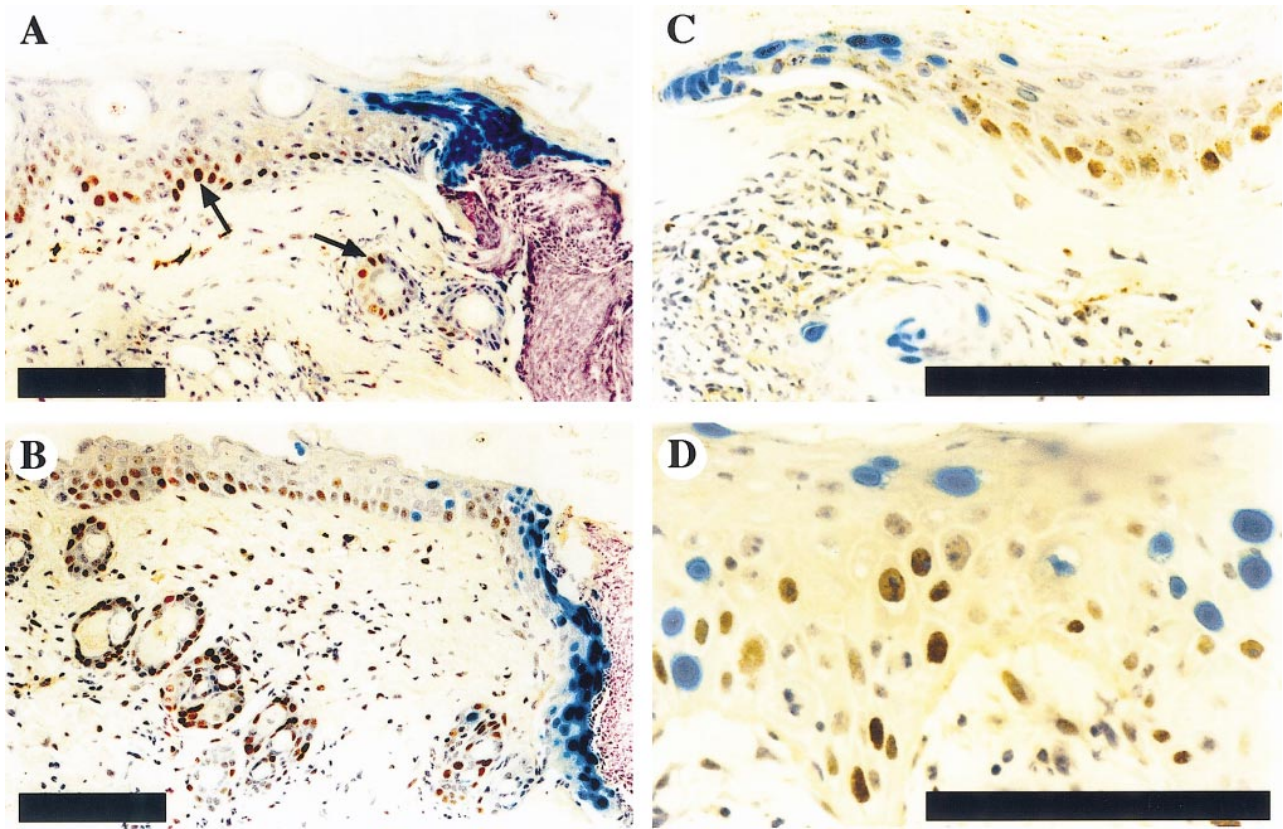


Figure 3. FiRE activation is found in migrating but not in proliferating cells. Proliferating cell nuclear antigen (PCNA) and X-Gal double staining were used. *A*) At the early time points of wounding (day 1), PCNA staining (brown color, arrow) is found more distal from FiRE activity in the basal keratinocytes and in hair follicle keratinocytes. *B*) In a more advanced phase (day 2), PCNA staining is more marked but not seen in the migrating cells with X-Gal staining. Higher magnifications of wounds at the start of migration (*C*) and when reepithelialized show robust PCNA staining (*D*), but not simultaneous with FiRE activity. Bars, 100 μ m.

the early time points (e.g., at day 1, when FiRE activity appears at the wound margin), PCNA staining was found to locate more distally, either in the basal keratinocytes and the hair follicle keratinocytes (Fig. 3*A*, arrow; Fig. 3*C*) or in the glandular epithelium. At a more advanced migratory phase (day 2), cell proliferation was more marked in the basal layer, yet in a totally different cell pool than FiRE activity (Fig. 3*B*). In the fully reepithelialized wound at day 7, where robust cell division was found in the basal keratinocytes, and in the stratum spinosum of the thickened epidermis, the β -galactosidase staining was detected in only a few cells of the differentiated granular layer (Fig. 3*D*). This suggested that the activation of FiRE is strictly restricted to the migrating keratinocytes during wound healing.

Syndecan-1 proximal promoter is not required for activation of FiRE

To investigate the impact of SynPr on the wounding induced activation of FiRE, we constructed an Lac-Z expression plasmid where the 2.2 kb syndecan-1 promoter in the FiRE plasmid was replaced by a 170 bp

fragment of the FiRE-TK (Fig. 4*A*, and Materials and Methods). Four independent DNA-positive transgenic mouse strains were obtained by microinjection. Linear wounds were made into the backs and tail of the mice. As with the FiRE-SynPr construct, the FiRE-TK construct was found to be activated at the wound sites in all four strains (Fig. 4*B*). Strong β -galactosidase staining was seen at the migrating keratinocyte sheet while the intact skin as well as the dermis remained negative for Lac-Z activity. Similar to the FiRE-SynPr, the FiRE-TK was also activated in the nearby wound site hair follicle keratinocytes. This demonstrated that FiRE does not require the syndecan-1 promoter to be properly activated during wound healing.

Activation of FiRE is inhibited by suramin and genistein

FiRE has previously been shown to be activated by FGF-1 and -2 in mesenchymal cells (17). Therefore, we hypothesized that the activation of FiRE would involve growth factors in vivo. To assess the molecular mechanisms inducing FiRE in wounded epithelium,

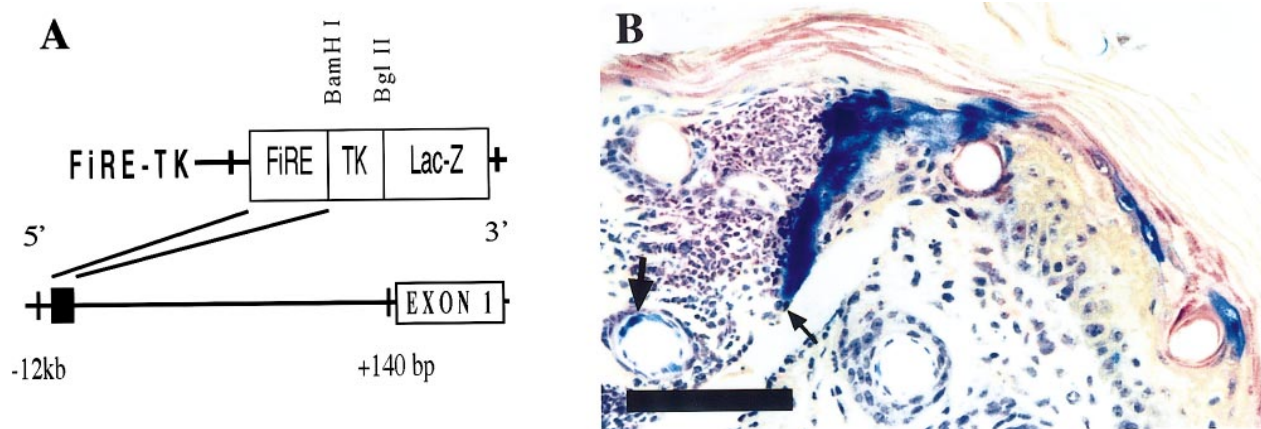


Figure 4. FiRE does not require syndecan-1 promoter. *A*) Schematic presentation of the FiRE thymidine kinase promoter-LacZ (FiRE-TK) construct. The syndecan-1 proximal promoter (SynPr; see Fig. 1*A*) from FiRE plasmid has been replaced by a 170 bp BamHI-BglII fragment of thymidine kinase basal promoter. *B*) A 2-day tail wound from a FiRE-TK mouse. The FiRE-TK is activated in migrating wound keratinocytes, similar to the FiRE construct. A thin arrow points to the tip of the migrating keratinocyte sheet and the thick arrow points to one of the hair follicle keratinocytes stained by β -galactosidase. Bar, 100 μ m.

we tested chemicals known to inhibit activation of cell surface receptor tyrosine kinases (RTKs) by growth factors in an organ culture model described in Materials and Methods. It is known that extracted skin pieces are viable for several days in cell culture conditions (26).

Suramin is a well-characterized heparin analog that blocks the binding of several growth factors (e.g., EGF and KGF) (3) to their receptors. It has been shown to have some retarding effects on wound healing without inhibiting reepithelialization (27). Gen-

istein, on the other hand, is a specific inhibitor of tyrosine kinases that, among other tyrosine kinases, blocks the growth factor receptors. The ability of these chemicals to inhibit FiRE activity was assayed in a model where mice tails were cut into consecutive pieces after culturing with or without the inhibitors and subsequently stained with X-Gal. Inhibition of the β -galactosidase activity in the healing surface of the cut tail ends was easily visible (Fig. 5). Numerical data were obtained by developing a method where FiRE activation was evaluated as a proportional inten-

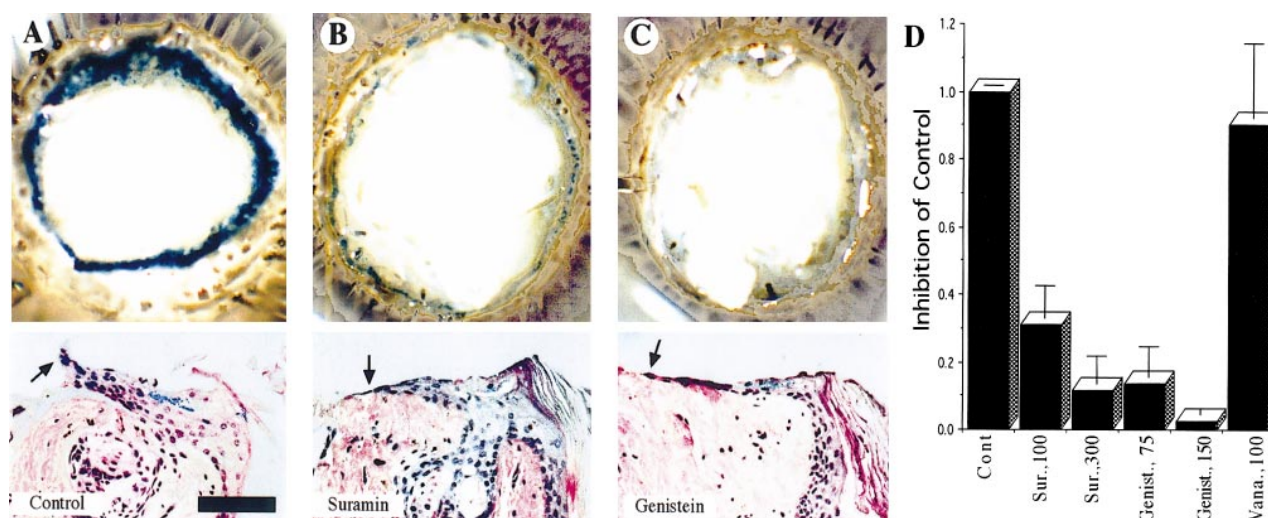


Figure 5. Activation of FiRE is inhibited by suramin and genistein. Mice tails were cut into pieces, followed by culture without (*A*) or with the growth factor antagonist suramin (100 μ M) (*B*), the tyrosine kinase inhibitor genistein (75 μ M) (*C*), or the tyrosine kinase phosphatase inhibitor vanadate (100 μ M). Blue staining indicates FiRE activity on the healing surface of cut tail end. Sections of the chemical-treated tail pieces were produced to reveal keratinocyte migration over the cut surface (lower panels). Black arrows point to the leading edge of the migrating keratinocyte sheet. Bar, 100 μ m. *D*) Inhibition of wounding induced FiRE activation by different chemicals was evaluated as the proportional area of β -galactosidase staining on the healing surface of cut tails and analyzed by the Image Analyzer System (see Materials and Methods). Suramin (Sur.), genistein (Genist.), and orthovanadate (Vana.) were used in the micromolar concentrations indicated. In each experiment, β -galactosidase activity in the chemical-treated wound edges was correlated with the nontreated (control) wounds. Means and standard deviations of five to eight independent experiments are shown.

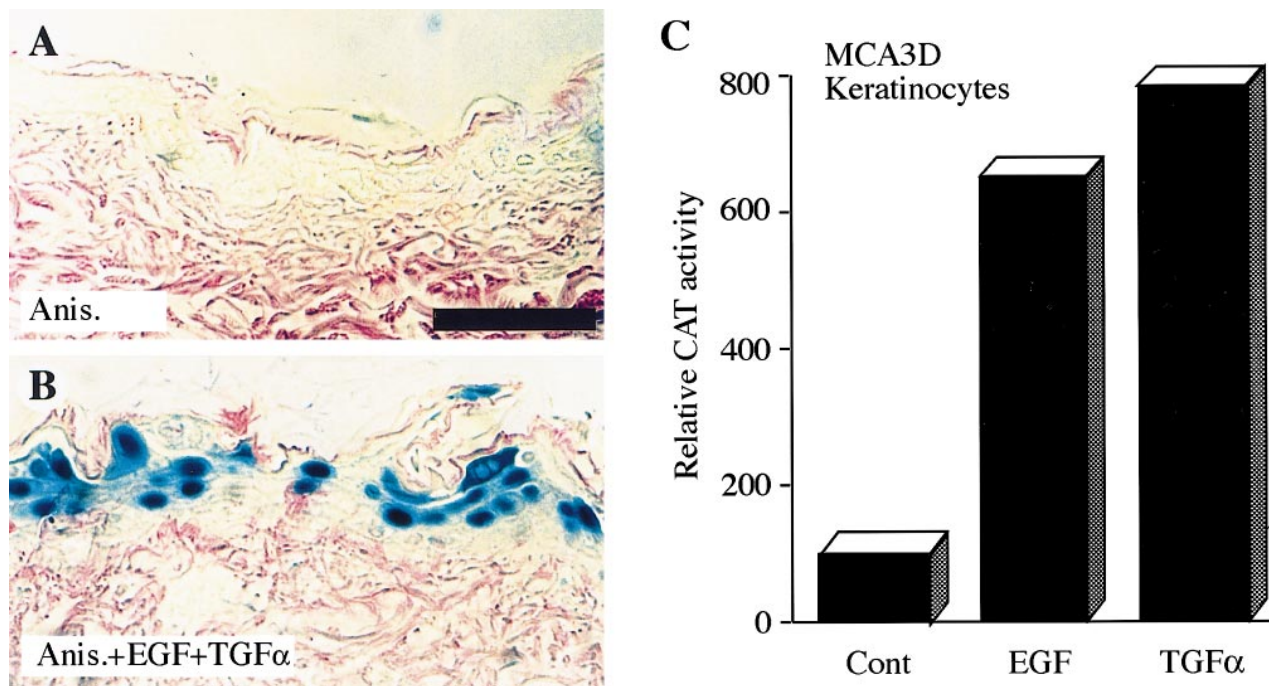


Figure 6. Activation of FiRE is induced by growth factor stimuli in resting skin. Skin pieces were tape stripped and cultured in DMEM and 2% CMS for 2 days in the presence of the indicated compounds. In resting skin, anisomycin (1 μ g/ml) (Anis.) alone cannot trigger FiRE activity (A), but FiRE activity is induced when given simultaneously (B) with a cocktail of growth factors (EGF and TGF- α , 200 ng/ml each). C) Activation of FiRE in MCA3D keratinocyte cell line. Cells were stably transfected with p-271FiRECAT plasmid (see Materials and Methods); after serum starvation, they were treated overnight with EGF or TGF- α , followed by determination of CAT enzyme activity. Bar, 100 μ m.

sity of the Lac-Z activity to the healing surface of the tail pieces (for detailed description of the procedure, please see Materials and Methods). Both suramin and genistein inhibited FiRE activation in a concentration-dependent manner (Fig. 5). Suramin (100 μ M) reduced the staining intensity to approximately 30%; with 300 μ M suramin, the activation of FiRE was almost completely blocked (Fig. 5B, D). The tyrosine kinase inhibitor genistein was also found to inhibit FiRE at 30 μ M. The enzyme activity was reduced to approximately 10% at 75 μ M and was totally abolished at 150 μ M (Fig. 5C, D). In contrast to growth factor receptor and tyrosine kinase blocking agents, the tyrosine phosphatase inhibitor vanadate had only a marginal effect on FiRE activity at 100 μ M (Fig. 5D). As many of the chemicals are known to be cytotoxic, we investigated whether the lack of FiRE activation is due to total blockage of cell migration. As judged by histological sections, none of the chemicals completely prevented keratinocyte migration (Fig. 5, lower panels). These data suggested that activation of FiRE in migrating keratinocytes might be dependent on growth factor-induced RTK activation.

Growth factors, together with anisomycin, activate FiRE in nonwounded skin

Since inhibition of growth factor action could block the wounding induced FiRE activity, we tested

whether the growth factors could elicit activation of FiRE in intact skin. As introducing various growth factors into the media of cultured nonwounded skins did not activate FiRE, we considered the possibility of a requirement for signaling cascades other than those triggered by growth factors. The Raf/MAPKK1/ERK (Raf/mitogen-activated protein kinase kinase/extracellular regulated kinase) cascade is one well-established pathway known to be induced by growth factors. Therefore, we also stimulated the stress-activated JNK/SAPK and p38 MAPK signaling pathways by anisomycin and UV irradiation. Pieces of skin were tape stripped, which is known to induce at least the EGF receptor (28), and treated with a combination of growth factors (a cocktail of EGF and TGF- α) and anisomycin in organ culture. FiRE was not activated if anisomycin (Fig. 6A) or growth factors (not shown) were added separately to the culture medium. However, treatment of the skin simultaneously with the growth factors and anisomycin resulted in the activation of FiRE in the keratinocytes of nonwounded skin (Fig. 6B). Equivalent results were obtained by treating the skin pieces simultaneously with UV irradiation (80 J / m²) and growth factors. Okadaic acid, known to activate several intracellular kinases, Fos and Jun proteins, and AP-1-driven promoters (29) by inhibiting protein phosphatases 1 and 2A, alone was a potent activator of FiRE in resting skin (Fig. 7B). The skin of SynPr strains was not in-

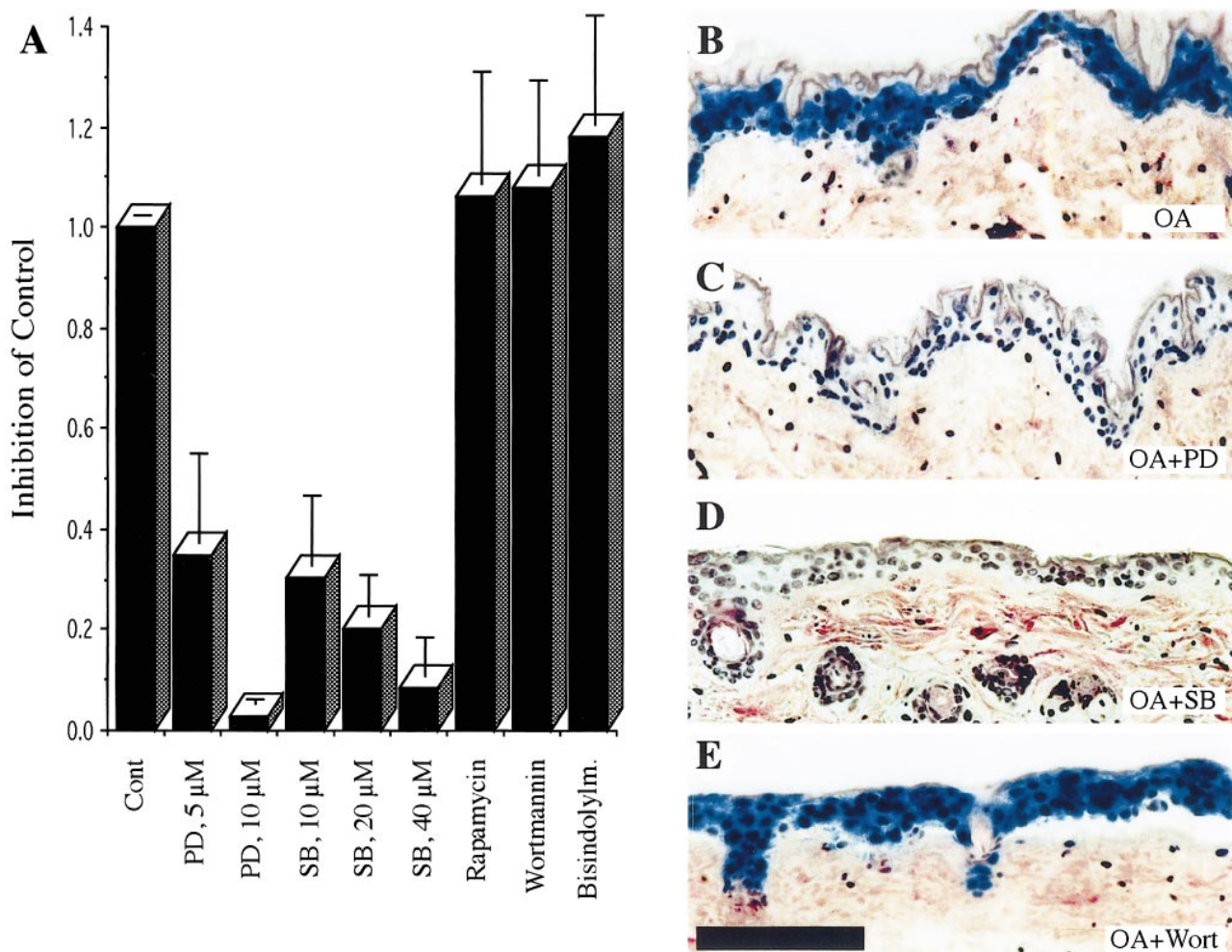


Figure 7. Blocking ERK or p38 MAP kinase inhibits FiRE activity. *A*) Compounds known to specifically inhibit different intracellular kinases were used in an experiment comparable to that shown in Fig. 5. PD 098059 (PD), a compound that inhibits activation of MAP kinases ERK-1 and -2 by MAPKK1, reduced activation of FiRE at 5 μ M and totally blocked it at 20 μ M. SB 203580 (SB), a specific inhibitor of stress-induced p38 MAPK, also acted as an inhibitor of FiRE at 5 μ M and completely blocked the activity at 40 μ M. High concentrations of the PI-3 kinase inhibitor wortmannin (5 μ M) had no effect on the wound-induced activation of FiRE. Similar to wortmannin, p70 S6 kinase inhibitor rapamycin (100 nM) and PKC inhibitor bisindolylmalamide (Bisindolylm.) (1.2 μ M) did not interfere with FiRE activity. Means and standard deviations of five to seven independent experiments are shown. *B–D*) Skin pieces were cultured in DMEM and 10% FCS for 2 days in the presence of the compounds indicated. *B*) Okadaic acid (100 nM) was a potent activator of FiRE in nonwounded skin. *C*) Pretreatment with PD 098059 (20 μ M) blocked okadaic acid-induced Lac-Z activity (*D*), as did SB 203580 (40 μ M). *E*) Wortmannin (2 μ M) had no effect of the okadaic acid-induced activation FiRE. Bar, 100 μ m.

ducible by any of these treatments (not shown). We also examined whether TGF- α , as well as EGF, could elicit activation of FiRE in cultured keratinocytes. Therefore, an immortalized keratinocyte cell line, MCA3D (30), was stably transfected by p-271Fi-RECAT, a plasmid that contains the FiRE element and 98 bp of syndecan proximal promoter in front of a CAT reporter gene. The transfected cells were grown without serum for 2 days and treated overnight with or without the growth factors, followed by determination of CAT enzyme activity. As shown in Fig. 6C, both EGF and TGF- α were able to increase CAT activity by severalfold, indicating that FiRE responds to these growth factors. A reporter plasmid with only the syndecan proximal promoter did not respond to

either EGF or TGF- α (not shown). All these results suggested that the activation of FiRE can be elicited by growth factors in keratinocytes, but also that the activation might depend on two separate signaling pathways: 1) growth factor-induced RTKs and 2) a stress-induced pathway.

Inhibiting either ERK or p38 MAP kinases blocks FiRE activity

To further investigate the signal transduction pathways downstream from the cell surface, we used well-characterized compounds known to specifically inhibit different intracellular kinases in an experiment comparable to that shown in Fig. 5 (Fig. 7A). PD

098059, a chemical that inhibits activation of MAP kinases ERK-1 and -2 by MAPKK1 at micromolar concentrations (31), was able to reduce activation of FiRE at 5 μ M and totally block it at 20 μ M, implying involvement of MAP kinases in this activation. A specific inhibitor of stress-induced p38/RK MAPK, SB 203580, shown to be selective for p38 MAPK at least up to 100 μ M (32), also acted as an inhibitor of FiRE in a concentration-dependent manner. The β -galactosidase staining was clearly reduced at 10 μ M and abolished at 40 μ M concentrations. In contrast to these two compounds, neither the phosphatidylinositol 3-kinase (PI-3 kinase) inhibitor wortmannin (33), even at a concentration of 2000 nM, nor the p70 S6 kinase inhibitor rapamycin, at 100 nM, had any effect on the wound-induced activation of FiRE. Likewise, the protein kinase C (PKC) -specific inhibitor bisindolylmaleimide (1.2 μ M) failed to inhibit FiRE activation (Fig. 7A). As well as an inhibitory effect on wounding induced Lac-Z activity, pretreatment with either PD 098059 or SB 203580 was able to abolish okadaic acid (Fig. 7C, D, respectively), whereas wortmannin up to 5 μ M had no inhibitory effect (Fig. 7E). These results suggest that although the ERK and p38 pathways might be mandatory for transcriptional activation of AP-1-regulated genes in migrating keratinocytes, the PI-3 kinase or the typical PKCs may not be necessary.

DISCUSSION

Several wounding induced elements have been described in plants, their activation possibly transmitted by ERK homologues (34). However, these have not been identified in mammals. In this study, we demonstrate wounding induced transcriptional activation of a growth factor-regulated and AP-1-driven enhancer (FiRE) of syndecan-1 gene during reepithelialization of keratinocytes in mammalian tissue. In adult tissue, a Lac-Z gene driven by the enhancer and the mouse syndecan-1 proximal promoter was expressed solely in the migrating, but not in proliferating, keratinocytes of healing wounds. Moreover, the Lac-Z gene driven by FiRE, together with thymidine kinase basal promoter, was expressed equally in the migrating keratinocyte sheet, demonstrating that the element is not necessarily restricted to syndecan-1. The activation of FiRE could be mimicked in an organ culture model of skin pieces by simultaneous treatment with a growth factor cocktail and anisomycin or by an application of okadaic acid alone. Furthermore, by using well-characterized, cell-permeable chemicals known to specifically inhibit different intracellular kinases, we showed that the activation of Lac-Z expression is dependent on at least two diverse signaling pathways.

Syndecan-1 mRNA and protein levels are induced from 10- to 20-fold in migrating keratinocytes at skin wound sites of adult mice (5). Syndecan-1 is also induced in murine and human endothelial cells, granulation tissue, and dermis (4, 5). Since the activation of FiRE is seen in the migrating keratinocytes, it might be responsible for the induced syndecan-1 expression in these cells. However, in other cells expressing syndecan-1 after wounding, no FiRE activity is seen. This could be due to other elements regulating syndecan-1 expression, but could also indicate that FiRE-induced β -galactosidase activity is not strong enough to be detected by X-Gal staining in these cells.

Besides syndecan-1, various AP-1-regulated genes including matrix remodeling metalloproteinases (35) and keratins (36) are expressed during trauma and wound healing. Transcription factors Fos and Jun have been proposed to be activated during wounding or wounding induced tumorigenesis (6, 7), and it is likely that Fos and Jun could mediate the growth factor-induced expression of several genes during keratinocyte migration. However, the induction of AP-1 might not to be sufficient to initiate transcription in some promoters, and there could be a requirement for other transcription factors in order to trigger strong expression and enhance the specificity of expression. This seems to be true for activation of FiRE, which is inactivated by deletion of binding sites for transcription factors other than AP-1 in, for example, NIH3T3 cells (17).

The substantial time (at least several hours) required to initiate FiRE activation and keratinocyte migration might be due to earlier signaling events necessary to induce all the mandatory cell surface components on the migratory cells. This could represent, for example, a growth factor-regulated cascade involving several steps, ending in the autocrine release of growth factors and activation of their cognate receptors on migrating keratinocytes. For example, KGF is released by fibroblasts and acts on keratinocytes, where it has been shown to induce the expression of TGF- α and EGF receptor (EGFR) (37). Activation of FiRE could be delayed until sufficient amounts of EGFR are available at the cell surface. EGF and TGF- α are known to induce proliferation of keratinocytes (9, 11), whereas the dividing keratinocyte population is completely FiRE negative. This might be due to the lack of activated stress receptors or maintained cell-cell contacts. On the other hand, FiRE-negative dermal cells might also lack several components demanded for activation, including growth factor receptors such as EGFR, insufficient activation of stress-induced signals, and activation of required transcription factors. The attenuation of FiRE after reepithelialization could be due not only to the down-regulation of local growth factor release, but also to the down-regulation of stress-activated signals

after reestablishing cell-to-cell and cell-to-matrix contacts.

In addition to RTK-activating growth factors such as EGF, which are known to regulate the ERK signaling cascade, the activation of FiRE is dependent on other extracellular stimuli, triggering the stress-activated p38 MAPK pathway. In cultured MCA3D keratinocytes, FiRE could be activated only by growth factor treatment, which might be due to the constitutive activity of p38 MAPK in these cells (P. Jaakkola, unpublished observation). Several different cellular stresses, such as UV irradiation and osmotic shock, have been shown to activate p38, though they are poor activators of ERKs (38). Besides the diverse stresses, some cytokines (including interleukins and TNF- α) are able to induce p38 (39). The requirement of UV irradiation or anisomycin, together with growth factors of the EGF family, implies that the growth factors are not responsible for activation of p38, whereas the growth factors are prime candidates for triggering signals of FiRE activation via ERK. What the signal (or signals) is that triggers the p38 in migrating keratinocytes during wound healing remains to be investigated. Several possibilities exist, including involvement of interleukins, the local release of cell degradation products, or the loss of cell to matrix contacts and subsequent changes in adhesion molecule expression. It has been suggested that both the growth factor and stress-induced activations could be triggered through RTKs (40). Although the growth factor receptors can also be the major starting point for stress-induced signaling pathway in some biological situations, in our model there is a requirement of both growth factor- and stress-regulated events to activate FiRE in nonwounded skin, which suggests that these stimuli do not trigger the same kinase activity of the receptors or subsequent downstream mediators. However, whether the two stimuli might still be triggered by different growth factor receptors or by activating different subset of signaling molecules by one type of growth factor receptor cannot be ruled out. Whatever the explanation is, it is implied that gene transcription requires a variety of signals during wound reepithelialization.

FiRE can be considered a healing process-related gene response element. Our initial analysis of the human homologue of FiRE has indicated a high similarity between the human and murine DNA sequences (J.-P. Pursiheimo et al., unpublished observation). Whether it is also activated in the context of other growth factor-regulated regeneration processes remains to be studied. Although found upstream from the gene of syndecan-1, the FiRE or similar elements might also function to up-regulate other genes in reepithelializing wound keratinocytes. This is suggested by the fact that FiRE is activated at wound sites regardless of the basal promoter it co-operates with. FiRE will be a highly useful tool for

growth factor-induced and healing-related gene expression studies, and it might also have therapeutic applications, for example, in poorly healing ulcerative wounds where FiRE-driven therapeutic agents, such as keratinocyte and fibroblast chemoattractants or mitogens, could be introduced into wound margin keratinocytes.

[FJ]

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REFERENCES

1. Woodley, D. T. (1996) in *The Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., ed) 2nd Ed, pp. 339–354, Plenum, New York
2. Martin, P. (1997) Wound healing—aiming for perfect skin regeneration. *Science* **276**, 75–81
3. Marchese, C., Cheddidi, M., Dirch, O. R., Csaky, K. G., Santanelli, F., Latini, C., LaRocelle, W. J., Torrisi, M. R., and Aaronson, S. A. (1995) Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin. *J. Exp. Med.* **182**, 1369–1376
4. Gallo, R., Kim, C., Kokenyesi, R., Adzick, N. S., and Bernfield, M. (1996) Syndecans-1 and -4 are induced during wound repair of neonatal but not fetal skin. *J. Invest. Dermatol.* **107**, 676–683
5. Elenius, K., Vainio, S., Laato, M., Salmivirta, M., Thesleff, I., and Jalkanen, M. (1991) Induced expression of syndecan in healing wounds. *J. Cell Biol.* **114**, 585–595
6. Marshall, G. M., Vanhamme, L., Wong, W. Y., Su, Y., and Vogt, P. K. (1992) Wounding acts as a tumor promoter in chickens inoculated with avian sarcoma virus 17. *Virology* **188**, 373–377
7. Martin, P., and Nobes, C. (1992) An early molecular component of the wound healing response in rat embryos—induction of c-fos protein in cells at the epidermal wound margin. *Mech. Dev.* **38**, 209–215
8. Bennet, N., and Schultz, G. (1993) Growth factors and wound healing: biochemical properties of growth factors and their receptors. *Am. J. Surg.* **165**, 728–737
9. Nanney, L. B., and King, L. E., Jr. (1996) *The Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., ed) 2nd Ed, pp. 171–194, Plenum, New York
10. Basilico, C., and Moscatelli, D. (1992) The FGF family of growth factors and oncogenes. *Adv. Cancer Res.* **59**, 115–165
11. Tsuboi, R., Sato, C., Kurita, Y., Ron, D., Rubin, J. S., and Ogawa, H. (1993) Keratinocyte growth factor (FGF-7) stimulates migration and plasminogen activator activity of normal human keratinocytes. *J. Invest. Dermatol.* **101**, 49–53
12. Werner, S., Smola, H., Liao, X., Longaker, M., Krieg, T., Hofschneider, P. H., and Williams, L. (1994) The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* **266**, 819–822
13. Brown, G. L., Nanney, L. B., Griffen, J., Cramer, A. B., Yancey, J. M., Cutsinger, L. J., Holtzin, L., Schultz, G. S., Jurkiewicz, M. J., and Lynch, J. B. (1989) Enhancement of wound healing by topical treatment with epidermal growth factor. *N. Engl. J. Med.* **321**, 76–79
14. Staiano-Coico, L., Krueger, J. G., Rubin, J. S., D'limi, S., Vallat, V. P., Valentino, L., Fahey, T., Hawes, A., Kingston, G., Madden, M. R., Mathwich, M., Gottlieb, A. B., and Aaronson, S. A. (1993)

- Human keratinocyte growth factor effects in a porcine model of epidermal wound healing. *J. Exp. Med.* **178**, 865–878
15. Davidson, J. M., and Broadley, K. N. (1991) Manipulation of the wound-healing process with basic fibroblast growth factor. *Ann. N.Y. Acad. Sci.* **638**, 306–315
16. Clark, R. A. F. (1996) *The Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., ed) 2nd Ed, pp. 3–50, Plenum, New York
17. Jaakkola, P., Vihinen, T., Määttä, A., and Jalkanen, M. (1997) Activation of an enhancer on syndecan-1 gene is restricted to the fibroblast growth factor-family members. *Mol. Cell. Biol.* **17**, 3210–3219
18. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L., and Lose, E. J. (1992) Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu. Rev. Cell Biol.* **8**, 365–393
19. Vihinen, T., Määttä, A., Jaakkola, P., Auvinen, P., and Jalkanen, M. (1996) Functional characterization of mouse syndecan-1 promoter. *J. Biol. Chem.* **271**, 12532–12541
20. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
21. Hanley, T., and Merlie, J. P. (1991) Transgene detection in unpurified mouse tail DNA by polymerase chain reaction. *Bio-Techniques* **10**, 56
22. Behringer, R. R., Crotty, D. A., Tennyson, V. M., Brinster, R. L., Palmiter, R. D., and Wolgemuth, D. J. (1993) Sequences 5' of the homeobox of the Hox-1.4 gene direct tissue-specific expression of Lac-Z during mouse development. *Development* **117**, 823–833
23. Vihinen, T., Auvinen, P., Alanen-Kurki, L., and Jalkanen, M. (1993) Structural organization and genomic sequence of mouse syndecan-1 gene. *J. Biol. Chem.* **268**, 17261–17269
24. Hinkes, M. T., Goldberger, O. A., Neumann, P. E., Kokenyesi, R., and Bernfield, M. (1993) Organization and promoter activity of the mouse syndecan-1 gene. *J. Biol. Chem.* **268**, 11440–11448
25. Takasaki, Y., Deng, J. S., and Tan, E. M. (1981) A nuclear antigen associated with cell proliferation and blast transformation: its distribution in synchronized cells. *J. Exp. Med.* **154**, 1899–1909
26. Haynes, J. H., Krummel, T. M., Flood, L. C., Cohen, I. K., and Diegelmann, R. F. (1990) Fetal skin organ culture. *J. Invest. Surg.* **3**, 349–355
27. Chamberlain, J., Shah, M., and Ferguson, M. W. J. (1995) The effect of suramin on healing adult rodent dermal wounds. *J. Anat.* **186**, 87–96
28. Stoscheck, C. M., Nanney, L. B., and King, L. E. J. (1992) Quantitative determination of EGF-R during epidermal wound healing. *J. Invest. Dermatol.* **99**, 645–649
29. Schonthal, A., Tsukitani, Y., and Feramisco, J. R. (1991) Transcriptional and post-transcriptional regulation of c-fos expression by the tumor promoter okadaic acid. *Oncogene* **6**, 423–430
30. Kulesz-Martin, M., Kilkenny, A. E., Holbrook, K. A., Digernes, V., and Yuspa, S. (1983) Properties of carcinogen altered mouse epidermal cells resistant to calcium induced terminal differentiation. *Carcinogenesis* **4**, 1367–1377
31. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* **270**, 27489–27494
32. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229–233
33. Arcaro, A., and Wymann, M. P. (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4 5-triphosphate in neutrophil responses. *Biochem. J.* **296**, 297–301
34. Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. *Science* **270**, 1988–1991
35. Rosenberg, G. A. (1995) Matrix metalloproteinases in brain injury. *J. Neurotrauma* **12**, 833–842
36. Navarro, J. M., Casatorres, J., and Jorcano, J. L. (1995) Elements controlling the expression and induction of the skin hyperproliferation-associated keratin K6. *J. Biol. Chem.* **270**, 21362–21367
37. Dlugosz, A., Cheng, C., Denning, M., Dempsey, P., Coffey, R., and Yuspa, S. (1994) Keratinocyte growth factor receptor ligands induce transforming growth factor alpha expression and activate the epidermal growth factor receptor signaling pathway in cultured epidermal keratinocytes. *Cell Growth Differ.* **5**, 1283–1292
38. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027–1037
39. Cuenda, A., Cohen, P., Buee-Scherrer, V., and Goedert, M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.* **16**, 295–305
40. Sachsenmaier, C., Radler-Pohl, A., Zinck, R., Nordheim, A., Herrlich, P., and Rahmsdorf, H. J. (1994) Involvement of growth factor receptors in the mammalian UVB response. *Cell* **78**, 963–972

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