Expression of the α₅ Integrin Subunit Gene Promoter Is Positively Regulated by the Extracellular Matrix Component Fibronectin through the Transcription Factor Sp1 in Corneal Epithelial Cells in Vitro*

Kathy Larouche‡, Steeve Leclerc, Christian Salesse§, and Sylvain L. Guérin¶
From the Oncology and Molecular Endocrinology Research Center, and §Ophthalmology Research Unit, CHUL/CHUQ and Laval University, Ste-Foy, Quebec G1V 4G2, Canada

The accumulation of fibronectin (FN) in response to corneal epithelium injury has been postulated to turn on expression of the FN-binding integrin αβ1α in this work, we determined whether the activity directed by the α5 gene promoter can be modulated by FN in rabbit corneal epithelial cells (RCEC). The activity driven by chloramphenicol acetyltransferase/α5 promoter-bearing plasmids was drastically increased when transfected into RCEC grown on FN-coated culture dishes. The promoter sequence mediating FN responsiveness was shown to bear a perfect inverted repeat that we designated the fibronectin-responsive element (FRE). Analyses in electrophoretic mobility shift assays provided evidence that Sp1 is the predominant transcription factor binding the FRE. Its DNA binding affinity was found to be increased when RCEC are grown on FN-coated dishes. The addition of the MEK kinase inhibitor PD98059 abolished FN responsiveness suggesting that alteration in the state of phosphorylation of Sp1 likely accounts for its increased binding to the α5 FRE. The FRE also proved sufficient to confer FN responsiveness to an otherwise unresponsive heterologous promoter. However, site-directed mutagenesis indicated that only the 3′ half-site of the FRE was required to direct FN responsiveness. Collectively, binding of FN to its α5β1 integrin activates a signal transduction pathway that results in the transcriptional activation of the α5 gene likely through altering the phosphorylation state of Sp1.

Corneal wounds account for a substantial proportion of all visual disabilities and medical consultations for ocular problems in North America. They can be superficial with damage limited to the epithelium or associated with a deeper involvement of the epithelial basement membrane and of the stromal lamella. Severe recurrent and persistent corneal wounds are most commonly secondary to ocular diseases and damage such as recurrent erosion, mild chemical burns, superficial herpetic infections, neuroparalytic cornea, autoimmune diseases, and stromal ulcerations due to viral or bacterial infections or to severe burns (1). Despite currently available treatments, many of these corneal wounds persist for weeks and months or else recur frequently and can progress to corneal perforation.

Tissue repair requires cell migration, proliferation, and adhesion. Cell adhesion and migration in turn require extracellular matrix (ECM) synthesis and assembly. ECM is a complex, cross-linked structure of proteins and polysaccharides. It organizes the geometry of normal tissues. Fibronectin (FN) is an ECM adhesion protein identified as a potential wound healing agent because of its cell attachment, migration, differentiation, and orientation properties (for a review see Refs. 2–4). In the unwounded rat eye, FN is observed by immunohistological staining at the level of the corneal epithelium basement membrane (5–7). Shortly after corneal injury, the basal cells that border the injured area and stromal keratocytes start producing massive amounts of FN (5, 8–11). FN promotes corneal cell migration both in vitro (12, 13) and in vivo (14) by acting as a temporary extracellular matrix to which corneal epithelial cells attach as they migrate over the wounded area (13, 15). Once the wound is re-epithelialized, the subepithelial immunohistochemical staining of FN progressively decreases (5, 16–18).

The increase in FN expression that has been reported to occur during corneal wound healing was postulated to be coordinated with the expression of its major integrin receptor α5β1 (5), as has also been shown for laminin and tenascin and their corresponding integrin receptor subunits α5 and αv, respectively (19–21). For instance, the integrin α5β1 was shown to be present during corneal wound healing after radial keratectomy (22). Direct evidence that FN can positively alter αβ1 integrin expression at both the protein and mRNA levels has been provided through FN antisense expression studies performed in the epithelium-derived human colon carcinoma cell line Moser (23) as well as in murine AKR-2B fibroblasts (24). Other indirect evidence linking expression of αβ1 to that of FN has also emerged from recent studies (25, 26).

As a consequence, it is not surprising that ECM, through its interactions with membrane-bound integrins, exerts profound influences on the major cellular program of growth, differentiation, and apoptosis by altering, through a number of signal

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¶ From the Oncology and Molecular Endocrinology Research Center, and Ophthalmology Research Unit, CHUL/CHUQ and Laval University, Ste-Foy, Quebec G1V 4G2, Canada.

1 The abbreviations used are: ECM, extracellular matrix; FN, fibronectin; RCEC, rabbit corneal epithelial cells; FRE, fibronectin-responsive element; EMSA, electrophoretic mobility shift assays; CAT, chloramphenicol acetyltransferase; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; hGH, human growth hormone; bp, base pair; OM, osteosarcoma M; LDLR, low density lipoprotein receptor; VEGF, vascular endothelial growth factor; AChR, acetylcholine receptor; FBS, fetal bovine serum.
transduction pathways, the transcription of genes whose specific functions are linked to these cellular functions. Binding of ECM components, such as FN, with their corresponding integrin receptors will trigger the activation of intracellular signalizing mediators such as focal adhesion kinase, mitogen-activated protein kinases (MAPKs), and Rho family GTPases (for a review see Ref. 27). Activation of the MAPK signal transduction pathway is of particular interest since it links integrin-mediated signaling to transcriptional regulation of genes that are crucial for cell growth and differentiation. The results presented hereby provide evidence that, by acting on α5 gene expression, such a route of signal transduction might alter cell adhesion properties as well. The downstream cascade of family members that are activated following transient activation of Ras GTP-binding proteins through receptor tyrosine kinases include MAPK/ERK kinase (designated MEK) and ERK1 (p44)/ERK2 (p42) (28). Activation of ERK1/ERK2 through phosphorylation causes their translocation to the nucleus, where they have been reported to phosphorylate and activate distinct transcription factors, such as ELK, c-Jun, and c-Myc (29–31), as well as members of the ETS family (such as PEA3) (32).

In the present study, we demonstrated that FN can alter the transcription of the α5 integrin subunit gene at the promoter level. Such a FN responsiveness was shown to be determined by the binding of the transcription factor Sp1 to a target site that is part of a perfect inverted repeat which, by itself, can confer FN responsiveness to an otherwise unresponsive heterologous promoter. Most of all, the FN-activated, integrin-mediated signal transduction pathway appears to require activation of ERK1/ERK2 since the Sp1 DNA binding affinity, and, as a consequence, the FN responsiveness of the α5 promoter were both found to be diminished by blocking their activation with the MEK kinase inhibitor PD98059. Together, these results demonstrate the novel finding that the α5 integrin subunit, through activation of the MAPK pathway, can autoregulate its own synthesis in a manner that is dependent on the extracellular concentration of FN.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Media—**Rabbit corneal epithelial cells (RCECs) were obtained from the central area of freshly dissected rabbit corneas as described previously (33) and then grown to low (near 15% coverage of the plates), intermediate (near 75% coverage), or high cell density (100% coverage or more than 48 h) under 5% CO2 in SHMEM medium supplemented with 5% FBS and 20 µg/ml gentamicin. When indicated, human plasma FN (obtained as described previously (34)) or ECM gel (basement membrane matrix from Engelbreth-Holm-Swarm mouse sarcoma, Fisher) was coated for 18 h at 37 °C on the culture dishes at (basement membrane matrice from Engelbreth-Holm-Swarm mouse plasma FN (obtained as described previously (34)) or ECM gel (basement membrane matrix from Engelbreth-Holm-Swarm mouse sarcoma, Fisher) was coated for 18 h at 37 °C on the culture dishes at

**FN Responsiveness of the α5 Integrin Gene Promoter**

**Fig. 1. a5 promoter activity in RCEC grown with or without FN.** A, cell density dependence of the α5 promoter FN responsiveness. RCEC were plated at low (1×10^5 cells per Petri), intermediate (5×10^5 cells per Petri), or high cell density (1.5×10^6 cells per Petri); H) on either uncoated or FN-coated (2 µg/cm^2) tissue culture dishes. Cells were transiently transfected 24 h later with the α5–954 recombinant plasmid and harvested 48 h post-transfection. CAT activities were measured and normalized to hGH as described under “Experimental Procedures.” Each value is expressed as the ratio of the CAT activity from RCEC grown on FN-coated Petri dishes over that of RCEC grown solely on plastic. Standard deviation is provided for each individual value. B, dose-dependent activation of the α5 promoter FN responsiveness in RCEC. RCEC (3×10^5 cells/Petri) were plated on culture dishes that had been coated with either none or increasing concentrations of FN (1–16 µg/cm^2). RCEC were then transfected with the α5–954 recombinant construct and harvested 48 h later. CAT activity was determined and normalized as described under “Experimental Procedures.” Each value is expressed as detailed in A.

The double-stranded oligonucleotides used in the present study were chemically synthesized using a Biosearch 8700 apparatus (Millipore). They contained the DNA sequence from the human α5 promoter comprised between positions −82 and −36 and designated the α5 FRE (5′-GATCCAGCGTGGGTGGAGCCTG-3′) (37), the high affinity binding site for the positive transcription factor Sp1 (5′-GATCATATCTGCCGGAGGGCGCCGACGACAG-3′) (38) or the Sp1-binding site (designated pL2A) identified in the basal promoter from the mouse p12 gene (5′-GATCCAGTGGTGAGCCTG-3′) (36).

**Transient Transfection and CAT Assay—**RCEC plated at either low (5×10^5 cells per 35-mm tissue culture plates), intermediate (5×10^5 cells per 35-mm tissue culture plates), or high (1×10^6 cells per 35-mm tissue culture plates) cell density were transiently transfected using the polycationic detergent LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer. Each LipofectAMINE-transfected plate received 1.5 µg of the test plasmid and 0.5 µg of the human growth hormone (hGH)-encoding plasmid pXGH5 (39). Drosophila Schneider cells were transfected according to the calcium phosphate precipitation procedure (36, 40) at a density of 1×10^6 cells per 60-mm culture plate.

Levels of CAT activity for all transfected cells were determined as described (40) and normalized to the amount of hGH secreted into the culture media and assayed using a kit for quantitative measurement of hGH (Immunocorp, Montréal, Quebec, Canada). Because the metallothionein-I promoter, which directs expression of hGH from the pXGH5 plasmid, proved to be highly inefficient in Drosophila cells, CAT activities from transfected Schneider cells were normalized to the amount of β-galactosidase encoded by the plasmid pA5V5-His/LacZ and cotransfected along with the CAT recombinant constructs. Each cell-containing plate therefore received 15 µg of the test plasmid, 4 µg of pA5V5-His/LacZ, and 1 µg of pPPAC (empty vector). In the cotransfection experiments performed with the Sp1 expression plasmid, the empty pPPAC plasmid pAC5/V5-His/LacZ was obtained from Invitrogen (Carlsbad, CA).
The presence of FN at 8 m sequence of a perfect inverted repeat that has been designated as the (100- and 500-fold) of synthetic double-stranded oligonucleotides bears performed using 10^9 shifted DNA-protein complexes generated. Competitions in EMSA were conducted by first incubating varying amounts (as specified in the figure legends) from RCEC grown on either untreated or ECM-coated dishes with not or without ECM. RCEC plated at an intermediate cell density (5 x 10^4 cells per Petri) on either regular or ECM-coated (10 µg/cm^2) FN over CAT – FN. Standard deviation was determined and normalized as described under “Experimental Procedures.” Each value is expressed as detailed in the legend to Fig. 1.

B. CAT activity directed by recombinant plasmids bearing 5’ deletions of the α5 promoter in RCEC grown with or without ECM. RCEC plated at an intermediate cell density (5 x 10^4 cells per Petri) for 24 h on either regular or ECM-coated (10 µg/cm^2) culture dishes were transiently transfected with the various 5’ deletion constructs from the α5 promoter (see Fig. 2A). CAT activity was measured and expressed as the ratio of CAT + ECM over CAT – ECM. Standard deviation is provided for each value.

was substituted for 1 µg of pPacSp1. The value presented for each individual test plasmid transfected corresponds to the mean of at least three separate transfections done in triplicate. To be considered significant, each individual value needed to be at least three times over the background level caused by the reaction buffer used (usually corresponding to 0.15% chloramphenicol conversion). Standard deviation is also provided for each transfected CAT plasmid.

Nuclear Extract Preparation—Crude nuclear extracts were prepared from RCEC grown solely on plastic or FN-coated culture dishes and dialyzed against DNAse I buffer (50 mM KCl, 4 mM MgCl₂, 20 mM K₃PO₄, pH 7.4, 1 mM β-mercaptoethanol, 20% glycerol) as described (41) except that a combination of protease inhibitors (peptatin A (0.5 µg/ml), leupeptin (5 µg/ml), chymostatin (5 µg/ml), antipain (5 µg/ml), aprotinin (5 µg/ml), benzamidine (5 mM)) (all reagents from Sigma) was added to all the buffers used in order to restrict proteolysis. Extracts were kept frozen in small aliquots at –80 °C until use.

Electrophoretic Mobility Shift Assays (EMSA) and Supershift Experiments—EMSAs were carried out using either the 27-bp α5 FRE or the high affinity Sp1 oligomer as 5’ end-labeled probes. Approximately 2 x 10⁶ cpm labeled DNA was incubated with crude nuclear proteins (as specified in the figure legends) from RCEC grown on either untreated or FN-coated culture dishes in the presence of 500 ng of poly(dI-dC)poly(dI-dC) (Amersham Pharmacia Biotech) in buffer D (5 mM HEPES (pH 7.9), 10% glycerol (v/v), 25 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol, 0.125 mM phenyl methosulfonyl fluoride). Occasionally, crude nuclear extracts from human HeLa cells were also used in EMSA as a positive control for comparison purposes. Incubation proceeded at room temperature for 10 min upon which time DNA-protein complexes were separated by gel electrophoresis through 6% native polyacrylamide gels run at 4 °C against Tris glycine buffer as described. Each value is expressed as detailed in the legend to Fig. 1.

B. Competitions in EMSA were conducted by first incubating varying amounts (as specified in the figure legends) from RCEC grown on either untreated or ECM-coated (10 µg/cm^2) FN over CAT – FN. Standard deviation was determined and normalized as described under “Experimental Procedures.” Each value is expressed as detailed in the legend to Fig. 1.

FN Responsive of the α5 Integrin Gene Promoter—The Activity Directed by the α5 Integrin Subunit Gene Promoter Is Positively Regulated by Fibronectin—Studies conducted by Rajagopal et al. (23) and Huang et al. (24) both provided evidence that the level of expression for the α5 integrin subunit was positively modulated by the presence of the extracellular matrix component fibronectin. We therefore exploited transient transfection of primary cultured RCEC using recombinant plasmids bearing the CAT reporter gene fused to various segments from the human α5 gene promoter and assayed for CAT activity in the presence of fibronectin (FN). The results (Figs. 2 and 3) provide evidence that the presence of FN is required for activation of the α5 integrin promoter in RCEC. The data presented in Figs. 2 and 3 show that the α5 integrin promoter is positively regulated by fibronectin (FN). The results (Figs. 2 and 3) provide evidence that the presence of FN is required for activation of the α5 integrin promoter in RCEC. The data presented in Figs. 2 and 3 show that the α5 integrin promoter is positively regulated by fibronectin (FN). The results (Figs. 2 and 3) provide evidence that the presence of FN is required for activation of the α5 integrin promoter in RCEC. The data presented in Figs. 2 and 3 show that the α5 integrin promoter is positively regulated by fibronectin (FN).
gene promoter (35) in order to evaluate whether such an FN-dependent increase in \( \alpha_5 \) mRNA could be determined by discrete cis-acting elements from the \( \alpha_5 \) gene upstream regulatory region. For this purpose, a recombinant plasmid bearing the \( \alpha_5 \) promoter up to position -954 (\( \alpha_5 \)-954) inserted upstream from the CAT reporter gene was transfected into RCEC plated either on plastic or FN-coated culture dishes (2 \( \mu \)g/cm\(^2\)) at varying cell densities. As Fig. 1A indicates, culturing RCEC on FN-coated Petris did not alter the activity driven by the \( \alpha_5 \)-954 plasmid when transfected at low cell density (near 15% coverage of the plates). However, at both intermediate (near 75% coverage) and high (100% coverage for more than 48 h) cell density, the activity of the \( \alpha_5 \) promoter was found to be 6.1- and 6.4-fold, respectively, higher when cells are grown on FN-coated culture plates rather than solely on plastic. Withdrawal of the serum contained into the culture medium (which normally contains 5% FBS) prior to cell seeding on FN-coated culture dishes had no statistical effect on the CAT activity directed by \( \alpha_5 \)-954 (results not presented).

The dose dependence of the \( \alpha_5 \) promoter FN responsiveness was next evaluated by transfecting RCEC plated at an intermediate cell density on culture dishes coated with either none or increasing concentrations of FN (from 1 to 16 \( \mu \)g/cm\(^2\)). As shown on Fig. 1B, the activity directed by the \( \alpha_5 \)-954 plasmid increased proportionally to the amount of FN coated on the culture dishes, reaching a drastic 18-fold stimulation at 16 \( \mu \)g/cm\(^2\) FN. No further increase in \( \alpha_5 \) promoter function was observed at FN concentrations above 16 \( \mu \)g/cm\(^2\) (results not presented). We therefore conclude that the activity of the human \( \alpha_5 \) promoter can be drastically increased when RCEC are grown on FN-coated culture dishes and that such a positive influence is obviously cell density-dependent.

**Fig. 4. Binding of nuclear proteins from RCEC to the \( \alpha_5 \) FRE in vitro.** A, EMSA analysis of the nuclear proteins from RCEC interacting with the \( \alpha_5 \) FRE. The double-stranded oligonucleotide bearing the \( \alpha_5 \) FRE was \( 5' \) end-labeled and incubated with varying concentrations (2–20 \( \mu \)g) of crude nuclear proteins from RCEC grown on either not coated (FN–) or FN-coated (FN+) culture dishes (8 \( \mu \)g/cm\(^2\)). The position of three DNA-protein complexes is shown (a–c) along with that of the free probe (U). P, labeled probe alone. B, competitions in EMSAs. The \( 5' \) end-labeled \( \alpha_5 \) FRE was incubated with 10 \( \mu \)g of crude nuclear proteins from RCEC grown on FN-coated culture dishes in the presence of either 100- or 500-fold molar excess of various unlabeled double-stranded oligonucleotide competitors (FRE, Sp1, NF1, and p12 A). U, free labeled probe; P, labeled probe alone; C, labeled probe with nuclear proteins but without unlabeled competitor.

A Distinct Cis-acting Element from the Basal Promoter of the Human \( \alpha_5 \) Gene Mediates FN Responsiveness in RCEC—Discrete cis-acting regulatory elements are known to mediate many of the regulatory effects that are triggered through signal transduction pathways by binding trans-acting nuclear proteins with distinctive regulatory properties. To determine more precisely the minimal \( \alpha_5 \) promoter sequence required to confer FN responsiveness, CAT recombinant plasmids bearing various 5’ deletions of the \( \alpha_5 \) promoter were transfected into RCEC grown at intermediate density on both plastic and FN-coated (2 \( \mu \)g/cm\(^2\)) culture dishes. Neither the deletion of the \( \alpha_5 \) promoter down to position -178 nor -92 could prevent the average 5-fold increase in \( \alpha_5 \) promoter activity observed when RCEC are grown on FN-coated plates. However, the further deletion of the \( \alpha_5 \) sequences down to position -41 almost totally abolished the FN responsiveness of the \( \alpha_5 \) promoter. A detailed examination of this 41-bp sequence revealed the presence of a perfect inverted repeat of the following sequence, 5’-GGAGTTTG-3’ (Fig. 2B). Therefore, FN responsiveness of the \( \alpha_5 \) promoter appears to be determined by a short stretch of DNA sequence contained between positions -41 and -92 relative to the \( \alpha_5 \) mRNA start site.

**Influence of ECM Components Other Than FN on the Activity of the \( \alpha_5 \) Promoter**—Apart from FN, proteins such as collagen IV, vitronectin, entactin, and laminin are also commonly found in the extracellular matrix. We examined whether components from the ECM other than FN can also alter the expression directed by the \( \alpha_5 \) promoter in RCEC. For this purpose, RCEC were grown to intermediate cell density on either untreated or ECM-coated culture dishes before they were transiently transfected with the \( \alpha_5 \) promoter DNA upstream regulatory sequence. However, optimal promoter activity observed when RCEC are grown on ECM-coated dishes (10 \( \mu \)g/cm\(^2\)) culture dishes. The CAT activity directed by the \( \alpha_5 \) promoter was raised to 4-fold when both FN (2 \( \mu \)g/cm\(^2\)) and the ECM gel (10 \( \mu \)g/cm\(^2\)) are coated together on the culture dishes. However, optimal promoter activation was obtained when FN (2 \( \mu \)g/cm\(^2\)) was coated alone on the culture plates (7.6-fold activation). Transient transfection of RCEC plated on ECM-coated culture dishes with the recombinant plasmids bearing the various 5’ deletions of the \( \alpha_5 \) promoter identified the ECM-responsive element somewhere between positions -178 and -954 (Fig. 3B). We conclude that components from the ECM other than FN had only a moderate effect on the \( \alpha_5 \) promoter activity and that their action is mediated through a cis-acting element distinct from that which determines FN responsiveness in RCEC.

**The Transcription Factor Sp1 Binds Specifically to the \( \alpha_5 \)
FRE in Vitro—In order to determine whether the FN responsiveness mediated by the −41/−92 α5 promoter segment (which also contains the −82 to −56 inverted repeat that has been designated as the fibronectin-responsive element (FRE)) depends on its recognition by nuclear transcription factors, EMSAs were performed. For this purpose, the synthetic oligomer bearing the α5 FRE was 5′ end-labeled and incubated with increasing amounts of crude nuclear proteins (2, 5, 10, and 20 μg) from RCEC grown either on plastic or FN-coated culture flasks (8 μg/cm2). As shown in Fig. 4A, three distinct DNA-protein complexes (designated a, b, and c) were observed upon autoradiography, complex a being the most abundant at 5, 10, and 20 μg of proteins (a few other fast-migrating complexes were also occasionally observed in EMSA but their formation proved to be highly inconsistent). The signal corresponding to both complexes a and c was usually found to be much stronger in the crude extract prepared from RCEC grown on FN-coated culture dishes. Specificity for the formation of these complexes was then evaluated by competition experiments in EMSA using, as unlabeled competitors, various double-stranded oligonucleotides bearing target sequences for known transcription factors. Formation of both complexes a and b could easily be competed off by a 100-fold molar excess of unlabeled FRE, whereas that of complex c was partly prevented at a 100-fold molar excess but nearly completely abolished at a 500-fold excess (Fig. 4B). Formation of both complexes a and b could not be prevented by an unrelated oligomer bearing the target sequence for HeLa CTF/NF-1 in adenovirus type 2. However, that of complex c was efficiently prevented when a 500-fold molar excess of the NF1 oligomer was used suggesting that binding of a member of the NF1 family of transcription factors likely accounts for the formation of this complex. Most of all, an oligomer bearing the high affinity binding site for the positive transcription factor Sp1 could compete for formation of complexes a and b even as efficiently as the FRE itself, a 100-fold molar excess being sufficient to almost totally prevent their formation in EMSA (Fig. 4B). As further evidence that Sp1 or any other member of this family (43) is the major transcription factor binding the α5 FRE, a synthetic oligomer bearing the target sequence for Sp1 that we identified in the basal promoter from the mouse p12 gene (and designated p12.A) (36) was also used as unlabeled competitor. This Sp1 site diverges from the Sp1 consensus by the lack of the central C residue (Fig. 2B) which is substituted by a T in the p12.A element. It is also relatively well preserved with the 3′ half-site of the α5 FRE (9 out of 12 residues) since the five G residues identified as critical for recognition of the p12.A element by Sp1 are also preserved in the α5 FRE (36) (see Fig. 10). As shown in Fig. 4B,
the p12.A element competed nearly as well as the FRE for the formation of both complexes a and b in EMSA. These results suggest that formation of complexes a and b likely results from the recognition of the labeled α5 FRE by distinct members of the Sp1 family of transcription factors and that complex c might result from the recognition of that same probe by a member of the NF1 family. A detailed examination of the DNA sequence from the α5 FRE indeed revealed the presence of a perfect half-palindromic site for NF1 (TGGCA; see Figs. 2B and 10) that has been previously reported to bind this transcription factor (44, 45).

We next performed supershift experiments in EMSA to establish clearly whether Sp1 was truly binding the FRE to yield complexes a and b in EMSA. The experiment was conducted at three different protein concentrations (5, 10, and 20 μg of crude nuclear proteins) in the presence of either none or 1 μl (corresponding to 1 μg) of an antiseraum raised against human Sp1. Again, formation of complex a but not that of complex c was found to be much stronger when the extract from RCEC grown on FN-coated Petri dishes was used at either 10 or 20 μg (but not at 5 μg; Fig. 5A), suggesting that Sp1 expression (or its corresponding DNA binding affinity) is increased when RCEC are cultured on FN-coated dishes. The further addition of the Sp1 antiseraum resulted in a strong reduction of complex a formation and yielded a new complex (a-Sp1Ab) with a lower electrophoretic mobility resulting from the recognition of complex a by the Sp1 antibody. The proportion of the signal supershifted by the Sp1 antibody was much stronger in the extract from RCEC grown on FN-coated culture dishes (at both 10 and 20 μg but not at 5 μg of proteins) than with RCEC grown solely on plastic, providing further evidence that either Sp1 expression, or its DNA binding affinity, is indeed increased in cells grown on FN-coated culture dishes. As Fig. 5B indicates, no supershifted complex could be obtained when the Sp1 antiseraum was substituted with the non-immune serum, which is used as a negative control in such experiments. Western blot analysis using the Sp1 antiseraum as the source of primary antibody revealed that RCEC express nearly the same amount of an antiseraum raised against human Sp1.

To answer whether the Sp1-dependent FN responsiveness—FN responsiveness to a heterologous promoter, a synthetic, double-stranded oligonucleotide bearing the α5 sequence from −82 to −56 (designated as FRE) was inserted upstream from the basal promoter of the mouse p12 gene. The p12 gene encodes a 12-kDa secretory protease inhibitor whose expression is mainly restricted to the ventral prostate, the coagulating gland, and the seminal vesicle (46). We have previously shown that the basal promoter from the p12 gene, which extends from position −108 to +7 in plasmid p12.108, is constitutively expressed to relatively high levels in most transfected cell types (36, 47). However, to avoid any interference by the Sp1 site identified in the middle of the p12 basal promoter, the FRE was inserted into a derivative from p12.108 that bears mutations into the p12 Sp1 target site (p12.108/M (36)) (Fig. 7A). When transfected into mid-confluent RCEC, only a weak difference (1.6-fold activation) was observed in the CAT activity directed by the parental plasmid p12.108/M when 8 μg/cm² FN was coated on the culture plates (Fig. 7A). However, insertion of either one (in plasmid p12/FRE) or two sense copies (in plasmid p12/2xFRE) of an oligomer bearing the −82/−56 α5 FRE immediately upstream from the p12 basal promoter resulted in 6.1- and 8.8-fold increase in CAT activity, respectively. Mutations introduced in the 5’ half-site of the inverted repeat contained on the FRE (see under “Experimental Procedures”) had no statistically significant effect on either the basal p12 promoter-driven activity when cells are grown on plastic or on the FN responsiveness when they are cultured on FN-coated culture dishes (32% reduction when compared with the level directed by the wild-type p12/FRE) (Fig. 7B). On the other hand, mutations that altered part of the 3’ half-site of the FRE and most of its downstream GC-rich sequence had no effect on the unstimulated, p12 promoter basal activity but totally abolished FN...
responsiveness when RCEC were cultured on FN-coated dishes. As expected, mutating both the 3'- and 5' half-sites from the α5 FRE had the same effect as mutating the 3' half-site alone. To confirm that the lack of FN responsiveness resulting from mutating the 3' half-site of the α5 FRE was the consequence of preventing Sp1 from properly interacting with its target sequence in the FRE, competition experiments in EMSA were performed. Crude nuclear proteins were prepared from mid-confluent RCEC grown on FN-coated culture dishes and incubated with the α5 FRE-labeled probe in the presence of varying concentrations of unlabeled oligonucleotides bearing the sequence from either the wild-type FRE or any of its mutated derivatives. As shown in Fig. 7C, incubation of the labeled probe with nuclear proteins from RCEC yielded the typical Sp1-FRE complex observed above (also denoted complex a in both Figs. 4 and 5). As expected, as little as a 100-fold molar excess of unlabeled wild-type FRE totally prevented formation of this complex. Similarly, a 100-fold molar excess of the 5' half-site-mutated FRE competed as well the unmutated FRE for the formation of the Sp1 complex providing evidence that these mutated positions did not interfere with the recognition of the oligomer by Sp1. On the other hand, derivatives of the FRE bearing mutations in the 3' half-site (altering either the 3' half-site alone or in combination with the 5' half-site) were totally inefficient in preventing formation of the Sp1-FRE complex, even when used at a 500-fold molar excess, therefore providing evidence that both mutated oligomers are unable to bind Sp1. We therefore conclude that the inverted repeat identified in the basal promoter of the α5 gene can confer Sp1-dependent FN responsiveness to an otherwise unresponsive heterologous promoter and that only the 3' repeat of the FRE, along with its downstream GC-rich sequence, is required for this effect to occur.

As further evidence that FN responsiveness mediated by the α5 FRE was determined through its recognition by Sp1, co-transfection experiments were therefore conducted into Drosophila Schneider cells. These cells have been reported to be deficient in producing this transcription factor, as well as many others expressed in higher eukaryotes, which make them an ideal system for studying gene expression or transcription factor functions (for a review see Ref. 48). Both the FRE-bearing α5-92 and the FRE-depleted α5-42 plasmids were cotransfected into Schneider cells either alone or with a recombinant plasmid (pPasSp1, a generous gift from Dr. Guntram Suske, Institute für Molecular Biology und Tumorforschung, Philipps Universität Marburg, Germany) containing the Sp1 cDNA under the control of the Drosophila actin gene promoter and therefore ensuring high levels of Sp1 expression in Schneider cells. Neither α5-41 nor α5-92 could determine high basal promoter activity when individually transfected in Schneider
cells. However, when cotransfected along with pPacSp1, a dramatic 75-fold increase in promoter activity was observed with the FRE-containing plasmid α5-92 but not with the FRE-deleted plasmid α5-41 (Fig. 8A). These recombinant constructs were then transfected either alone or with pPacSp1 into Schneider cells (Fig. 8B). The parental plasmid p12.108/M, although encoding substantial amounts of CAT in Schneider cells, responded only weakly (3.5-fold activation) to the presence of Sp1. However, the further addition of the α5 FRE in p12.108/M resulted in a strong increase (52-fold) in the CAT activity normally directed by p12.108/M. As with the transfection experiments conducted in RCEC (see Fig. 7, A and B), mutations introduced in the 5′ half-site of the FRE (in plasmid p12/FREm5′) had only a modest effect on the Sp1-mediated activation of the p12 promoter (Fig. 8B). On the other hand, no Sp1-mediated activation could be observed upon mutating either the 3′ half-site alone or both the 3′ and 5′ half-sites of the FRE (in the plasmids p12/FREm3′ and p12/FREm5′ + 5′, respectively). These results are consistent with those of the competition experiment shown in Fig. 7C and provide clear evidence that Sp1 does bind to the 3′ half-site of the FRE in order to influence positively the activity of its downstream promoter (in this case, either the α5 or the p12 promoter).

**Activation of ERK1/ERK2 Mediates the FN Responsiveness of the α5 Promoter**—Culturing murine Swiss 3T3 or rat REF52 fibroblasts on substrata coated with either FN or with a synthetic peptide containing the RGD sequence has been shown to result in the activation of mitogen-activated protein kinases (MAPKs) (49), such as extracellular signal-regulated kinases (ERKs), which have been shown to be recruited to the ECM ligand/integrin-binding site (50). Sp1 has been recently recognized as one of the few target transcription factors phosphorylated by ERK kinases (51–53). We have shown above that its ability to interact with the α5 FRE is strongly increased upon activation of the FN/αβ1 integrin-mediated signal transduction. To determine whether the Sp1-mediated FN responsiveness directed by the α5 FRE was due to the activation of the Ras-Erk signaling pathway through the interaction of the α5β1 integrin with its ECM ligand FN accounts for the increase in α5 promoter activity when RCEC are grown on FN-coated dishes (Fig. 9A). However, the addition of as little as 10 μM of the PD98059 inhibitor (many studies have used doses 5–10-fold higher of this inhibitor (51–53)) totally abolished this FN responsiveness, the level of CAT activity returning to the unstimulated level. Identical results were also obtained with the recombinant plasmid p12/FRE, which bears one sense copy of the α5 FRE inserted upstream from the basal promoter of the p12 gene (see Fig. 7, A and B). Again, the nearly 3-fold increase in the α5 FRE-mediated FN responsiveness of the p12 promoter was totally abolished when cells were cultured in the presence of the inhibitor (Fig. 9B). Crude nuclear extracts were prepared from RCEC grown either on plastic or FN-coated culture dishes in the presence of either none or 10 μM of the PD98059 inhibitor and then used in EMSAs. Upon incubation with the α5 FRE-labeled probe, a clear Sp1 signal that increased severalfold when RCEC were grown on FN could be observed with nuclear extracts from RCEC that have not been exposed to the MEK-1 inhibitor (compare 1st and 3rd lanes in Fig. 9C). However, cultivating RCEC in the presence of the inhibitor totally abolished formation of the α5αβ1 complex (Fig. 9C) even when cells were grown solely on plastic. We therefore conclude that activation of the Ras-Erk signaling pathway through the interaction of the α5β1 integrin with its ECM ligand FN accounts for the increase in α5 promoter activity when RCEC are grown on FN-coated culture dishes and that this effect is most likely dependent on the altered phosphorylation state of Sp1 by activated ERK1/ERK2.

**DISCUSSION**

Debridement of the corneal epithelium is known to promote wound healing by stimulating migration and differentiation of both the basal corneal epithelial cells that border the injured area and the precursor cells from the corneal limbus. Induction of the migration process is known to be influenced by the
massive production of FN by both the stromal keratinocytes
and the basal cells that border the injured area (5, 8–15).
Moreover, recent studies provided evidence that the level of
expression for the mRNA encoding the \( \alpha_5 \) integrin subunit was
positively modulated by the presence of such FN (23, 24). The
present study was therefore conducted in order to investigate
whether FN, through its \( \alpha_5 \beta_1 \) receptor-mediated signal trans-
duction pathway, can alter the transcriptional activity directed
by the promoter of the \( \alpha_5 \) integrin subunit gene. We provided
clear evidence that FN can indeed alter the transcriptional
activity of the \( \alpha_5 \) promoter by altering the DNA binding affinity
of the positive transcription factor Sp1 for a short \( \alpha_5 \) promoter
segment located between positions \(-77 \) and \(-61 \) that has been
designated as the \( \alpha_5 \) FRE. The \( \alpha_5 \) FRE bears a perfect inverted
repeat of the following sequence, \( 5'-GGAGTTTG-3' \). However,
site-directed mutagenesis provided evidence that only the 3’
half-site (along with its nearby 3’ GC-rich base pairs (TCCCC))
was required for FN responsiveness to occur. This short stretch
of sequence from the \( \alpha_5 \) promoter was found to be highly homologous
to a 12-bp sequence from the murine acetylcholine receptor
(\( \alpha_5 \)ChR) \( \delta \)-subunit gene that was reported to be absolutely
required for muscle-specific expression of \( \alpha_5 \)ChR (54)
(see Fig. 10). This cis-acting element, which is comprised be-
tween positions \(-106 \) and \(-95 \), was postulated as being the
target sequence for the transcription factor myogenin (54).
However, myogenin has not been reported as a target protein
that might be subjected to differential phosphorylation by pro-
tein kinases.

The substantial variations we have observed in the ability of
Sp1 to bind the \( \alpha_5 \) FRE in RCEC grown with or without FN
might either result from alteration in the binding affinity of
Sp1 or from modification in the amount of Sp1 protein produced
by RCEC under both culture conditions. However, our inability
to detect any significant variations in the absolute amount of
Sp1 between RCEC grown with or without FN in Western blot
analyses rather favors the former hypothesis. Our results are
consistent with those recently reported by Alroy \textit{et al.} (55) who
could not see any variation in Sp1 protein levels despite an
increased binding of Sp1 to the Neu differentiation factor re-
sponse element from the promoter of the acetylcholine receptor
e upon stimulation with Neu differentiation factors. Variations
in the \( \alpha_5 \) promoter activity might then be triggered by modify-
ing the affinity of Sp1 for the FRE target site by altering its
state of phosphorylation through nuclear proteins that belong
to the MAPK family, such as ERK-1 (p44) and ERK-2 (p42).
Alteration of the state of phosphorylation for the transcription
factor Sp1 has been reported to alter, either positively or neg-
atively, its DNA-binding properties \textit{in vitro} (52, 55, 56). Li \textit{et al.}
(51) recently reported that Sp1 might also be a target for ERK
proteins. Indeed, they identified a 16-bp sequence (repeat 3)
that mediates responsiveness of the human low density lip-
oprotein receptor (LDLR) to oncostatin M (OM) through a

\[ \begin{align*}
\text{FIG. 9. The integrin-mediated FN} \\
\text{responsiveness is abolished by the} \\
\text{MEK/kinase inhibitor PD98059. A, the} \\
\text{recombinant plasmid} \alpha_5-92 \text{ was tran-} \\
\text{siently transfected into RCEC grown on} \\
\text{plastic (–FN) or FN-coated (+FN; 8 \mu g/} \\
\text{cm}^2 \text{) culture dishes with either none or 10} \\
\text{\mu M of the MEK/kinase inhibitor PD98059.} \\
\text{Cells were harvested 48 h later, and CAT} \\
\text{activity was determined and normalized} \\
\text{as detailed under “Experimental Proce-} \\
\text{dures.” B, same as in A except that the} \\
\text{recombinant plasmid p12/FRE (see Fig. 7) was} \\
\text{substituted to} \alpha_5-92 \text{ for the transfec-} \\
\text{tion experiments. C, the double-stranded} \\
\text{oligonucleotide bearing the} \alpha_5 \text{FRE was 5'} \\
\text{end-labeled and incubated with crude nu-} \\
\text{clear proteins (5 \mu g) from RCEC grown on} \\
\text{either none (–) or FN-coated (+) culture} \\
\text{dishes (8 \mu g/cm}^2 \text{), in the presence of ei-} \\
\text{ther none or 10 \mu M PD98059. Formation} \\
\text{of the Sp1-FRE complex was then moni-} \\
\text{tored by EMSA as detailed in Fig. 4 ex-} \\
\text{cept that the concentration of the poly-} \\
\text{acrylamide gel was lowered to 4%. The} \\
\text{position of the Sp1-FRE complex is shown} \\
\text{(Sp1) along with that of the free probe (U).} \\
\text{P, labeled probe alone.}
\end{align*} \]
signal transduction pathway that involves phosphorylation of ERK-1 and ERK-2 by the upstream kinases MEK-1 and MEK-2. The sequence from the LDLR repeat 3 also bears an intact copy of the GGAAGTT motif (on the non-coding strand) identified in the α5 FRE (see Fig. 10). Most of all, it also contains the GC-rich sequence (TCCCC) located downstream of the 3’ repeat that proved to be required for the FN responsiveness directed by the α5 FRE. Interestingly, only Sp1 and the Sp1-related protein Sp3 have been shown to bind repeat 3 (51). However, these authors have been unable to detect any OM-induced alterations in Sp1 binding by EMSA or in the ratio of hyper- versus hypophosphorylated Sp1 by Western blot analyses (51). As Fig. 5A reveals (and to some extent also Fig. 4A), no significant changes in Sp1 binding to the α5 FRE could be observed between nuclear extracts obtained from RCEC grown with or without FN when only 5 µg of crude nuclear proteins were used. However, raising the amount of proteins to either 10 or 20 µg clearly revealed a much stronger binding of Sp1 to the FRE when RCEC are grown on FN-coated culture dishes, which is also supported by a more intense supershift of the Sp1/Sp3-binding sites identified in the promoter of the mouse p12 gene (p12.A), the 16-bp repeat 3 element from the human LDLR, and sequences from the murine AChR-β subunit gene promoter that also bears a binding site for the transcription factor myogenin (MO) (underlined). Arrows indicate the position of each α5 FRE half-inverted repeats, and black dots indicate the position of those G residues whose methylation by dimethyl sulfate interferes with the recognition of the p12.A element by Sp1. The DNA sequences that show homology to both the NF1 and Sp1 target sites are indicated.

Not all Sp1/Sp3-binding sites are subjected to regulation by the MAPK pathway. Proper positioning of the Sp1/Sp3 target site relative to the TATA box has been postulated as being particularly critical for OM-mediated transcriptional activation of the LDLR gene (57). Indeed, although LDLR repeat 1 also bears an Sp1-binding site that is critical for basal transcriptional activity, it is not affected by the presence of OM, unlike the more proximal Sp1 site from repeat 3 (57). Liu et al. (57) postulated that OM may induce the expression of an Sp1-dependent coactivator that would bridge Sp1 bound to a properly positioned target site to the general transcriptional machinery. Alternatively, OM-mediated transduction pathway, through activation of ERK1/ERK2, might also lead to post-translational phosphorylation of Sp1 and account for LDLR transcriptional activation (57), which has been recently shown to occur for the gastrin gene promoter (52). Although the α5 promoter has no TATA box, it has been shown to contain an initiator site located at position -45 that is likely sufficient to bind the general transcriptional machinery (35). The α5 FRE is therefore located approximately 15 bp upstream from the putative initiator site, a positioning nearly identical to that observed for the LDLR Repeat 3 Sp1 site and the LDLR TATA-like sequence (17 bp from the most 5’ located TATA-like sequence) (57, 58). As for the LDLR repeat 1, the putative high affinity Sp1-binding sites identified in the α5 promoter between positions -178 and -92 did not contribute much to the FN-mediated responsiveness of the α5 promoter since they could be deleted without any significant effect on the CAT activity. Based on the results shown in the present study, we suggest that both phosphorylation of Sp1 and proper positioning of its target site relative to the general transcriptional machinery are required to transduce properly the signal triggered by extracellular FN. Whether the need for an Sp1-dependent co-activator is required to produce the proper response remains to be demonstrated.

Expression of integrin subunit genes other than α5 has also been reported to be positively influenced by the binding of Sp1 to their promoter sequence. Indeed, the transcriptional activity directed by the α6 integrin subunit gene promoter was reported to be dependent on its recognition by both Sp1 and AP2 (59). Transcription directed by the promoter of both the β1/CD18 and the αHb integrin subunits has recently been reported to be positively regulated through the synergistic action of both Sp1 and members of the Ets family of transcription factors, such as GABP (60–61). Two tandemly repeated Sp1-binding sites were also identified in the promoter of the α2 integrin subunit gene (62). Interestingly, phosphorylation of Sp1 appears to be required for formation of the Sp1 DNA-protein complex in vitro.

Characterization of an FN-responsive element such as the α5 FRE is a first step toward the understanding of the nuclear events taking place upon activation of the signal transduction pathway normally triggered by membrane-bound FN integrins. Our results provide a link between the extracellular ligand/ integrin-mediated signal transduction and the nuclear events leading to expression of the α5β1 integrin subunit gene. Most of all, it also provides further support to the major signaling pathway activated by the α5β1 integrin and for which a model was recently proposed (63).

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