Involvement of the Sp3 Transcription Factor in Induction of p21<sup>Cip1/WAF1</sup> in Keratinocyte Differentiation*

(Received for publication, August 14, 1996, and in revised form, October 8, 1996)

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The cyclin-dependent kinase inhibitor p21 is induced in several in vitro terminal differentiation systems as well as in differentiating tissues in vivo. To determine the mechanism responsible for p21 induction during differentiation of mouse primary keratinocytes, we performed a deletion analysis of the p21 promoter. The minimal region of the p21 promoter required for its induction in keratinocyte differentiation consists of a contiguous stretch of 78 base pairs, which contains a GC-rich region as well as the TATA box. We determined that transcription factors Sp1 and Sp3, present in primary keratinocyte nuclear extracts, bind the GC region concomitantly. Expression studies established that both Sp1 and Sp3 activate the p21 promoter, but showed that only Sp3 overexpression enhances promoter inducibility during differentiation. Furthermore, disruption of the GC-rich region dramatically decreases transcription factor binding as well as promoter activity and inducibility upon differentiation. The overexpression of either Sp1 or Sp3 restores the basal activity of the disrupted promoter, but only Sp3 can restore its inducibility. These findings show that both Sp1 and Sp3 can contribute to the basal activity of the p21 promoter, and establish Sp3 as a specific transcription factor involved in the induction of p21 promoter during keratinocyte differentiation.

Induction of terminal differentiation is a complex process involving the regulation of many genes, including those directly responsible for cell cycle withdrawal and growth arrest. The activity of the cyclin-dependent kinases (CDKs)<sup>1</sup> is tightly regulated during passage through the cell cycle by cyclin association, subunit phosphorylation, and interaction with CDK inhibitors (1, 2). Induction of the CDK inhibitor p21 occurs in several in vitro terminal differentiation systems (3), as well as in differentiating tissues in vivo (4, 5), suggesting that this molecule plays an important role in regulating terminal differentiation-associated growth arrest.

Control of p21 induction upon differentiation is of great interest, as it may involve mechanisms common to most cell types and others that are cell type-specific. In all the differentiation systems analyzed so far the induction of p21 occurs by p53-independent mechanisms (5–9). During differentiation of myoblasts to myotubes, p21 up-regulation has been reported to be under the control of MyoD (6), although other myogenic regulatory factors may also be involved, at least in MyoD knockout mice (5). In differentiating primary keratinocytes, the induction of p21 depends on the function of the nuclear phosphoprotein p300 (9), a novel transcriptional coactivator, which is thought to act as a bridge between specific transcription factors and the basal transcription complex. Little else is known about the regulation of p21 expression during normal differentiation.

Recent data indicate that the GC-rich region of the p21 promoter, located next to the TATA box, binds the Sp1 transcription factor and can exert important regulatory functions (10, 11). The Sp1 DNA binding domain consists of three zinc finger motifs, which recognize the GC box (12). Sp1 can interact with TATA-binding protein-associated factors (TAFs; Ref. 13) as well as a number of specific transcription factors (see Refs. 14 and 15 and references therein).

In addition to Sp1, three related genes, Sp2, Sp3, and Sp4, have recently been identified (16, 17). The most well characterized homolog is Sp3, which has a zinc finger DNA binding domain that is highly conserved with Sp1 and recognizes GC and GT motifs with an identical affinity. Sp3, like Sp1, has two glutamine-rich activation domains, but these are not functionally interchangeable with the glutamine-rich activation domains of Sp1 (16). The expression of Sp3 in the Drosophila cell line SL2, which is devoid of Sp1 activity, is reported to both repress and activate Sp1-responsive elements in a promoter-dependent manner (16, 18).

Sp1 has been implicated in the induction of the p21 promoter upon TGFβ treatment of the HaCaT keratinocyte cell line (11) and phorbol ester treatment of the leukemic cell line U937 (10). In both cases, GC boxes alone were sufficient to confer responsiveness to the inducing agent, although the functional involvement of Sp1 relative to other GC binding factors was not established. Similarly, it was not clear how the GC boxes account for the specific induction of the p21 promoter relative to other promoters containing similar elements.

Here, we show that the minimal region essential for induction of the p21 promoter in differentiating mouse keratinocytes is GC-rich and overlaps with the region implicated in the TGFβ response in HaCaT cells. However, we demonstrate that this GC box alone, although essential, is not sufficient for promoter activation upon keratinocyte differentiation and that continuity of the entire region with the adjacent TATA box is required. Furthermore, we show that both Sp1 and Sp3 contribute to p21 promoter activity in keratinocytes, but that, of these transcription factors, only Sp3 is directly involved in promoter induction upon differentiation. The general implications of these findings

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*This work was supported by National Institutes of Health Grants AR39190 and CA16038 (to G. P. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Sp3 Regulates p21 Promoter in Keratinocyte Differentiation

RESULTS

p21 Induction during Differentiation Does Not Involve E Box Binding Transcription Factors—Mouse primary keratinocytes can be induced to terminally differentiate by increasing the concentration of extracellular calcium in the medium from 0.05 mM to 2 mM. This differentiation program includes the induction of a number of biochemical and morphological markers observed in vivo, as well as growth arrest (27). The use of a primary system eliminates the complications of secondary changes associated with cell immortalization.

for transcriptional control of this critical cell cycle regulatory gene in differentiation will be discussed.

MATERIALS AND METHODS

Cell Culture—Primary keratinocytes were prepared from newborn Sencar mice and grown in minimal essential medium with low calcium concentrations (0.05 mM), as described previously (19). Differentiation was induced by addition of calcium chloride to a final concentration of 2 mM.

Plasmids—The 2.4-kilobase pair p21 promoter (20) was a gift from B. Vogelstein (Johns Hopkins Oncology Center). Smaller regions of the promoter were obtained by restriction enzyme digestion at the unique PstI site at position −168 relative to the TATA box, and by polymerase chain reaction amplification using the original promoter construct as a template and primers containing the desired promoter sequence. These promoter fragments were cloned into the luciferase reporter pGL3 basic (Promega). The mutant p21 promoters were generated by synthesizing the desired p21 promoter sequences and linking them to either the 35- or 53-bp p21 promoter TATA box in the pGL3 basic reporter. To construct the heterologous promoters, the 50-bp GC-rich (−50 to −1) p21 promoter sequence was linked to either minimal Elb (−4 to +9) or SV40 (−10 to +40) TATA boxes cloned in the pGL3 basic reporter (Promega). The human Sp3 cDNA expression construct pBOS (21), the human Sp3 cDNA expression construct pCMV4Sp3Flu (18), the Id1 and Id2 expression constructs (22), the MyoD expression construct (23), and the 3luc900 reporter plasmid (24) have been described previously.

Transient Transfections and Luciferase Assays—Primary keratinocytes, 5 days after plating, were transfected in duplicate with plasmid DNA using the DEAE-dextran technique, as described previously (9). In order that all dishes received the same total amount of DNA, the pUC19 plasmid was used as a carrier. Unless otherwise indicated, transfected keratinocytes were either kept in low calcium medium or exposed to high calcium conditions for the last 24 h of the 72-h incubation period. The transfected cells were harvested at 72 h after transfection and the luciferase activity determined as described (25). The production of light was measured with a luminometer (EG&G Berthold) and expressed as relative light units (RLU). An expression plasmid for human growth hormone (pXGH5; Nichols Diagnostics Institute) was cotransfected (1 μg/60-mm dish) with the luciferase reporter constructs. Growth hormone levels determined using the HGH-TGES kit (Nichols Diagnostics Institute). Luciferase activities were normalized for either growth hormone (RLU/ng of growth hormone) or total protein concentrations (RLU/10 μg of extract). The -fold induction of the promoter relative to basal promoter activity was determined.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts from either proliferating keratinocytes or keratinocytes induced to differentiate by increased extracellular calcium concentration (2.0 mM) for 24 h, were prepared essentially as described (26), except samples were prepared in a Dounce homogenizer (100 strokes) during hypotonic and nuclear lysis. Oligonucleotides corresponding to the desired p21 promoter sequences (−50 to −1) were synthesized, annealed, labeled with 35S-32PATP using T4 polynucleotide kinase, and purified on polyacrylamide gels. Binding reactions contained 5 μg of nuclear extract in a buffer of 50 mM NaCl, 4 mM dithiothreitol, 1 mM EDTA, 1 mM MgCl2, 0.1% Nonidet P-40, 1 μg of poly(dI-dC), and 5% glycerol. The reactions were incubated for 30 min at 30°C before loading on 5% polyacrylamide gels (29:1 acrylamide/bisacrylamide) in low ionic strength TBE buffer.

Competition experiments were performed by adding unlabeled oligonucleotides, with either the same sequence or an unrelated sequence, to the binding reactions 10 min prior to addition of the labeled oligonucleotide. For supershift experiments using antibodies either 0.2 or 1.0 μg of specific anti-Sp1 (Santa Cruz Biotechnology) or Sp3 (a gift from J. M. Horowitz (Duke University Medical Center); Ref. 18) polyclonal antiserum were added to the binding reaction and a 10-min incubation on ice performed before addition of the radiolabeled oligonucleotide.

We have reported previously that calcium-induced differentiation of primary keratinocytes is associated with increased levels of p21 RNA transcripts and protein (9). In parallel with these observations, we found that a 2400-bp fragment of the p21 promoter linked to a luciferase reporter, when transiently transfected into mouse primary keratinocytes, was significantly induced upon differentiation in a p53-independent manner (9).

In the differentiation of skeletal myoblasts, p21 has been reported to be induced as a consequence of MyoD function (6). Basic helix-loop-helix factors of the MyoD family bind to consensus (CANNTG) E box target sites (28) and are not limited to muscle, as specific members of this family are found in neuronal cells (29). The observation that mammary epithelial cell differentiation can be inhibited by expression of Id, a general antagonist of MyoD-like factors (30), suggests that members of this family may also be involved in epithelial differentiation control. In order to determine whether MyoD family factors are involved in the induction of the p21 promoter during keratinocyte differentiation, Id1 or Id2 expression constructs were co-transfected with the 2400-bp p21 promoter (pW-2400) into mouse primary keratinocytes and the luciferase activities measured in both proliferating and differentiating cells. The expression of either Id1 or Id2 did not inhibit the induction of the p21 promoter, which occurs during keratinocyte differentiation (Fig. 1A). Expression of exogenous MyoD only slightly
activated the p21 promoter in keratinocytes under basal and differentiating conditions (Fig. 1B). In contrast, a MyoD-responsive promoter was markedly activated in keratinocytes by cotransfection of the MyoD expression construct, but activity of this promoter did not increase in differentiating keratinocytes (Fig. 1C). MyoD-like activities are therefore unlikely to be involved in the induction of the p21 promoter during keratinocyte differentiation.

**Mapping of the Calcium/Differentiation-responsive Element in the p21 Promoter**—In order to identify the mechanism(s) responsible for activation of the p21 promoter in our system, primary keratinocytes were transiently transfected with a series of p21 promoter deletion mutants linked to a luciferase reporter gene, together with a growth hormone reporter construct. The transfected cells were either kept in growth medium or induced to differentiate by addition of extracellular calcium for the last 24 h of the experiment. The promoter activities were determined by measurement of luciferase activity (as described under “Materials and Methods”) and normalized for growth hormone production.

As reported previously, the activity of the 2400-bp p21 promoter construct (pW-2400) was consistently increased 5–10-fold after calcium-induced differentiation (9). To define the position of the differentiation-responsive region within the promoter, the unique PstI site at position −168 relative to the TATA box was used to generate the construct pW-225. The basal activity and calcium inducibility of this small fragment were similar to the 2400-bp promoter, indicating that the core promoter and its differentiation-responsive sequences co-localize within a region of 225 bp (−168 to +57 bp; Fig. 2A). This region contains a number of potential transcription factor recognition sequences, including six Sp1 binding sites (GC boxes) and three E boxes, as well as the TATA box. The constructs pW-107 (lacking the E box at −116 bp) and pW-78 (lacking the E boxes at +26 and +41, as well as the pair of Sp1 sites at −73 and −63 bp) were generated using a polymerase chain reaction-based approach. These deletions did not affect the calcium inducibility, although the overall promoter activity declined 3–5-fold. The basal activity of the promoter was further reduced, and the calcium inducibility abolished by an additional 25-bp deletion at the 5′ end of the fragment to generate pW-53, indicating that the region between −50 and −25 bp is essential for calcium responsiveness (Fig. 2A). A smaller p21 promoter construct of 35 bp (pW-35), containing the TATA box but lacking the entire 50-bp GC region, was also inducible.

The region from −50 to −25 bp of the promoter is GC-rich and contains a fully conserved Sp1 binding site. The same GC-rich region was shown to be sufficient for induction of the p21 promoter in response to TGFβ in HaCaT cells (11). To determine whether the −50 to −25 bp region is also sufficient for the calcium induction, we linked this region to the minimal p21 promoter (construct pW-35\textsuperscript{GC}), transiently transfected primary keratinocytes, and determined its calcium responsiveness. The construct pW-35\textsuperscript{GC} was not induced by calcium treatment (Fig. 2B), suggesting that although essential this GC region alone is not sufficient for calcium induction during differentiation.

In order to test whether the intact 50-bp GC-rich region is sufficient for calcium induction, this region was fused to either a minimal adenoviral E1b (pE1b-50) or SV40 (pSV40-50) heterologous promoter. Activity of these chimeric promoters was not induced upon keratinocyte differentiation (Fig. 2B). Similarly, when the 50-bp GC-rich region was fused to the minimal p21 promoter at position −7 bp (pW-78M\textsuperscript{−7}), creating a 6-bp insertion between the two elements, no induction of promoter activity upon differentiation was detected.

To determine whether interaction(s) between the four potential SP1 sites within the GC-rich region are required for basal promoter activity and/or calcium induction, the spacing between these sites was disrupted. A mutant promoter with a 6-bp insert at position −25 bp (pW-78M\textsuperscript{−25}), which perturbs the spacing between the region −50 to −25 bp and the remainder of the minimal p21 promoter, had reduced basal activity compared to the wild type promoter (pW-78) and was also not inducible upon keratinocyte differentiation (Fig. 2B). Thus, taken together, our data suggest that the entire contiguous −50 to +28 bp region of the p21 promoter, which includes the TATA box, is required for its responsiveness to calcium-induced differentiation.

**The Differentiation-responsive Minimal p21 Promoter Is Specifically Activated by Extracellular Calcium**—We have shown previously that endogenous p21 transcripts as well as the activity of the 2400-bp p21 promoter are specifically increased upon induction of keratinocyte differentiation by increased extracellular calcium and not by treatment with either TGFβ (a pure growth inhibitory agent) or TPA (a partial inducer of differentiation) (3, 9). It was important to determine whether the minimal calcium-responsive p21 promoter (pW-78), containing the entire contiguous −50 to +28 bp region of the p21 promoter, maintained the same specificity as the 2400-bp p21 promoter. In fact, we found that the minimal p21 promoter (pW-78) was specifically activated in primary mouse keratinocytes in response to increased extracellular calcium in a concentration-dependent manner, and no induction was observed upon treatment of these cells with either TGFβ or TPA treatment (Fig. 3).

Calcium can induce keratinocyte differentiation in a dual manner, acting at the cell surface as well as intracellularly. In particular, critical aspects of the early biochemical response, such as specific tyrosine phosphorylation events, are specifically induced by increases in extracellular calcium and not by calcium ionophore-mediated increases in intracellular calcium levels (31). In parallel with these findings, the minimal p21 promoter (pW-78) was not induced in keratinocytes in low calcium medium by treatment with the calcium ionophores A23187 or ionomycin (Fig. 3). Thus the minimal differentiation-responsive region of the p21 promoter (−50 to +28) is specifically activated by increases in extra- but not intracellular calcium, and not by other agents inducing keratinocyte growth arrest and/or a more restricted program of differentiation.

**Identification of the Nuclear Factors Involved in the Calcium Response**—Deletion analysis of the p21 promoter established that the minimal differentiation-responsive element consisted of a 50-bp GC-rich region and the adjacent TATA box. We wished to determine whether the increased promoter activity was due to changes in binding of proteins to the GC-rich region. Electrophoretic mobility shift assays (EMSA) were performed with an oligonucleotide corresponding to the 50-bp GC-rich region, and nuclear extracts prepared from primary mouse keratinocytes under growing conditions or induced to differentiate by calcium for 24 h. A major DNA-protein complex was formed with nuclear extracts from both low and high calcium conditions, which was sequence-specific, as it could be completely competed away by an excess of unlabeled homologous oligonucleotide but not with an unrelated oligonucleotide (Fig. 4A). The retardation of the complex formed with nuclear extracts from growing versus differentiating keratinocytes was similar, but the intensity reproducibly increased 1.5–3-fold as determined by densitometric analysis (compare experiments in Figs. 4, A and B, and 5C), consistent with cellular proteins from differentiating cells binding to increasing amounts of the free
probe (rather than additional proteins binding to the same fragment).

As described in the previous section, the mutant promoter (pW-78M⁻²⁵), containing a 6-bp insert between the first and second GC box, is not induced during keratinocyte differentiation. In parallel with that finding, EMSA showed that binding of proteins to the mutant 50-bp GC-rich oligonucleotide was greatly reduced in comparison to the wild type sequence (Fig. 4B).

The 50-bp GC-rich region contains four potential Sp1 sites and can bind recombinant human Sp1 in EMSA (data not shown). We determined whether the proteins present in keratinocytes recognize this region and are induced during differentiation.

**Fig. 2.** The minimal calcium/differentiation-responsive region of the p21 promoter maps to a 78-bp GC-rich fragment, which includes the TATA box region. A, serial deletion mutants of the p21 promoter linked to a luciferase reporter construct were transiently transfected (0.5 μg/60-mm dish) together with a growth hormone expression construct (1 μg/60-mm dish) into primary keratinocytes in low calcium medium. Transfected keratinocytes were either kept in low calcium medium (white boxes) or exposed to high calcium concentrations (black boxes) for the final 24 h prior to termination of the experiment (72 h after transfection). Luciferase activity was normalized for growth hormone production. The -fold induction of promoter activity in differentiating versus growing keratinocytes is indicated. B, insertion mutants of the p21 promoter, or heterologous promoters where the GC-rich region of the p21 promoter was linked to either minimal adenoviral E1b (pE1b-50), SV40 (pSV40–50), or its own (pW-78M⁻²⁷) TATA binding region, were transiently transfected into primary keratinocytes (0.5 μg/60-mm dish) together with a growth hormone expression construct (1 μg/60-mm dish). Transfection with the intact p21 minimal promoter (pW-78) was included. Luciferase activities were normalized for growth hormone and -fold induction determined as described above.
tinocyte nuclear extracts which bind this region included Sp1. The addition of Sp1 antiserum (but not a nonspecific serum) to the binding reaction of keratinocyte nuclear extract from either low or high calcium conditions caused the major complex to be supershifted (Fig. 5, A and B). However, the supershift with anti-Sp1 antibodies was not complete, and a weak band remained, which migrated at essentially the same position as the original complex. Therefore we tested whether the related GC-binding protein, Sp3, also binds to this region. A supershift of the major complex was obtained with nuclear extracts derived from keratinocytes in both low and high calcium conditions, as shown previously (31). The -fold induction of luciferase activity was determined as described in Fig. 1.

Thus the 50-bp GC region, which is required together with the TATA box for induction of the p21 promoter during keratinocyte differentiation is specifically bound by proteins present in keratinocyte nuclear extracts in amounts which increase upon differentiation. Binding of these proteins is disrupted by a 6-bp insert, which also abolishes promoter responsiveness. The transcription factors Sp1 and Sp3 bind to this region, both individually and in a concomitant fashion.

Functional Involvement of the Sp3 Transcription Factor in Keratinocyte Differentiation—Deletion analysis and binding studies suggested that the induction of the p21 promoter upon differentiation may involve the Sp1 and Sp3 transcription factors. As a functional test of this possibility, we assessed the affect of Sp1 or Sp3 overexpression on both the wild type p21 promoter (pW-78) and the mutant pW-78M−25 promoter, which has a 6-bp insert in the GC region that abolishes its inducibility. The activity of the wild type promoter was induced by Sp1 overexpression in keratinocytes under basal conditions, but no further induction was observed upon differentiation (Fig. 6). Sp3 overexpression also induced promoter activity under basal conditions. However, in contrast to the Sp1 results, significant enhancement of the induction of the p21 promoter during differentiation was observed. A similar effect was observed when Sp1 was co-expressed with Sp3 (Fig. 6).

The basal activity of the mutant promoter (p-78M−25) was increased by Sp1 expression, but calcium inducibility was not reestablished. In contrast, Sp3 expression not only increased the basal activity of the mutant promoter but also restored its responsiveness to calcium-induced differentiation (Fig. 6). Concomitant expression of Sp1 and Sp3 had a similar effect (Fig. 6). Thus both Sp1 and Sp3 contribute to the basal activity of the p21 promoter, but of these factors, only Sp3 appears to be rate-limiting and/or specifically involved in the induction of this promoter upon differentiation.

DISCUSSION

The expression of the CDK inhibitor p21 can be induced in cultured cells by the exposure to a number of diverse agents including growth factors (8, 32), TGFβ (33), phorbol esters (10, 32), retinoids (34), calcium (9), and vitamin D3 (7). Therefore, a number of signal transduction pathways may converge to induce p21 expression by activation of its promoter. To determine the mechanism by which induction of the p21 promoter occurs during calcium-induced keratinocyte differentiation, we per-

FIG. 3. Extracellular calcium specifically activates the differentiation-responsive minimal p21 promoter. The minimal 78-bp p21 promoter fused to the luciferase gene reporter (pW-78) was transiently transfected (2 µg/60-mm dish) with a growth hormone expression construct (1 µg/60-mm dish) into primary keratinocytes. These cells were treated with either calcium, TGFβ1, TPA, calcium ionophore A23187, or ionomycin for 24 h, prior to termination of the experiment 72 h after transfection. The ability of the calcium ionophores to increase intracellular calcium levels in primary keratinocytes was demonstrated previously (31). The -fold induction of luciferase activity was determined as described in Fig. 1.

FIG. 4. The GC-rich region of the p21 promoter is specifically bound by keratinocyte nuclear proteins under growing and differentiating conditions. A, EMSA was performed using radiolabeled oligonucleotide corresponding to regions −50 to −1 of the wild type p21 promoter, which contains a sequence essential for induction of the p21 promoter in differentiation, as shown in Fig. 2. The labeled oligonucleotide was incubated with no nuclear extract (lane 1), an equal amount of nuclear extract (5 µg) derived from proliferating keratinocytes (lane 2), or keratinocytes induced to differentiate by calcium for 24 h (lanes 3–5). Specificity of binding was verified by incubating the labeled oligonucleotide with nuclear extract from differentiating cells in the presence of a 50-fold excess of unlabeled specific competitor (lane 4) or 50-fold excess of unlabeled nonspecific competitor (lane 5). B, EMSA were performed using either radiolabeled oligonucleotide corresponding to regions −50 to −1 of the wild type p21 promoter (lanes 1–3) or the corresponding mutant oligonucleotide containing a 6-bp insert at position −25 (lanes 4 and 5). The oligonucleotides were incubated with no nuclear extract (lane 1), the same amount of nuclear extract (5 µg) from either proliferating cells in low calcium medium (lanes 2 and 4), or from cells induced to differentiate by calcium for 24 h (lanes 3 and 5). The major DNA protein complex is indicated (I).
formed a promoter deletion analysis. The minimal region of the p21 promoter required for its induction in keratinocyte differentiation was mapped to a contiguous stretch of 78 bp, which contains a GC-rich region as well as the TATA box. This region maintained the same specificity of induction to calcium-trig-...

We have shown previously that induction of the p21 promoter in differentiating keratinocytes depends on the transcriptional coactivator p300 (9). p300 interacts with a number of transcription factors, including CREB (35), which is involved in the induction of genes responsive to increased intracellular calcium (36). Given that calcium is a primary inducer of keratinocyte differentiation, the calcium-mediated activation of CREB/p300 factors could provide a mechanism for p21 promoter induction. However, CREB binding sites are not present within the minimal differentiation-responsive region of the p21 promoter, and induction of the p21 promoter in keratinocytes cannot be triggered by a calcium ionophore-induced increase in intracellular calcium levels.

Tyrosine phosphorylation is an early and specific event re-...
mouse keratinocyte differentiation. The TGFβ-responsive element alone was sufficient to mediate TGFβ-induced transcription when linked to a heterologous promoter and transactivated into HaCaT cells. In contrast, we have found that this GC-rich region is essential but not sufficient for induction of the p21 promoter upon calcium-induced differentiation, and that no induction of the p21 promoter occurs after TGFβ treatment of mouse primary keratinocytes. In spite of these differences, which may be due to the use of cells of human versus mouse origin, and/or primary cells versus an established cell line, the possibility that TGFβ and differentiation signaling pathways converge at the point of p21 promoter induction is an exciting one, which will deserve further attention.

Sp1 and Sp3 transcription factors were demonstrated to bind to the GC-rich region of the p21 promoter in HaCaT cells (11), although the functional significance of this binding was not established. In other systems expression of Sp1 activates promoters, whereas Sp3 expression can either activate or repress promoter activity, depending on the individual promoter and cell line (16, 18). The activation or repression of different promoters by Sp3 may therefore depend on subtle differences between GC boxes and an interplay with other transcription factors binding to similar DNA elements, most notably Sp1.

During keratinocyte differentiation, binding of proteins to the calcium-responsive GC-rich element of the p21 promoter consistently increased. Supershift experiments showed that both Sp1 and Sp3 bind to this region, in a concomitant fashion, and that the relative amount of binding of one factor versus the other is unaltered upon differentiation. Direct functional studies indicate that Sp1 and Sp3 play an important role in p21 promoter regulation in keratinocytes. A small insertion between two fully conserved Sp1 binding sites in the GC-rich region of the promoter disrupts protein binding, reduces basal promoter activity, and abrogates promoter inducibility during differentiation. The decreased basal activity of the disrupted p21 promoter can be overcome by overexpression of either Sp1 or Sp3, consistent with decreased binding affinity to the disrupted GC-rich region being compensated for by increased amounts of available factors. Aside from enhancing basal activity, Sp3 but not Sp1 overexpression restores inducibility of this disrupted promoter to calcium. Similarly, Sp1 overexpression increases the activity of the intact minimal promoter under basal conditions, but inhibits further promoter activation during differentiation. In contrast, Sp3 overexpression not only enhances the basal activity of the intact promoter, but superactivates it upon differentiation. Thus, on the basis of our findings, it can be proposed that both Sp1 and Sp3 bind to the minimal p21 promoter and contribute to basal promoter activity, possibly through synergistic interactions. However, only Sp3 is rate-limiting and/or contributes to promoter induction upon differentiation.

The differentiation-responsive region that we have mapped requires the Sp1/Sp3 sites to be contiguous with the TATA box for the p21 promoter to be induced. This suggests that more than simple transcription factor activation is involved in the induction. Evidence is accumulating that the basal transcription apparatus is capable of being regulated in cells, in part through interaction of TAFs with transcription factors (38). A recently identified component of the basal transcription complex is the coactivator p300, reported previously to interact with TATA-binding protein (39) and to be important for p21 promoter induction (9). It is tempting to speculate that Sp3 may interact with this and other TAFs, and that these associations may play a critical role in induction of the p21 promoter in differentiation.

Acknowledgments—We thank Janice Brissette, Caterina Missero, and Enzo Calautti for helpful comments on this manuscript and Jean-nie Chin for technical help in the initial phase of these experiments. We also thank G. Blebel, J. M. Horowitz, A. Lasorella, F. Tato, and N. Rosenthal for their gifts of reagents.

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