p51/p63 Controls Subunit $\alpha_3$ of the Major Epidermis Integrin Anchoring the Stem Cells to the Niche*

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p51/p63, a member of the tumor suppressor p53 gene family, is crucial for skin development. We describe here identification of ITGA3 encoding integrin $\alpha_3$ as a target of its trans-activating function, proposing that p51/p63 allows epidermal stem cells to express laminin receptor $\alpha_3\beta_1$ for anchorage to the basement membrane. When activated by genotoxic stress or overexpressed ectopically in non-adherent cells, p51/p63 transduced a phenotype to attach to extracellular matrices, which was accompanied by expression of ITGA3. Motifs matching the p53-binding consensus sequence were located in a scattered form in intron 1 of human ITGA3, and served as p51/p63-responsive elements in reporter assays. In addition to the trans-activating ability of the TA isoform, we detected a positive effect of the $\Delta N$ isoform on ITGA3. The high level $\alpha_3$ production in human keratinocyte stem cells diminished upon elimination of p51/p63 by small interfering RNA or by Ca$^{++}$-induced differentiation. Furthermore, a chromatim immunoprecipitation experiment indicated a physical interaction of p51/p63 with intron 1 of ITGA3. This study provides a molecular basis for the standing hypothesis that p51/p63 is essential for epidermal-mesenchymal interactions.

The p51/p63 gene (1, 2) codes for tumor suppressor p53-like nuclear proteins whose structures, tissue localization, and biological significance are well conserved among diverse organisms from humans to zebrafish (2–6) as reported to date. Studies showed enhanced expression of p51/p63 in squamous cell carcinomas of head, neck (7, 8), and lung (9), but detected less tight association of p51/p63 with other cancers (10). The gene knockout mice exhibited severe defects in skin, limb, and maxillofacial tissues (3, 6), reflecting the normal embryonic p51/p63 expression localized in epidermis, apical ectodermal ridge of the limb buds, and surface ectoderm at the branchial arches.

Germ line p51/p63 mutations in humans are associated with the ectoductyly, ectodermal dysplasia, and cleft palate syndrome (11) and other malformation syndromes (12). Immunohistochemical analyses with skin tissues revealed nuclear expression of the p51/p63 protein confined to the basal layer of epidermis (2). By a clonal analysis of human keratinocytes for proliferative potential and protein composition, it was determined that p51/p63 is specifically expressed in keratinocyte stem cells and transit amplifying cells, predominantly and less predominantly, respectively (13). Consistently, p51/p63-null mice exhibited striking epidermal defects: absence of keratinocyte stratification and differentiation, lack of normal basal cells expressing keratin 14, and exposed dermis (3, 6). Thus, true biological activities of p51/p63 essential for skin development should be identified in keratinocyte stem cells.

More than 6 isoforms arise from p51/p63 by using the TA- and $\Delta N$-type transcriptional initiation sites and by RNA splicing to form the C-terminal $\alpha_3\gamma$, $\beta_1\gamma$, and $\beta_1\gamma$ variants (2). Reflecting the structural similarity to p53 in the DNA binding domain, many of the genes inducible by p53 are also responsive to the p51/p63 proteins (1, 2, 14). Each of the TA isoforms acts more or less as a trans-activation factor, whereas the $\Delta N$ isoforms lacking the TA domain exhibit dominant-negative activities against p53 and the p51/p63 TA isoforms in reporter assays (2). More recently, however, a report revealed that a $\Delta N$ isoform of p73, another p53 homolog involved in neurogenesis, acts both as a positive and negative regulator of transcription (15). Furthermore, the TA isoforms of p51/p63 are unstable because of degradation by proteasome under normal conditions, but can accumulate in response to DNA damage to induce gene expression (16–18).

p51/p63 has been implicated in transcriptional events related to cell growth and differentiation. Those include down-regulation of the epidermal growth factor receptor expression (19), trans-activation of Jagged-1 encoding a Notch ligand (20), induction of $\beta$-globin expression indicative of erythroleukemic cell differentiation (16, 17), and activation of REDD-1 implicated in redox stress responses (21). With all these findings, however, essential molecular and cellular mechanisms that are assigned to p51/p63 for epidermal stem cell regulation still remain obscure.

We report here that p51/p63 trans-activates the ITGA3 gene coding for integrin subunit $\alpha_3$, also referred to as CD49c and VLA3-$\alpha$. The $\alpha_3$ subunit pairs with $\beta_1$ to form the $\alpha_3\beta_1$ (VLA3) complex which falls into the category of major epidermis integrins with other members, $\alpha_2\beta_1$ and $\alpha_4\beta_1$ (22–24). Integrin $\alpha_3\beta_1$...
is expressed predominantly in keratinocyte stem cells (23), and functions as a receptor for laminin, a major extracellular matrix (ECM) protein in the basement membrane (also referred to as basal lamina). The critical interactions between the dermal stem cells and their niche (25) may be facilitated by p51/p63 through its ability to induce ITGA3.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—U937 cell culture has been described (26). ECM binding assays were performed using culture dishes coated with laminin, fibronectin, collagen, or polylysine (BioCoat, BD Biosciences). Sixteen hours after incubation with actinomycin D, dishes were once washed with phosphate-buffered saline, and adherent and non-adherent cells were counted. Neonatal human keratinocyte (NHK) preparations from foreskin were from Cambrex. The basal medium was supplemented with human recombinant epidermal growth factor (0.2 ng/ml), insulin (10 μg/ml), hydrocortisone (1 μg/ml), gentamicin (0.1 mg/ml), amphotericin B (100 μg/ml), and bovine pituitary extract (Keratinocyte Growth Medium BulletKit, Cambrex). Experiments with NHK were accomplished within three subculturing cycles, during which cells maintained a high proliferating potential: more than 80% of the cells grew to form 16- to 32-cell colonies on day 3 after attachment to the basement (day 0).

**PCR amplification and RT-PCR**—Total RNA was purified with RNazol (Ambion) in combination with DNase I (Invitrogen) treatment. One-step RT-PCR was performed with the SuperScript One-Step RT-PCR system (Invitrogen). After the RT reaction with 0.2–0.5 μg of RNA and PCR primers for 30 min at 50–54 °C, synthesized DNA was directly amplified by 28–34 cycles of PCR consisting of annealing at 55–60 °C, elongation at 72 °C, and DNA strand dissociation at 94 °C. 18 S RNA was amplified for 16 cycles. The following primer pairs were used: p21/ctd, 5'-GTCCTCTTGAGGCCCCAGG-3' (forward) and 5'-GTTCAAAGACATGACTACGCT-3' (reverse); glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGATGTTC-3'; integrin α3, 5'-TCTCGGCTTCAACTCGGTGCTCTT-3' and 5'-GTTGACAGCTGAGCTGACACG-3'; integrin α5, 5'-TGGAGGTGGTACTGACACAC-3' and 5'-TGGAGGCGGCGGACGAC-3'; integrin α6, 5'-TCCACCCGGCACCAGACCT-3' and 5'-TCACTCAGAGGACGACCAAA-3'; integrin α10, 5'-CTCCGGTACGACGCCAGCT-3' and 5'-CCAACTTCGATGGGTCCAGTT-3'; glyceraldehydes-3-phosphate dehydrogenase (GAPDH), 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGATGTTC-3'. Primer sequences were confirmed with the ABI 377 sequencer. Plasmids were purified from DH5α by a GenElute Endotoxin-free Plasmid Kit (Sigma).

**Western Blotting**—We described Western blotting previously (17). An anti-integrin α3 antibody (Biogenesis, 5355–1305), an anti-p51/p63 antibody (AA4, Santa Cruz), an anti-p21 antibody (H164, Santa Cruz), a rabbit anti-human p53 antibody (FL939, Santa Cruz), and an anti-β-actin antibody (AC-15, Sigma) were purchased. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 7, 8.5, or 10% gels were transferred to polyvinylidene difluoride (Millipore) membrane, pore size 0.45 μm. For detection of p21/ctd, 12% polyacrylamide gel and polyvinylidene difluoride membranes with 0.2-μm pores were used.

**Transfection**—Plasmids were transfected into HeLa and Saos-2 cells with 0.2–0.5 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen), or with Lipofectamine 2000 plus 0.1% Triton X-100 in phosphate-buffered saline containing 5% nonfat dry milk and 5% goat serum (30 min at room temperature), and incubated with a primary antibody (for 16 h at 4 °C). Samples probed with fluorescein isothiocyanate- or rhodamin-labeled secondary antibody, for 1 h at 22 °C, were washed and mounted in an anti-fade medium (Dako, Japan). Photomicrographs were obtained with a Leica DMRB microscope equipped with an air-cooled camera (Synsys) controlled by QFSISH software (Leica).

**Chromatin Immunoprecipitation (ChIP)**—HEK293 cells seeded on 15-cm plates were transfected with a plasmid for expression of human influenza virus hemagglutinin epitope (HA)-tagged p51A/TAp63γ (Hap51A) (1). After 48 h, cells were fixed with formaldehyde, and the nuclei were purified. Sheared chromatin was immunoprecipitated with an anti-HA antibody (Zymed Laboratories Inc., 71-5500). Protein G-agarose blocked with salmon sperm DNA, RNAse A, proteinase K, a proteinase inhibitor mixture, mini-columns for DNA purification, and buffers were supplied in the ChIP-IT kit by Active Motif. Adsorption of the antibody-protein-DNA complexes to protein G-agarose, washing, reversal of cross-links, removal of RNA, protein digestion, and DNA purification were performed following the manufacturer’s protocol. A 500-bp segment (bp) of the GAPDH gene promoter locus was amplified by PCR with primers: 5'-TCTCCGCTTCAACTCGGTGCTCTT-3' and 5'-GTTGACAGCTGAGCTGACACG-3'; and 5'-TGGAGGCGGCGGACGAC-3'. For detection of the ITGA3 intrinsic sequences encompassing the first and second p53/p51/p63-binding consensus sequences, a 390-bp segment was amplified with primers: 5'-TCTCGGCTTCAACTCGGTGCTCTT-3' and 5'-GTTGACAGCTGAGCTGACACG-3'.

**RESULTS**

**Induction of an ECM-binding Phenotype and Integrin α3 Expression**—As we reported earlier, p51A/TAp63γ, a potent start codon of the ΔN CDNAs. siRNAs were transfected into NHK by an Amaxa Nucleofector apparatus (Amaxa Biosystems) with human keratinocyte nucleofector solution.

**p51/p63 Expression in HEK293 Cells**—We transfected HEK293 cells with pFRT/LacZeo and selected zeocin-resistant clones. The pFRT-positive cells were next transfected with tetracyclin repressor expression plasmid pCDNA6/TR, and blasticidin S-resistant clones were obtained. The pCDNA5/FRT/TTO vector with each of the p51/p63 isoform cDNAs was co-transfected with pOG4 into HEK293 clones having pFRT and pCDNA5/TR. Stable hygromycin-resistant clones were maintained as cell lines that induce p51/p63 expression in response to tetracyclin (2 μg/ml).

**Plasmids**—Overexpression of p51/p63 with the pCDMV vector was described, as were luciferase expression constructs, pRGC-luc (18) and pGL-ES (32). To construct p1+2 luc, a synthetic double strand DNA having sequences 594–612 and 877–901 derived from intron 1 of human ITGA3 was inserted into the multiple cloning site of a Luc expression vector, pGL3-promoter. p3+4 luc was constructed similarly, so that nucleotides 2465–2490 and 6437–6461 were inserted in tandem between the Nhel and Xhol sites located 5' to the SV40 promoter. The (3+4) segment was inserted at Nhel/Xhol sites of p1 (+ 2) to create p1+2+3+4 luc. Human genomic DNA was purified from peripheral blood cells obtained from a healthy volunteer donor (Y. T.). A DNA region encompassing intron 1 of ITGA3 was amplified by PCR with primers targeting the p51/p63-siRNA2 were synthesized (Greiner Japan), in which nucleotide number 1 corresponded to the A residue of the translation start codon of the ΔN CDNAs.
trans-activator isoform of p51/p63, accumulated in response to DNA damage to induce p21waf1 in a mouse cell line (16, 26). In this study, we propagated U/p51A and U/p53 cell lines derived from p53-deficient U937 human lymphoid cells after transfection with an Rous sarcoma virus promoter-driven, low-level expression vector for p51A/TAp63 and p53, respectively (16).

In U/p51A cells, p51A/TAp63 accumulated 8–24 h after exposure to 5 nM actinomycin D without an alteration in the mRNA level (Fig. 1A). The RT-PCR and Western blot analyses also showed activation of the p21waf1 and GADD45 expression with an increase in the p21waf1 protein amount. U/p53 cells incubated with actinomycin D also stabilized p53, inducing p21waf1. Control U937 cells caused a slight up-regulation of p21waf at 16 h, probably by a p53-independent mechanism (33).

We examined those cells for attachment to basements coated with laminin, fibronectin, or collagen (Fig. 1B). More than 90% of the U/p51A cells incubated with actinomycin D attached to laminin-coated dishes within 16 h. Approximately 55 and 16% of the cells bound to fibronectin and collagen, respectively, with little evidence of their binding to polylysine. In contrast, only a 9% fraction of the drug-treated U/p53 cells bound to laminin, and a yet smaller fraction to fibronectin. Control U937 cells
adhered to neither of the extracellular matrix proteins under the conditions used.

To find the molecular basis of the p51A-caused cell attachment to ECM, cellular RNA was analyzed for expression of integrin genes by RT-PCR (Fig. 1C). With two different primer pairs, α3 (#1), and α3 (#2), an increase in ITGA3 mRNA was evident at 8–24 h after exposure to actinomycin D. By contrast, expression of the αε, α5, α6, and α3 genes remained constant through the time course. Although β1 gene expression increased slightly, reaching maximum at 8 h, the response was not specific to U/p51A. Consistent with RT-PCR analysis, the 145-kDa α3 (Ref. 34) content increased in U/p51A cells during the period (Fig. 1C, bottom). Because the α3 subunit associates with β1 to form the α3β1 heterodimer whose major and minor ligands are laminin and fibronectin/collagen (35), respectively, we hypothesized that p51/p63 promotes ITGA3 expression directly or indirectly to cause cell-ECM attachment.

**Transient or Drug-controlled p51/p63 Expression also Activates ITGA3**—We performed transient overexpression of p51/p63 in U937 cells using a cytomegalovirus promoter vector with a liposome-based transfection carrier. The p51A/TAp63y expression vector, but not the vector-only plasmid, caused laminin binding activity (Fig. 2A and B). Reflecting the transfection efficiency, 10%, determined by a G418-resistant colony formation assay, a ~10^-fold fraction of the cells bound to laminin (Fig. 2B). Although the apparent increases in the mRNA and protein of α3 were not so great (2–3-fold) when the entire culture was analyzed (Fig. 2A), we could speculate that there was a greater increase in α3 expression in the attached cell population (10%). Furthermore, ΔNp51ΔNp63γ lacking the trans-activation domain also generated cells adherent to laminin (Fig. 2B), which was accompanied by ITGA3 activation as detected by mRNA and protein analyses (Fig. 2A). In contrast to the poor retention of 57-kDa p51A/TAp63γ having the N-terminal sequences that determine its fate for degradation by proteasome (16, 18), ΔNp51A/ΔNp63γ was stable enough to form an intense band at 52 kDa in Western blotting (Fig. 2A). Thus, neither the laminin-adherent phenotype nor the α3 induction shown in Fig. 2 required a cellular signaling event caused by actinomycin D. When expressed to a certain level, the TA and ΔN proteins seemed to induce ITGA3.

Human embryonic kidney (HEK) 293 cells were genetically engineered so that established cell lines, 293-1, -2, and -5 expressed cDNAs of p51A/TAp63γ, p51B/TAp63α, and ΔNp51A/ΔNp63γ, respectively, from the same position on the chromosome upon induction with tetracycline. Twelve hours after induction, each cell lines achieved a high level production of p51A/TAp63γ (57 kDa) (16), p51B/TAp63α (85 kDa) (17), or ΔNp51A/ΔNp63γ (52 kDa) (36) (Fig. 2C, left). Expectedly, the ITGA3 mRNA increased ~10-fold in p51A/TAp63γ-expressing 293-1 cells (Fig. 2C, right). p51BTAp63α- or ΔNp51A/ΔNp63γ-expressing cells also caused a 3- or 2-fold activation of ITGA3, respectively, whereas control HEK293 cells showed no detectable change. Consistently, the 145-kDa integrin α3 protein...
increased in tetracycline-induced 293-1, 293-2, and 293-5 cells, reflecting the relative amounts of the ITGA3 mRNA (Fig. 2C, left). Processed integrin α3 light chain (30 kDa) was not detectable. Supporting the experiments with U937 cells (Figs. 1 and 2), these results indicated that ITGA3 is inducible by both the TA and ΔN isoforms of p51/p63.

*p51/p63-responsive Elements in the First Intron of ITGA3—* We found sequences matching RRRC(T/A)(T/A)GYYY at positions 597, 881, 2475, and 6422 in intron 1 of ITGA3 on human chromosome 17 (at 17q21.33), in which position numbers are relative to nucleotide 1 at the 5’ end of intron 1 (Fig. 3). Another motif was present 2594 bp upstream of the transcription start site of the 1.4-kb 5’-terminal intron segment containing the nearby TATA box. The decanucleotide motif corresponded to a half-site of the full consensus sequence occupied by a p53 tetramer (a dimer of a dimer), RRRC(T/A)(T/A)GYYY-N(0–13)-RRRC(T/A)(T/A)GYYY, where N(0–13) indicates a spacer of 0 to 13 bases in the classical definition (37). Because a half-site is bound by a p53 dimer to cause moderate transcriptional activation (40), the four motifs scattered in intron 1 were speculated to serve as the cis-acting elements for the regulation by p51/p63.

We constructed luc expression plasmids, p(1+2)luc, p(3+4)luc, and p(1+2+3+4)luc, by placing the 1st (597), 2nd (881), 3rd (2475), and 4th (6422) motifs upstream of the SV40 promoter in different combinations (Fig. 3A). Trans-activation assays were performed by co-transfection of the luc plasmids with p53, p51A/TA(p63)γ, ΔNp51A/ΔNp63γ, or control expression vector (pRC-CMV) in HeLa cells derived from human cervical epithelia (Fig. 3B). p51A/TA(p63)γ activated the (1+2), (3+4), and (1+2+3+4) promoters by 4.5-, 1.8-, and 3.4-fold, respectively. p53 also caused a 3.4-, 1.4-, and 3.8-fold increase with those plasmids. The alignment of the 1st and 2nd motifs was more effective than that of the 3rd and 4th motifs in this assay. ΔNp51A/ΔNp63γ positively affected transcription from either of the plasmids, although the ΔN isoform certainly exerted a dominant-negative type activity in our assay system with the ribosomal gene cluster promoter as reported previously (36). The activation profile detected with p(1+2)luc was consistent with the endogenous ITGA3 responses to p51/p63 observed in U937 and HEK293 cells (Figs. 1 and 2).

We next performed reporter assays with the (α3-1)luc plasmid that had the 1.4-kb 5’ terminal intron segment containing the 1st and 2nd motifs immediately downstream of the luc-coding region in the 3’ to 5’ orientation (Fig. 3, C and D). (α3-1)luc increased its luciferase expression by 1.2-, 3.2-, and 1.9-fold in the presence of p53, p51A/TA(p63)γ, and ΔNp51A/ΔNp63γ, respectively. Combined expression of p51A/TA(p63)γ and ΔNp51A/ΔNp63γ
and Δp51A/Δp63γ caused a 2.5-fold activation of (α3-1)luc, implying an interaction between the TA and ΔN isoforms. When a single mutation, G603T, and a double mutation, G603T/G887T, were introduced into (α5-1)luc to render M1(α5-1)luc and M1M2(α5-1)luc, respectively, the efficiency of transactivation by p51A/TAp63γ dropped to 1.4- and 1.2-fold. These results indicated that the 1st and 2nd half-site motifs cooperatively play an essential role in response to p51A/TAp63γ. The ΔN isoform seemed to act on the same sites to cause moderate activation. On the other hand, (α5-4)luc, in which the 1.4-kb segment was placed in the 5′ to 3′ orientation, produced a high background of luciferase activity, and was less sensitive to p53 and p51/p63 (Fig. 3D), implying that G/C-enriched sequences in the 5′ terminal region of the insert affected the heterologous viral promoter activity in the vector.

Concurrent Expression of p51/p63 with α3 in Epidermis Development—Immunostaining of skin sections from mouse embryos on day 14 (E14) showed p51/p63 protein localization in the inner layer of the double-layered surface ectoderm or the periderm (Fig. 4A, far left). The p51/p63 nuclear stain intensified in the basal layer of epidermis on E16 when epidermis stratification was in progress. In newborn mice, however, the overall p51/p63 stain significantly decreased, leaving p51/p63-positive cells in clusters that corresponded to the patches of keratinocyte stem cells (13). The double immunofluorescence analysis showed that nuclear p51/p63 stain (fluorescein isothiocyanate) coincided with peripheral α5 (Rhodamin) stain in the basal cells of the E14, E16, and newborn tissues (Fig. 4A, three right panels). Furthermore, the α5 label was also markedly weakened at birth. In their temporal and spatial expression profiles, p51/p63 and α5 were closely related to each other in mouse skin development.

The α3 Expression Is Associated with p51/p63 in Keratinocyte Stem Cells—We cultured NHK to analyze p51/p63 and integrin α3 expressions. More than 90% of the cells in the NHK culture were able to replicate to form a colony of 16 or 32 descendant cells within 3 days after plating with medium containing 0.1 mM Ca2+ (low Ca2+). As judged by the high growth potential, epidermal stem cells were predominant in the culture. By changing the extracellular calcium concentration, we could control growth and differentiation of the epidermal cells (41–43). When the Ca2+ concentration was raised to 1 mM (high Ca2+), those cells ceased replicating and underwent differentiation as described. Seven days after incubation with high Ca2+, we detected mRNA for involucrin, a keratinocyte differentiation marker (Fig. 4B).

Both the TA and ΔN type transcription occurred in NHK (Fig. 4B, day 0). Western blotting also detected the p51/p63 proteins, p51A/TAp63γ (57 kDa), p51B/TAp63αβ (85 kDa), Δp51A/Δp63γ (52 kDa), and Δp51B/Δp63αβ (80 kDa), as identified by experimental expression in U937, HEK293 (Fig. 2), and HeLa cells (36). The ΔN-type transcripts gradually decreased on days 4 and 7, whereas the TA-type mRNA did not change significantly up to day 7. However, Western blotting revealed that both the TA and ΔN proteins gradually diminished during this period (Fig. 4B). The protein degradation system controlling p51/p63 may be enhanced in cells incubated with high Ca2+. Thus, depletion of p51/p63 during keratinocyte differentiation (13) seemed to involve transcriptional and posttranscriptional mechanisms.

In NHK (day 0), 145-kDa full-length α5 appeared predominant in the Western blot with the antibody reactive with the cytoplasmic peptide. On day 4 of Ca2+ induction, the 30-kDa light chain of α5 (44), instead of the 145-kDa protein, formed an intense band, indicating that the α5 maturation process had become active (Fig. 4B). We did not detect a decrease in the ITGA3 expression by RT-PCR for at least 4 days after the Ca2+ input. On day 7, when the p51/p63 protein content had mark-
edly decreased, the α3 mRNA and protein levels had declined markedly. In contrast, p21\textsuperscript{med} gene expression was up-regulated on days 4 and 7 compared with day 0, possibly because of the decline in the ΔN isoforms that negatively regulate p21\textsuperscript{med}. The level of integrin α3 mRNA and protein decreased, the α3 protein shift from the perinuclear regions to the cell-cell borders. Control double labeling with an anti-pan-keratin antibody indicated that the siRNA did not affect the total level of cytokeratins. Thus, the integrin α3-inducing ability of p51/p63 seemed vital in keratinocyte stem cells.

Detection of an Interaction of p51/p63 with Intron 1 of ITGA3—
To assess whether p51/p63 can directly interact with the 1st intron of ITGA3 on the chromosome, a ChIP experiment (48) was carried out with HEK293 cells transfected with an HAp51A expression vector and control untransfected cells. Expression of HAp51A was confirmed by Western blotting with anti-HA antibody and 4A4 anti-p51/p63 antibody (Fig. 6A). An increase in 145-kDa integrin α3 was also detectable in the HAp51A-expressing cells.

For immunoprecipitation, we used three different antibodies: control non-immune IgG, an anti-HA antibody, and an antibody against TFIIB, a general transcription factor. 4A4 was not reactive with either of the p51/p63 isoforms unless proteins were fully denatured, and was not useful for ChIP. DNA fractions recovered from the immunoprecipitates were examined by PCR for a GAPDH promoter locus and an ITGA3 intron 1 segment (553–942) encompassing the 1st and 2nd half-sites. From both the HAp51A-transfected and untransfected cells, the GAPDH promoter segment was more abundantly precipitated by the anti-TFIIB antibody than by the control and anti-HA antibodies (Fig. 6B, left panels). Constant transcription of the housekeeping gene was thus detectable by this ChIP experiment. On the other hand, the ITGA3 segment was more enriched in the anti-TFIIB and anti-HA precipitates from the HAp51A-expressing cells than in the control non-immune IgG precipitates. Neither the anti-TFIIB or anti-HA precipitate...
from untransfected cells was enriched for the ITGA3 sequences (Fig. 6B, right panels). These results not only indicated a physical interaction of p51/p63 with intron 1 of ITGA3, but also verified that the gene transcription is induced by p51/p63.

**DISCUSSION**

We have shown (i) induction of integrin α3 by DNA damage-caused p51/p63 protein activation and transient and controlled p51/p63 overexpression, (ii) transduction of an ECM-binding phenotype relevant to integrin αβ1 in a non-adherent leukemic cell line, (iii) presence of p51/p63-responsive sites in the 1st intron of human ITGA3, (iv) suppression of α3 by p51/p63 knockdown with siRNAs and by differentiation in the NHK culture, and (v) association of p51/p63 with intron 1 of ITGA3 on the chromatin. Transcriptional activation of ITGA3 may be one of the pivotal roles of p51/p63 in epidermis development.

Integrin α3β1 is a major epidermis laminin receptor whose expression is confined to the stem cells (23). Although Itga3-null mice caused occasional skin blistering where the epidermis separated from the dermis (49), conditional null mice caused occasional skin blisters where the epidermis displayed extreme skin blistering, hair defects, and basement membrane disassembly, indicating more critical roles for the partners of β1 in skin development (50). The terminal differentiation program was, however, preserved even in the absence of β1 (50). A double mutation in α3 and α6 impaired basement membrane assembly and epidermal cell compaction in the apical ectodermal ridge, and thereby caused abnormal limb patterning (51). Defects in the urogenital tracts and other organs were also observed in the Itga3−/−Itga6−/− mice. Thus, most of the abnormalities in the integrin α or β knock-out embryos are closely related to the phenotypes of the ectoductuity, ectodermal dysplasia, and cleft palate syndrome (11, 12) and p51/p63 deletion (3, 6). Our p51/p63 knockdown experiment with siRNA provided evidence that p51/p63 is a dominant factor inducing the α3 expression in keratinocyte stem cells. Because of the localized, abundant expression in undifferentiated keratinocytes (23), the p51/p63 proteins may control ITGA3 efficiently in those cells. However, the more striking skin phenotypes observed in the p51/p63-null mice imply cooperation of α3 with other signaling and/or structuring molecules under control of p51/p63.

Most of the known p53-responsive promoters have a full-site that forms a stable complex with a p53 tetramer (38). However, a half-site can accommodate a p53 dimer (39), and serves as a minimal p53-responsive element when placed adjacent to a Sp1 site (40). Furthermore, a full p53-binding site with a 33-base minimal p53-responsive element when placed adjacent to a Sp1 site (40). Additionally, a full p53-binding site with a 33-base minimal p53-responsive element when placed adjacent to a Sp1 site (40), whereas a half-site can accommodate a p53 dimer (39), and serves as a functional dimerization domain was detectable by a yeast two-hybrid assay, indicating dimer formation (50). The mechanism of ITGA3 regulation by p51/p63 may involve topological arrangement of the separate half-sites in intron 1, interactions of p51/p63 with other transcriptional regulatory factors, and association between different p51/p63 isoforms.

The mouse α3 gene also contains three half-site motifs in intron 1, in which the 1st and 2nd motifs are located in the 5′ terminus, suggesting a similar regulatory mechanism in intron 1 by p51/p63. The promoter/enhancer region extending to −4 kb has been extensively studied (32, 54) to determine that the Ets binding site at −133 is critical for gene expression in MKN1 gastric carcinoma cells. However, the 4-kb promoter/enhancer region lacked a putative p51/p63-binding site, and did not respond to p51/p63 in our assay (data not shown).

When assayed with the promoters of p53 target genes, the ΔN isoforms suppressed the trans-activating ability of p51A/TAp63γ (2), by which the ΔN proteins were originally determined to be dominant-negative-type isoforms. A recent study demonstrated that a ΔN isoform of p73 is able to both positively and negatively regulate p53 target genes (15). p53 activates the human BAX promoter using Sp1 as a cofactor (40), whereas p51A/TAp63γ suppresses epidermal growth factor-receptor gene expression by an interaction with Sp1 (19). Gene regulation by the TA and ΔN forms of p51/p63 now appears more elusive than originally characterized. To exert the positive effect, the ΔN proteins may interact with an activation factor whose expression could be cell-type specific.

αβ1 is essential for cell spreading on the basement membrane, ECM assembly, hemidesmosome stability, establishment/maintenance of the cytoskeletal organization, epidermal proliferation, and control of cell migration (24, 25, 50, 55–57). It may be through the αβ1−/−-caused epidermal-mesenchymal interactions that p51/p63 facilitates generation of an undifferentiated basal cell population. In addition to the epidermis, p51/p63 expression occurs in the basal cells of epithelia of the mammary gland (58), oral tissues (59), uroterovix (60), and bladder (30), where the α3−/−-inducing role of p51/p63 may also function. Furthermore, the overexpression of p51/p63 in squamous cell carcinomas of head, neck, and lung (7, 9) might contribute to the high-level α3 expression that determines cellular capability of invasion and metastasis (61–63). It is interesting to envisage that p51/p63, a hypothetical ancestor gene to tumor suppressor p53 (64), had evolved as an inducer of the critical interaction between growing epithelial cells and their niche.

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