Slug Regulates Integrin Expression and Cell Proliferation in Human Epidermal Keratinocytes*

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The human epidermis is a self-renewing epithelial tissue composed of several layers of keratinocytes. Within the epidermis there exists a complex array of cell adhesion structures, and many of the cellular events within the epidermis (differentiation, proliferation, and migration) require that these adhesion structures be remodeled. The link between cell adhesion, proliferation, and differentiation within the epidermis is well established, and in particular, there is strong evidence to link the process of terminal differentiation to integrin adhesion molecules expression and function. In this paper, we have analyzed the role of a transcriptional repressor called Slug in the regulation of adhesion molecule expression and function in epidermal keratinocytes. We report that activation of Slug, which is expressed predominantly in the basal layer of the epidermis, results in down-regulation of a number of cell adhesion molecules, including E-cadherin, and several integrins, including α3, β1, and β4. We demonstrate that Slug binds to the α3 promoter and that repression of α3 transcription by Slug is dependent on an E-box sequence within the promoter. This reduction in integrin expression is reflected in decreased cell adhesion to fibronectin and laminin-5. Despite the reduction in integrin expression and function, we do not observe any increase in differentiation. We do, however, find that activation of Slug results in a significant reduction in keratinocyte proliferation.

The human epidermis is a self-renewing epithelial tissue composed of several layers of keratinocytes. Cells are continually lost from the tissue surface and require constant replacement by a process known as terminal differentiation. Terminal differentiation involves cells in the basal layer of the epidermis withdrawing from the cell cycle and migrating into the suprabasal differentiated layers (1). This migration process, whereby cells detach from a specialized extracellular matrix (ECM) known as the basal lamina and move upwards through the epidermis, requires that cellular junctions with the underlying basal lamina (focal adhesions and hemidesmosomes) and with neighboring cells (adherens junctions and desmosomes) be regulated. There is considerable evidence that integrin adhesion molecules play key roles in regulating epidermal function. In normal epidermis, integrins (with the exception of αvβ8) are not expressed in the suprabasal, differentiating layers of the epidermis and are instead generally confined to the basal layer (1, 2). Keratinocyte stem cells express high levels of integrins in vivo and in vitro, and disruption of integrin-ECM interactions results in initiation of terminal differentiation in vitro (3–5).

The Snail family of transcriptional repressors are conserved throughout evolution, with well documented roles in a number of vertebrate and invertebrate developmental processes. These include control of mesoderm differentiation and neurogenesis in Drosophila and left-right asymmetry in vertebrates (reviewed in Ref. 6). All Snail family proteins are zinc-finger-containing transcriptional repressors that bind to DNA at E-box motifs (CANNTG) on target gene promoters (7, 8). Originally, Snail family members were thought to be determinants of developmental processes, such as mesoderm induction, but increasingly the evidence suggests that Snail family members control developmental processes by regulating genes involved in cell adhesion and migration rather than inducing these processes directly (6).

Recently, a number of reports have implicated Snail family members in tumor progression. Snail has been reported to be up-regulated in a number of human tumors, including breast, colon, and gastric cancer, and there is a strong correlation between Snail expression and degree of invasiveness in these tumors (9–11). Another Snail family member, Slug, has also been shown to be up-regulated in breast cancer and in malignant mesothelioma (12, 13). One role of Snail family proteins in human tumors may be to repress E-cadherin expression, thus contributing to the invasive, migratory phenotype of these cells (13, 14). This would be consistent with mouse tumor models, where Snail has been shown to be active in the invasive region of the tumor (14).

Whereas much is known about the function of Snail proteins during development, and their potential role in malignant progression is becoming more apparent, relatively little is known about their role in normal adult tissue. Slug mRNA has been regulated kinase; MAPK, mitogen-activated protein kinase; BrdUrd, bromodeoxyuridine.

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¶The abbreviations used are: ECM, extracellular matrix; 4-HT, 4-hydroxytamoxifen; Q-PCR, quantitative PCR; GFP, green fluorescent protein; ER, estrogen receptor; CFE, colony forming efficiency; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; BrdUrd, bromodeoxyuridine.
detected in a variety of adult human tissues, and recently expression of a Slug-β-galactosidase fusion protein has been detected in mouse epidermis (15, 16). In this study, we have used conditional activation of Slug in human keratinocytes to analyze the role of Slug in the epidermis. We provide evidence that components of focal adhesions (α3β1 integrin) and hemidesmosomes (β4 integrin) are novel targets for Slug and that activation of Slug results in inhibition of keratinocyte proliferation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Antibodies used were involucrin (SY-5; Abcam, Cambridge, UK), Slug (G-18; Santa Cruz Biotechnology), E-cadherin (HECD-1), actin (1A; Sigma), PAK (450-11A), α-tubulin (DM 1A; Sigma), PAKα (C-19; Santa Cruz Biotechnology), ERK (9102; Cell Signaling), phospho-ERK (pTEpY; Promega), Rb (9302; Cell Signaling Technology), phospho-Rb (9308S; Cell Signaling Technology), and caspase-3 (552037; BD Biosciences). Secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). All other reagents were purchased from Sigma.

Cell Culture—Primary human keratinocytes were cultured using the method of Rheinwald and Green as described elsewhere (17). In some experiments Slug activity was induced by the addition of 100 nM 4-hydroxytamoxifen (4-HT). 293T HEK cells were cultured and then incubated for 1 h with a goat polyclonal Slug antibody (2 μg/ml). Sections were then incubated with peroxidase-linked rabbit anti-goat (1:100), and immunoreactivity was visualized using diaminobenzidine reagent followed by counterstaining with hematoxylin. Omission of primary antibody was employed as a negative control. For immunocytochemistry, keratinocytes, cultured on acid-washed glass coverslips, were fixed, permeabilized, and stained with appropriate primary and secondary antibodies as described previously (18). Entry of cells into S phase was analyzed by incorporation of BrdUrId using a commercially available kit (Roche Applied Science). Immunostained cells were visualized using a Leica DMRB microscope equipped with a Hamamatsu ORCA camera, and images were captured and processed using OpenLab software (Improvement).

SDS-PAGE and Western Blotting—Protein lysates were prepared in RIPa buffer, separated by SDS-PAGE, and subjected to Western blot as described elsewhere (21).

Adhesion Assays—Keratinocytes were resuspended in serum-free medium and plated in 96-well plates (Immunul II; Thermo Electron) coated with either recombinant fibronectin (319-10 diluted in phosphate-buffered saline or a laminin-5-enriched substrate and blocked in bovine serum albumin/phosphate-buffered saline for 3 h. After incubation for 1 h, non-adherent cells were removed by washing in phosphate-buffered saline, and numbers of adherent cells were assessed by analysis of endogenous hexosaminidase activity (22). Laminin-5 and fibronectin-10 were produced as described elsewhere (23, 24).

Real Time Q-PCR Analysis—RNA for real time quantitative polymerase chain reaction (Q-PCR) analysis was extracted from keratinocyte cultures using a Qiagen RNEASY mini with on column DNase digestion (Qiagen). RNA was eluted into 50 μl of RNase free water, and concentration was determined by spectrophotometry. cDNA was synthesized from 1 μg of RNA using random hexamers and single strand RT polymerase II

| Primer/Probe | Sequence |
|--------------|----------|
| E-cadherin   | Forward: 5′-GCCGCCACCTCGAGGAGA-3′ |
|              | Probe:   5′-AATTTTACTGGTCACCGCCGAGG-3′ |
|              | Reverse: 5′-TTGCGACCTCGTAATCCT-3′ |
| α3 integrin  | Forward: 5′-GTCCTTGCGTTATCTC-3′ |
|              | Probe:   5′-TTGCCGACCTGGTAGCATGA-3′ |
|              | Reverse: 5′-GACGAAATCTCGAAATCTATCCTC-3′ |
| β1 integrin  | Forward: 5′-TTCCATGGCCTCACTGAGG-3′ |
|              | Probe:   5′-TTGCCAGTTGTGGATCGACTGAGCTG-3′ |
|              | Reverse: 5′-ACCAGCAACACCTGGTACATCCTC-3′ |
| β4 integrin  | Forward: 5′-ACTATTCGATTTGGAGGTC-3′ |
|              | Probe:   5′-AAAGGCAGACTCCCTAGCCACAGG-3′ |
|              | Reverse: 5′-TCAGATCCAGGCTCTCATG-3′ |

| Primer/Probe | Sequence |
|--------------|----------|
| E-cadherin   | Forward: 5′-GCCGCCACCTCGAGGAGA-3′ |
|              | Probe:   5′-AATTTTACTGGTCACCGCCGAGG-3′ |
|              | Reverse: 5′-TTGCGACCTCGTAATCCT-3′ |
| α3 integrin  | Forward: 5′-GTCCTTGCGTTATCTC-3′ |
|              | Probe:   5′-TTGCCGACCTGGTAGCATGA-3′ |
|              | Reverse: 5′-GACGAAATCTCGAAATCTATCCTC-3′ |
| β1 integrin  | Forward: 5′-TTCCATGGCCTCACTGAGG-3′ |
|              | Probe:   5′-TTGCCAGTTGTGGATCGACTGAGCTG-3′ |
|              | Reverse: 5′-ACCAGCAACACCTGGTACATCCTC-3′ |
| β4 integrin  | Forward: 5′-ACTATTCGATTTGGAGGTC-3′ |
|              | Probe:   5′-AAAGGCAGACTCCCTAGCCACAGG-3′ |
|              | Reverse: 5′-TCAGATCCAGGCTCTCATG-3′ |
(Invitrogen), as per the manufacturer’s instructions. cDNA synthesis and equal RNA loading were checked by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers. Target primer and probe sequences were chosen with the assistance of Primer Express (PerkinElmer Life Sciences), and Blast searches were performed to confirm gene specificity. To avoid amplification of contaminating genomic DNA, primers and probes spanned exon-exon boundaries. Primers were synthesized by Sigma. Dual labeled FAM-TAMRA (5-carboxyfluorescein-6-carboxytetramethylrhodamine) probes were synthesized by Eurogentech. Nucleotide sequences of primers and probes are listed in Table 1. 18 S ribosomal RNA control kit, including primers and VIC-labeled probe, was used as internal control (PerkinElmer Life Sciences). Sample cDNAs were subjected to Q-PCR in triplicate using an ABI Prism 7000 sequence detection system (Applied Biosystems). Cycle threshold (Ct) values were calculated using identical threshold values for all experiments and 18 S Ct values subtracted as control for loading (dCt). Relative expression was compared with keratinocytes expressing GFP:ER treated with 4-HT for 72 h, and -fold change

FIGURE 1. Slug is expressed in basal layer of the human epidermis. 7-μm frozen sections of human epidermis were stained using a polyclonal Slug antibody (A and B). As a control, primary antibody was omitted (C). The basal (arrowhead) and cornified (star) layers of the epidermis are indicated. The scale bars represent 50 μm.

FIGURE 2. Establishment of keratinocytes expressing GFP:ER and GFP:Slug:ER fusion proteins. Stable keratinocyte cell lines expressing GFP:Slug:ER or control GFP:ER constructs were established by retroviral transduction. A, mRNA isolated from cells expressing GFP:ER or GFP:Slug:ER was subjected to RT-PCR using GFP and ER primers. mRNA from parental cells was used as a control. M, size markers (kb). B, cells stably expressing GFP:ER or GFP:Slug:ER were cultured in the presence or absence of 100 nm 4-HT for 72 h before being examined under a fluorescence microscope. Scale bar, 25 μm.
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was calculated by conversion through 2^ddCt. Data shown represent the mean and S.E. of three separate experiments.

**Reporter Assays**—The wild-type human E-cadherin promoter sequence (−301/+21) cloned into pGL3-basic luciferase reporter (EproWT) was a gift from Prof. van Roy (8), and the mouse α3 integrin minimal promoter cloned into pGL3basic (pGL-ES) was a gift from T. Tsuji (25). pGL-ES-EBM, containing the mutated, nonfunctional E-box sequence was generated by site-directed mutagenesis of the E-box sequence in pGL-ES using the following primer sequence: 5′-AGGGTTCCCGATCGGTGTCTGAGAGA-3′ (mutated bases are underlined) (25). pGL3 basic, containing the firefly luciferase gene without a promoter sequence, was used as a control, with the Renilla luciferase-expressing plasmid (pRL TK) used as a transfection control. 293T HEK cells were transiently transfected with 0.3 μg of either EproWT, pGL-ES, pGL-ES-EBM, or pGL3-basic in the presence of 1 μg of pcDNA3 control or pcDNA3-Slug vector and 0.1 μg of pRL TK. Firefly and Renilla luciferase activities were assayed 48 h post-transfection using the dual reporter assay kit Stop ‘N’ Glow (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity as a transfection control. Promoter activity is expressed as relative luciferase activity compared with that obtained in pGL3basic control-transfected cells.

**Electrophoretic Mobility Shift Assays**—Electrophoretic mobility shift assays were performed as described elsewhere (26). Briefly, 293T HEK cells were transiently transfected with pMSCV-Slug-IRES-GFP. Cells were subsequently lysed, and the nuclear and cytosol fractions were purified. 10 μg of nuclear extract was allowed to bind to 100 fmol/μl 5′ biotin-labeled α3 integrin oligonucleotide dimers with or without E-box mutations, in the presence or absence of unlabeled competitor at 20 pmol/μl, and in the presence or absence of 2 μl of anti-Slug antibody as appropriate. Reactions were electrophoresed on a 6% acrylamide gel under nondenaturing conditions and transferred to nylon membrane, and biotin-labeled oligonucleotides were detected using horseradish peroxidase-conjugated streptavidin. Binding oligonucleotide sequences were taken from positions 254 to 218 of the murine α3 promoter and are listed in Table 2.

**RESULTS**

**Slug Is Expressed Predominantly in the Basal Layer of the Epidermis**—The expression and function of Slug in adult tissue is poorly characterized. We analyzed expression of Slug in human skin by immunohistochemistry of sections taken from foreskin. Expression of Slug was observed in both the dermis and in all layers of the epidermis, with high levels of expression in the basal layer of the epidermis (Fig. 1). Expression of Slug was not confined to the...
nucleus, with strong expression also observed in the cytoplasm of basal and suprabasal cells. This is consistent with data from Slug-β-galactosidase transgenic mice, where expression of the Slug-β-galactosidase fusion protein was detected in the basal layers of the epidermis and in other epithelial tissues (16). The same report also identified high level Slug expression in hair follicles absent from these sections. Strong Slug immunoreactivity was also detected in the outermost, anuclear cornified layers of the epidermis. However, this staining is probably nonspecific and has been observed with a number of other nonrelated primary antibodies (data not shown).

**Slug Regulates E-cadherin Expression in Epidermal Keratinocytes**—To analyze the function of Slug in keratinocytes, we established, by retroviral transduction, cell lines stably expressing a protein consisting of full-length Slug fused to GFP and the ligand-binding domain of the estrogen receptor (ER). In this system, the GFP:Slug:ER fusion protein is constitutively expressed but is inactive due to the binding of a heat shock protein (HSP90) to the ER ligand-binding domain. The addition of the estrogen homologue, 4-HT, results in displacement of HSP90 and activation of Slug (27). We used retroviral transduction and selection in puromycin to establish keratinocyte cell lines expressing pBabePuroGFP:Slug:ER (GFP:Slug:ER) and pBabePuroGFP:ER (GFP:ER). We established expression of the respective constructs by RT-PCR (Fig. 2A) and by analyzing GFP fluorescence (Fig. 2B). We observed expression of GFP in over 80% of cells, and the GFP:Slug:ER fusion protein was localized to the nucleus in the presence and absence of 4-HT (Fig. 2B and data not shown). Having established these cell lines, we analyzed expression of E-cadherin in epidermal keratinocytes following activation of Slug. E-cadherin is a known Slug target in other cell types and thus provides a suitable functional readout for the activity of Slug in our cells (28). Following the addition of 4-HT, we observed significant, but not complete, loss of E-cadherin from sites of cell-cell adhesion in cells expressing GFP:Slug:ER but not in control cells expressing a GFP:ER fusion (Fig. 3A). Western blot analysis of the same cells also revealed a loss in E-cadherin expression in cells expressing GFP:Slug:ER following treatment with 4-HT to activate Slug (Fig. 3B). 4-HT treatment of control cells expressing GFP:ER resulted in no loss of E-cadherin expression, indicating that the loss in expression was a consequence of Slug activation and not a 4-HT-associated effect. The loss in E-cadherin expression at the protein level was mirrored by a decrease in E-cadherin mRNA levels as determined by real time Q-PCR (p < 0.01; Student’s t test) (Fig. 3C). In promoter reporter assays, we also observed repression of E-cadherin promoter activity following co-transfection of 293T cells with plasmids encoding full-length Slug and the E-cadherin promoter fused to luciferase (p < 0.001; Student’s t test). Expression of PAKα was also assessed as a loading control. C, real time Q-PCR was performed on RNA isolated from GFP:ER- and GFP:Slug:ER-expressing cells cultured for 72 h in the presence of 100 nM 4-HT. Data shown are the mean and S.E. from three separate experiments. D, 293T HEK cells were transiently transfected with 0.3 μg of pGL-ES (α3 promoter) or pGL-ES-EBM (α3 promoter E-box mutant), 0.1 μg of pRL-TK (transfection control), and 1 μg of either pcDNA3-Slug or pcDNA3, cultured for 48 h, and lysed, and luciferase activity was analyzed. Data shown are the mean and S.E. from six separate experiments.
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(Fig. 3D). This loss of E-cadherin expression and repression of transcription is consistent with previous reports identifying E-cadherin as a Slug target (7). The reduction in E-cadherin expression, rather than complete loss, is also consistent with the report that Slug is a weaker repressor of E-cadherin than Snail (28). These data confirm expression and activity of Slug and identify a role for Slug in the epidermis.

Regulation of α3 Integrin Expression by Slug—We next analyzed whether Slug could regulate expression of α3, β1, and β4 integrins, all of which are constitutively expressed in vivo and in vitro in keratinocytes (1). Analysis of α3, β1, and β4 integrin sequences revealed the presence of potential E-boxes in all three integrin promoters, suggesting that they might be potential Slug targets (25, 29, 30). Activation of Slug resulted in loss of α3 from sites of cell-cell contact (Fig. 4A), presumably as a consequence of the dramatic reduction in total α3 protein expression as determined by Western blotting (Fig. 4B). No change in α3 localization or expression was observed in control keratinocytes treated with 4-HT (Fig. 4, A and B). Further experiments, involving real time Q-PCR analysis, revealed a significant decrease in α3 mRNA following activation of Slug (p < 0.05; Student’s t test) (Fig. 4C). Co-transfection of 293T cells with a Slug cDNA expression vector and an α3 promoter reporter construct revealed a significant decrease in α3 promoter activity (p < 0.001; Student’s t test) (Fig. 4D). Control experiments using an α3 promoter construct in which the E-box has been mutated indicated that Slug-mediated repression of α3 transcription requires an intact E-box (Fig. 4D).

Slug Binds to the α3 Integrin Promoter in an E-box-dependent Manner—We used an electrophoretic mobility shift assay to analyze whether Slug could bind the α3 promoter, which contains one E-box motif (25). 293T HEK epithelial cells were transiently transfected with pMSCV-Slug-IRE-GFP, and 48 h post-transfection, cells were lysed, and nuclear fractions were isolated. Protein from the nuclear fraction of Slug-transfected cells efficiently bound to the wild-type probe, giving a retarded complex halfway up the gel (Fig. 5A). This retarded complex was not observed with a mutated E-box probe, suggesting that mutation of the E-box was sufficient to block formation of the retarded complex and that the E-box is required for effective binding of the nuclear extract protein. The labeled retarded complex was significantly reduced when wild-type probe was mixed with excess unlabeled competitor probe, demonstrating the specificity of this retarded complex in binding to wild-type probe. A supershift band was observed when an anti-Slug antibody was added, confirming that Slug was present in the retarded complex. Specificity was confirmed by loss of the supershift when excess unlabeled wild-type probe was introduced (Fig. 5B). This experiment confirms that Slug specifically binds to the α3 integrin promoter in an E-box-dependent manner.

Activation of Slug Results in Decreased Expression of β1 and β4 Integrins—Having observed that activation of Slug results in decreased α3 expression, we analyzed expression of its heterodimeric integrin partner, β1. We observed loss of β1 integrin from sites of cell-cell adhesion and reduced levels of total β1 protein following activation of Slug in keratinocytes (Fig. 6, A and B). As in the case of α3, we also observed significantly decreased levels of β1 mRNA (p < 0.005; Student’s t test) (Fig. 6C). We also analyzed expression of β4 integrin, which, together with α6, forms the integrin heterodimer α6β4 found in the hemidesmosome structures that link basal keratinocytes to the basal lamina. We observed significant loss of β4 integrin expression at both the protein and mRNA level (p < 0.001; Student’s t test), suggesting that Slug can regulate a number of different adhesion molecules and complexes in epidermal keratinocytes (Fig. 6, D and E).

Regulation of Keratinocyte Function by Slug—Having observed decreased integrin expression following Slug activation, we analyzed the ability of keratinocytes to attach to fibronectin and laminin-5. In cells expressing active Slug, we observed a significant (p < 0.005 at 25 µg/ml coating concentrations; Student’s t test) decrease in cell attachment to recombinant fibronectin (Fig. 7A). In a similar manner, we observe a significant (p < 0.001; Stud-
dent’s \( t \) test) reduction in attachment to laminin-5 in cells expressing active Slug (Fig. 7B). This indicates that the decrease in integrin expression observed following activation of Slug has functional consequences in terms of decreased cell adhesion to ECM.

To determine whether activation of Slug had any further effects on keratinocyte function, we used a number of different parameters to analyze proliferation and differentiation following activation of Slug. We used colony forming efficiency (CFE) assays to determine whether activation of Slug affects the pro-
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![Graph](image)

**FIGURE 7. Adhesion of keratinocytes to fibronectin and laminin-5 is reduced following activation of Slug.** A, GFP:ER (closed circles) and GFP:Slug:ER (open circles) keratinocytes were cultured in 100 nM 4-HT for 72 h before being plated onto increasing concentrations of recombinant fibronectin type III domains 9 and 10 for 1 h. Following removal of nonattached cells, adhesion was determined. B, GFP:ER and GFP:Slug:ER keratinocytes were cultured in 4-HT for 72 h before being plated onto a laminin-5-enriched substrate for 1 h. Following removal of nonattached cells, adhesion was determined. For both A and B, the data shown are the mean and S.E. of three separate experiments.

Differentially expressed keratinocytes. Keratinocytes expressing GFP:ER or GFP:Slug:ER were plated at low density on mitotically inactivated fibroblasts and cultured in the presence of 4-HT for 14 days. In control GFP:ER-expressing cells, CFE was ~3%, consistent with previously published data (4, 31). In contrast, activation of Slug resulted in a highly significant (p < 0.001; Student’s t test) reduced CFE (0.6%) (Fig. 8, A–C). Consistent with a decreased CFE, we also observed an equally significant (p < 0.001) decrease in BrdUrd uptake in GFP:Slug:ER-expressing cells treated with 4-HT to activate Slug when compared with control GFP:ER-expressing cells. During the 24-h labeling period, 25% of cells expressing active Slug incorporated BrdUrd compared with 76% of control GFP:ER-expressing cells (Fig. 8D). We found no evidence that the decrease in CFE or BrdUrd was a consequence of increased cell death with no evidence for caspase cleavage or nuclear fragmentation (Fig. 8E and data not shown). The ERK/MAPK pathway plays a critical role in keratinocyte cell proliferation, and, consistent with the evidence for decreased cell cycle progression, we observed decreased ERK activity following activation of Slug (Fig. 8F). Analysis of other cell cycle markers revealed decreased Rb phosphorylation, consistent with cell cycle arrest (Fig. 8F). Taken together, these data indicate that Slug regulates cell cycle progression in keratinocytes.

DISCUSSION

We have established that conditional activation of the transcriptional repressor Slug in human epidermal keratinocytes results in reduced expression of a number of adhesion molecules, reduced cell adhesion to ECM, and dramatically decreased cell proliferation. However, although loss of adhesion molecule expression is a key feature of epidermal differentiation, we observe no effect on keratinocyte differentiation. We have identified α3, β1, and β4 integrins as novel Slug targets. In normal epidermis, the principal integrin heterodimers expressed by keratinocytes are α2β1, α3β1, and α6β4. Other integrins, such as α5β1, αvβ6, and α9β1 are upregulated during wound healing (1). Whereas we have not directly analyzed expression of α2, down-regulation of β1 would obviously affect expression of α2β1 on the cell surface, since α2 only partners with β1. In a similar manner, expression of α6 in hemidesmosomes will be affected by the down-regulation of its heterodimeric partner β4. In addition, the presence of E-boxes in the α2 and α6 promotor sequences would also suggest both as potential targets for Slug (32–34). Thus, all of the integrins constitutively expressed in the epidermis are potentially regulated by Slug. Furthermore, of those integrins up-regulated during wound healing, both αv and α5 possess E-box sequences, as do other integrins not expressed in the epidermis, such as α7, suggesting a possible global regulatory mechanism involving Slug (35, 36).

With respect to the epidermis, the down-regulation of E-cadherin and integrins clearly has implications for migratory processes, such as wound healing in normal tissue and tumor invasion in diseases such as squamous cell carcinoma. A recent report described increased Slug expression in keratinocytes at wound margins and increased cell motility and wound healing in cultured HaCaT keratinocytes exogenously expressing Slug (37). However, in our primary keratinocytes, we observed no obvious increase in cell motility following activation of Slug. In normal cultures, colonies remain intact, with no cell migration away from the colony (Figs. 3, 4, and 6). In addition, in scratch wounding assays, we see no evidence for increased wound closure following activation of Slug (data not shown). This apparent discrepancy may reflect dif-
FIGURE 8. Activation of Slug results in reduced cell proliferation. A–C, GFP:ER (A) and GFP:Slug:ER (B) keratinocytes were plated at low density and cultured for 14 days in the presence of 4-HT before being fixed and stained with rhodanile blue. Numbers of colonies were scored. Scale bar, 10 mm. Results from three separate experiments (mean and S.E.) are presented in C. D, GFP:ER and GFP:Slug:ER cells were cultured in the presence of 4-HT for 72 h, with BrdUrd added to culture media for the final 24 h of treatment. Cells incorporating BrdUrd were detected by indirect immunofluorescence. Data presented are the mean and S.E. of three separate experiments. E, expression and integrity of caspase-3 were analyzed by Western blotting of lysates prepared from cells cultured in the presence or absence of 4-HT for 72 h. F and G, expression of ERK, phospho-ERK (pERK), Rb, phospho-Rb (pRb), α-tubulin, and the differentiation marker involucrin were analyzed by Western blotting of lysates prepared from GFP:ER and GFP:Slug:ER cells cultured in the presence or absence of 4-HT for 72 h.
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different cellular backgrounds or may be related to the degree of adhesion molecule down-regulation.

Our data indicating that activation of Slug results in reduced cell proliferation are consistent with studies in chick embryos, where ectopic expression of Slug in the developing neural tube results in reduced cell proliferation (38). This raises the important question of how Slug regulates cell cycle progression. One possibility is that Slug directly represses transcription of genes required for cell cycle progression. However, there is at present little direct evidence for such regulation by Slug. Our data are, in part, consistent with that obtained in Madin-Darby canine kidney cells transfected with the related protein Snail. These cells exhibit decreased BrdUrd incorporation and reduced Rb phosphorylation, and it was proposed that Snail acts to block cell cycle progression through G1/S by repressing cyclin D transcription (38). Thus, it is possible that Slug functions in a similar manner. However, in our cells, cyclin D expression is not affected following activation of Slug (data not shown). Furthermore, we observe decreased ERK phosphorylation following activation of Slug, whereas Snail-expressing cells have increased levels of ERK phosphorylation (38). This suggests that there may be fundamental differences in the regulatory mechanisms employed by Snail and Slug.

There is a substantial body of data to implicate integrins, cadherins, and cytoskeletal tension in control of cell cycle progression (reviewed in Ref. 39), and an alternative explanation for how Slug might regulate cell cycle progression is that the reduced cell proliferation we observe might be a consequence of down-regulating adhesion molecule expression. With regard to the epidermis, this would be consistent with studies in knockout mice, where targeted loss of β1 integrin in the mouse epidermis results in a number of abnormalities, including reduced keratinocyte proliferation (40, 41). Interestingly, the proportion of integrins lost appears to be critical, since loss of α3β1 or α6β4 alone or in combination is not sufficient to affect proliferation (42). Furthermore, recent experiments in α3- and conditional β4-null mice indicate that, as long as cell adhesion in the epidermis is not compromised, proliferation and differentiation proceed normally, and cells are protected from apoptosis (43, 44).

Given the established links between cell adhesion and induction of keratinocyte differentiation, one obvious question is why loss of integrin and cadherin expression following activation of Slug does not result in increased keratinocyte differentiation. One possible explanation is that whereas we see decreased expression of E-cadherin, α3, β1, and β4 integrins, we do not observe complete loss of these adhesion molecules. Thus, it may be that there are sufficient integrins binding ECM to prevent differentiation from occurring. This would be consistent with data indicating that the number, rather than proportion, of β1 integrin adhesion receptors occupied is the important factor in determining whether differentiation occurs (45). Alternatively, it may be that their loss is compensated for by other preexisting adhesion molecules or by up-regulation of other adhesion molecules. Evidence for this comes from Drosophila, where Snail is required for gastrulation. Snail represses E-cadherin expression during invagination of the ventral furrow, yet the epithelial cells still migrate as a sheet due to up-regulation of other adhesion molecules, such as N-cadherin (6, 46, 47). Thus, it may be that the decrease in adhesion molecule expression is sufficient to affect cell adhesion and proliferation but not to induce terminal differentiation.

Signaling through the ERK/MAPK pathway plays a key role in epidermal function. Activation of ERK has been observed in basal proliferating keratinocytes of normal epidermis and in hyperproliferative disease, such as psoriasis, and activation of ERK has been shown to promote keratinocyte proliferation in vitro (48, 49). In a similar manner, constitutive expression of an activated form of the kinase upstream of ERK, MEK1, results in hyperproliferation and inhibition of keratinocyte differentiation (49, 50, 51). Integrin-dependent adhesion via α6β4 seems to play a key role in ERK activation, in that ligation of α6β4 results in activation of ERK, transcription from the serum response element, and cell cycle progression (52). In a similar fashion, α3β1 has been shown to promote keratinocyte cell survival in an ERK-dependent manner (43). Thus, it is possible that the decreased ERK activity we observe following activation of Slug is a consequence of down-regulating expression of one or more of these integrins. Inhibition of ERK activity or expression of a kinase-dead form of MEK1 is also associated with increased keratinocyte differentiation (48, 51). However, in our experiments, whereas activation of Slug results in inhibition of cell proliferation, consistent with decreased ERK activity, we find no evidence that Slug promotes keratinocyte terminal differentiation.

In conclusion, we have demonstrated a novel role for the Slug transcriptional repressor in the regulation of integrin expression and function and in control of keratinocyte proliferation.

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