Role of the Nude Gene in Epithelial Terminal Differentiation

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Loss-of-function mutations in Whn (Hfh11, Foxn1), a winged-helix/forkhead transcription factor, cause the nude phenotype, which is characterized by the abnormal morphogenesis of the epidermis, hair follicles, and thymus. To delineate the biochemical pathway of Whn, we investigated its upstream regulation and downstream effects using primary keratinocytes from wild-type and transgenic mice. The transgenic animals express whn from the involucrin promoter, which is active in keratinocytes undergoing terminal differentiation. In wild-type cultures, as in the epidermis, Whn was induced during the early stages of terminal differentiation and declined during later stages. In transgenic keratinocytes, whn overexpression altered the terminal differentiation program, stimulating an early differentiation marker (keratin 1) and suppressing later markers (profilagrin, loricrin, and involucrin). These results suggest a role for Whn in the stepwise or temporal regulation of differentiation, as Whn can ensure that the differentiation program is carried out in proper sequence. Before the start of differentiation, Whn levels were suppressed by the p42/p44 mitogen-activated protein kinase cascade, and this signaling pathway was rapidly inactivated as differentiation began. Thus, as keratinocytes commit to terminal differentiation, mitogen-activated protein kinase signaling decreases, which permits the induction of Whn; Whn then activates early features of the differentiation program. Key words: Foxn1/Hfh11/keratinocyte/mitogen-activated protein kinase/Whn. J Invest Dermatol 118:303–309, 2002

The epidermis and hair follicles are related epithelial structures with many similarities in their development, organization, and self-renewal (reviewed in Fuchs, 1990; Hardy, 1992). Both originate from proliferative cell populations that exhibit relatively few differentiation features and generally border the dermis. These proliferative cells act as the progenitors of postmitotic cell types, which undergo terminal differentiation and form each structure’s functional components (e.g., the stratum corneum and hair). Though distinct from each other, the epidermal and follicular differentiation programs lead to comparable cellular changes, such as the acquisition of a flattened shape, the accumulation of keratins, and the breakdown of the nucleus. To produce a differentiated cell, these programs proceed through an ordered series of events, implying stepwise or temporal regulation of the process. While many factors are known to influence the formation of cutaneous epithelia, much remains unknown about the commitment of a progenitor cell to terminal differentiation, and the subsequent stepwise control of the differentiation program.

Nude mice and rats are characterized by the abnormal development of the epidermis, the lack of visible hair, and the absence of a thymus (Flanagan, 1966; Pantelouris, 1973; Köpf-Maier et al., 1990). This phenotype results from inactivating mutations in a single gene, originally designated whn (winged-helix nude) or hfh11 (hepatocyte nuclear factor 3/forkhead homolog 1) (Nehk et al., 1994, 1996; Segre et al., 1995), and recently renamed foxn1 (forkhead box n1) (Kaestner et al., 2000). The human and murine Whn proteins are 82% identical (Schorpp et al., 1997), and a nonsense mutation in human whn is associated with congenital alopecia and severe T cell deficiency (Frank et al., 1999). Thus, loss of Whn activity in humans results in a disease that closely resembles the nude phenotype, demonstrating the conservation of Whn function.

Whn is a member of the winged-helix or forkhead family of transcription factors, which share a highly conserved stretch of 100 amino acids (Kaufmann and Knochel, 1996). This conserved region forms a modified helix–turn–helix domain that mediates DNA binding (Kaufmann and Knochel, 1996). The negatively charged C-terminal domain of Whn can stimulate transcription when fused to the Gal4 DNA binding domain (Brissette et al., 1996; Schüddekopf et al., 1996; Schlake et al., 1997), indicating that Whn functions as a transcriptional activator.

In murine epidermis and hair follicles, nude mutations impair terminal differentiation, as several differentiated structures (such as the stratum corneum, inner root sheath, hair cortex, and hair cuticle) fail to form properly (Köpf-Maier et al., 1990). During skin development, epithelial cells induce whn expression as the first signs of terminal differentiation appear (Lee et al., 1999). In mature skin, whn expression is maintained at sites displaying the early stages of terminal differentiation, such as the first suprabasal layer of the epidermis and the supramatrical region of the hair bulb. Though mainly restricted to postmitotic cells, whn expression is also detected within progenitor cell compartments, marking a subset of epithelial cells in the basal epidermal layer, outer root sheath, and hair matrix. Based on the distribution of Ki-67, a nuclear marker of proliferation, a small number of multiplying cells express whn (Lee et al., 1999). Thus, whn expression seems to encompass the transition from proliferation to differentiation, and whn activation may be one of the first steps in the terminal differentiation pathway. Consistent
with its epithelial specificity in the skin, whn expression is also detected in the epithelial cells of several other organs, such as the thymus, nail, tongue, palate, nasal cavity, and teeth (Lee et al., 1999). In addition, whn homologs were identified in species that do not possess hair follicles or a thymus, such as amphioxus (Schlake et al., 1997) and fruit flies (Strodicke et al., 2000; Sugimura et al., 2000). Given whn’s phylogenetic distribution as well as its murine expression pattern, whn may control a common or fundamental property of epithelia (Lee et al., 1999).

In previous studies, transgenic mice were generated in which whn was placed under the control of the involucrin (inv) promoter (Prowse et al., 1999). Involucrin, a component of the cornified envelope (Rice and Green, 1979), is present in many stratified epithelia and serves as a marker of terminal differentiation in the epidermis and hair follicles (Rice and Green, 1979; Walts et al., 1985; de Viragh et al., 1994). Consistent with the inv expression pattern, the inv–whn mice develop severe, often lethal, abnormalities in the skin and urinary tract. These abnormalities include flaky, shiny skin, misshapen, truncated hair shafts, and a loss of epidermal barrier function, all of which indicate defects in differentiation. Thus, whn overexpression, like the loss of whn function, disrupts the terminal differentiation of the epidermis and hair follicles.

In this study, we examined Whn’s role in differentiation using murine keratinocyte cultures. Similar to its cutaneous expression pattern, endogenous Whn is induced in primary keratinocytes to initiate terminal differentiation. In inv–whn cultures, whn overexpression stimulates an early differentiation marker and suppresses later markers, implicating Whn in the stepwise or temporal control of the differentiation program. Furthermore, the mitogen-activated protein kinase (MAPK) cascade functions as an upstream regulator of Whn, as this pathway suppresses Whn levels in proliferating keratinocytes and becomes inactivated with the onset of differentiation. Thus, this study identifies an early sequence of events in the conversion of progenitor keratinocytes to a differentiated phenotype, as the inactivation of MAPK leads to the induction of Whn, which in turn stimulates initial features of differentiation.

MATERIALS AND METHODS

Cells For analyses of whn expression and function, primary keratinocytes were prepared from newborn inv–whn mice and their wild-type littermates. Pups were genotyped by polymerase chain reaction of tail DNA with primers corresponding to nucleotides 774–796 and 1449–1470 of the mouse whn cDNA (Nehls et al., 1994). Keratinocytes were grown in minimal essential medium containing a low calcium concentration (0.05 mM), 4% chexol-treated fetal bovine serum, and 10 ng epithelial growth factor per ml. Cells were differentiated in 304 wells for 5 days in medium containing 10 mM 1,4-piperazine-diethanesulfonic acid (PIPES), pH 7.0, 100 mM Tris, pH 8.0, 150 mM NaCl, 1% nonidet P40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 2 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4 (lysis buffer). Immunofluorescence analysis was carried out as described previously (Hennings et al., 1997) and fruit flies (Strodicke et al., 2000). Assays were carried out in 50 μl ADB containing 4 μM protein kinase C inhibitor peptide, 0.4 μM protein kinase A inhibitor peptide, 4 μM R24571, 15 μM MgCl2, 100 μM adenosine triphosphate, 0.4 μg/ml myelin basic protein (all from Upstate Biotechnology). MAPK was immunoprecipitated as described above. Whn protein bound to protein G-sepharose beads was washed twice in MAPK assay buffer A, and washed once with Assay Dilution Buffer [ADB; 20 mM 3-[N-morpholino]-propanesulfonic acid (MOPS), pH 7.2, 25 mM β-glycerophosphate, 5 mM ethyleneglycol-bis-[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid, 1 mM Na3VO4, and 1 mM dithiothreitol]. Assays were carried out in 4 μM protein kinase C inhibitor peptide, 0.4 μM protein kinase A inhibitor peptide, 4 μM R42571, 15 μM MgCl2, 100 μM adenosine triphosphate, 0.4 μg/ml myelin basic protein (all from Upstate Biotechnology), and 10 μCi [γ-32P]adenosine triphosphate (3000 Ci per μM, NEN). Reactions were incubated at 30°C with shaking for 20 min, briefly centrifuged, and spotted (30 μl) in the center of a 2 cm × 2 cm square of P81 paper (Pall Gelman, Ann Arbor, MI). Autoradiography was carried out three times in 0.75% phosphoric acid, once in acetone, and scintillation counting.

For analysis of MAPK phosphorylation, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 1 mM ethylenediamine tetracetic acid, 1 mM ethyleneglycolbis-[β-aminoethyl ether]-N,N,N’,N’-tetraacetic acid, 0.5 mM Na3VO4, 0.1% β-mercaptoethanol, 5 μM 32P-labeled GAPDH probe (a 1.3 kb fragment, a 1.4 kb fragment, and a 3 kb fragment), 25 units of calf intestinal alkaline phosphatase (Gibco BRL), and the enhanced chemiluminescence (ECL) detection system (NEN, Boston, MA).

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Northern blot analysis Total RNA was isolated from keratinocytes growing on mica spin columns (Qiagen, Valencia, CA). Thirty micrograms of total RNA was separated on a 1.2% agarose/formaldehyde gel and transferred to Hybond N membranes (Amersham Pharmacia Biotech). Blots were hybridized with a 32P-labeled whn probe corresponding to nucleotides 97–2038 of the mouse cDNA sequence. 32P-labeled GAPDH probe (a 1.3 kb hybridized PstI fragment; Tso et al., 1985) was used as a loading control.

3H-thymidine labeling Cells were incubated with 0.5 μCi 3H-thymidine (specific activity 5.0 Ci per mmol; Amersham Pharmacia Biotech) for 3 h in 24-well plates. After washing twice with phosphate-buffered saline, proteins were precipitated with 10% trichloroacetic acid and resolubilized in 0.2 M NaOH. Incorporation of 3H-thymidine was quantitated by scintillation counting.

Phosphatase assay Following immunoprecipitation as described above, Whn protein bound to protein G sepharose beads was washed in buffer containing 50 mM Tris, pH 8.0, 10 mM MgCl2, 150 mM NaCl, 0.1% Triton X-100, 0.05% SDS, and 2 mM phenylmethylsulfonyl fluoride. Phosphatase reactions were incubated at 37°C for 4 h in buffer containing 50 mM Tris, pH 8.0, 10 mM MgCl2, 150 mM NaCl, and 25 units of calf intestinal alkaline phosphatase (CIAp, Gibco BRL, Gaithersburg, MD). To demonstrate specificity, reactions were performed in the presence or absence of 20 mM Na3VO4.

Phosphoamino acid analysis Keratinocytes were metabolically labeled with carrier-free H3[32P]PO4 (1 mCi per ml; Amersham Pharmacia Biotech) in phosphate-free Dulbecco’s minimal Eagle’s medium (Gibco BRL) containing 1.8 mM CaCl2 for 2 h. Whn protein was then immunoprecipitated as described above. Proteins were separated on 7.5% SDS–polyacrylamide gels, transferred to Immobilon membranes, and visualized by autoradiography. Membrane regions containing 32P-labeled

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Whn were excised, rehydrated in methanol, washed twice with water, hydrolyzed with HCl (110°C, 2 h), and lyophilized (Kamps and Selton, 1989). Individual 32P-labeled phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis as previously described (Cooper et al., 1984).

RESULTS

Induction of Whn during terminal differentiation To determine the *whn* expression pattern *in vitro*, primary keratinocyte cultures were analyzed for Whn protein and RNA levels. In medium containing low calcium concentrations (50 μM), mouse keratinocytes exhibit many of the features of basal epidermal cells, including continuous proliferation even after achieving confluence (Hennings et al., 1980). While consisting mainly of proliferative cells, low calcium cultures also generate more differentiated cells that are ultimately shed into the medium (Roop et al., 1987). Upon increasing the calcium content of the medium to 2 mM (Hennings et al., 1980; Hennings and Holbrook, 1983), confluent cultures arrest growth and initiate a program of terminal differentiation similar to that observed in the epidermis. Whn protein levels were examined using polyclonal antibodies raised against full-length murine Whn. Similar to other regulatory proteins, the absolute amount of Whn is relatively low in wild-type keratinocytes. Thus to measure Whn levels, the protein was immunoprecipitated from concentrated cell extracts, and the immunoprecipitations were then analyzed on immunoblots. Cell extracts were prepared from wild-type keratinocytes under proliferating (low calcium) and differentiating (2 mM calcium) conditions. As shown in Fig 1(A) (left time course), Whn was weakly detected in growing cultures and strongly induced during keratinocyte differentiation. Whn levels increased rapidly following the addition of calcium, reaching at 6 h, and then decreased by 24 h after treatment. At all time points, Whn resolved into multiple, closely migrating species, indicating post-translational modification of the protein. In an identical experiment using nude primary keratinocytes, no Whn protein was detected, demonstrating the specificity of the antibodies (data not shown). As shown by northern analyses, Whn transcript paralleled the protein profile, although the transcript's changes were less dramatic (Fig 1C). Thus, Whn mRNA and protein are induced during the early stages of terminal differentiation in primary keratinocytes. This expression pattern is essentially identical to that observed in the epidermis, suggesting that *whn* is regulated by similar mechanisms *in vitro* and *in vivo*.

To investigate the effects of *whn* overexpression, Whn protein levels were examined in primary keratinocytes derived from *inv-whn* mice. In the epidermis, *inv-whn* expression is confined to suprabasal (postmitotic) keratinocytes (Prowse et al., 1999), consistent with the *inv* expression pattern (Rice and Green, 1979; Walts et al., 1985; de Viragh et al., 1994). In wild-type keratinocyte cultures, involucrin is induced by calcium treatment (Calautti et al., 1995) but is also detected in a subset of attached cells under low calcium conditions (Brissette et al., 1996), indicating the initiation of differentiation. Like the analysis of endogenous *whn* expression, Whn was immunoprecipitated from *inv-whn* cell extracts and visualized on immunoblots. As shown in Fig 1(A, right time course), Whn was easily detected under low calcium conditions and strongly induced by calcium treatment. At each time point, the protein resolved into multiple species, similar, if not identical, to the Whn of wild-type keratinocytes. As transgenic cultures contain relatively high amounts of Whn, the immunoprecipitations were performed with only 2 mg of total protein extract. In contrast, the detection of Whn in wild-type extracts required 8 mg of total protein (Fig 1A, left time course). At all time points, *inv-whn* cultures possessed higher levels of Whn than wild-type cultures. In addition, the time course of *whn* expression differed between the
stimulation of K1.

inv±whn cells (indicated times). Total cell extracts were normalized for protein content, were induced to differentiate by the addition of 2 mM calcium for the transgenic and wild-type keratinocytes. By 24 h after calcium

In their detached populations, transgenic cultures possess lower levels of differentiation program. (Figure 2. Overexpression of whn alters the terminal differentiation program. (A) Wild-type and inv–whn keratinocytes were induced to differentiate by the addition of 2 mM calcium for the indicated times. Total cell extracts were normalized for protein content, electophoresed on 7.5% SDS–polyacrylamide gels, and immunoblotted with antibodies to profilaggrin, K1, or K5. Compared with wild-type cells, transgenic keratinocytes exhibit a stimulation of K1 and an inhibition of profilaggrin. K1 stimulation is observed in both growing and differentiating cultures. (B) Detached cell populations were collected from confluent wild-type and transgenic cultures maintained in low calcium medium. The cells were harvested under low calcium conditions as high calcium prevents keratinocyte detachment. Collected cells were pelleted by centrifugation and then processed as in (A). Immunoblots were probed with antibodies specific for involucrin, loricrin, K1, or K5. In their detached populations, transgenic cultures possess lower levels of loricrin and involucrin than wild-type cultures. Similar to the attached inv–whn cells, the detached transgenic keratinocytes also display a stimulation of K1.

transgenic and wild-type keratinocytes. By 24 h after calcium addition, Whn declined significantly in wild-type cultures but remained abundant in inv–whn cultures. Thus, high Whn levels persisted longer in transgenic cultures, resulting in strong whn expression during later stages of the differentiation program.

The Whn protein encoded by the transgene is tagged at the N-terminus with a Flag epitope, enabling the exogenous protein to be distinguished from endogenous Whn (Prowse et al., 1999). To examine the relative amounts of tagged and endogenous Whn in inv–whn cultures, transgenic cell lysates were depleted of the tagged Whn by two consecutive immunoprecipitations with antibodies to the Flag epitope. The remaining Whn in these lysates was then immunoprecipitated with the Whn antibodies described above. Figure 1(B) compares the Flag and Whn immunoprecipitations by immunoblot analysis. As expected, the Flag immunoprecipitations depleted most of the Whn from the lysates, leaving behind low levels of the protein. Thus, transgene expression was directly responsible for the observed increase in Whn.

As shown in Fig 1(A, B), Whn (both endogenous and tagged) resolves as multiple bands during SDS–polyacrylamide gel electrophoresis, suggesting that the protein normally exists in several forms. Since some, if not all, of these forms may result from post-translational modification, we determined whether Whn undergoes phosphorylation. Whn was immunoprecipitated from either transgenic (Fig 1D) or wild-type (not shown) keratinocytes, and the protein was incubated with phosphatase. On immunoblots, phosphatase treatment converted the multiple Whn forms to a single, faster-migrating species, strongly suggesting that the different Whn forms are generated by phosphorylation. Inclusion of sodium vanadate (Na3VO4), a phosphatase inhibitor, in the reaction prevented the phosphatase from altering the mobility of the slower migrating species. To confirm Whn phosphorylation directly, keratinocytes were labeled metabolically with 32P inorganic phosphate (32PO4), and the phosphorylation status of Whn was determined by immunoprecipitation and SDS–polyacrylamide gel electrophoresis. As shown in Fig 1(E), Whn was labeled by the 32PO4, and the labeled protein displayed a banding pattern similar to that observed on immunoblots. Moreover, the 32P-labeled Whn generated several phosphorylated fragments when digested with trypsin and resolved by two-dimensional chromatography (phosphopeptide mapping; data not shown). Thus, consistent with its migration on immunoblots, the Whn protein is modified by phosphorylation at multiple sites. To identify the phosphorylated residues, Whn was subjected to phosphoamino acid analysis, and as shown in Fig 1(F), phosphorylation was predominantly on serine residues. On immunoblots, Whn reacted with antibodies to the phosphoryl forms of serine and threonine, indicating that the protein may be phosphorylated on threonine residues as well (data not shown). Thus, phosphorylation produces multiple species of Whn, suggesting complex modulation of this protein’s activity. In all, this study represents one of the first biochemical analyses of the Whn protein.

Effects of Whn on terminal differentiation As keratinocytes differentiate in the epidermis, the cells migrate towards the surface and proceed through an ordered series of morphologic and biochemical changes. Early terminal differentiation markers, present in deeper layers of the epidermis, include keratins 1 and 10, whereas later markers, associated with the more superficial layers, include filaggrin, loricrin, and involucrin (reviewed in Fuchs, 1990). In culture, wild-type keratinocytes induce both early and late markers in response to calcium (Hennings et al., 1980; Hennings and Holbrook, 1983), thus exhibiting a differentiation program similar to the epidermis. To assess Whn’s role in terminal differentiation, marker profiles were compared in wild-type and inv–whn primary cultures.

Under proliferating (low calcium) conditions, wild-type keratinocytes displayed low levels of K1, and following calcium treatment, this marker increased rapidly, persisting over a 24 h time course (Fig 2A). In contrast, transgenic cultures possessed abundant K1 both before and after the induction of differentiation with calcium (Fig 2A). At all time points, K1 levels were higher in inv–whn keratinocytes than in wild-type cells, and the upregulation of this marker was particularly dramatic under low calcium conditions. Thus, the overexpression of whn stimulated an increase in K1, even under conditions that promote proliferation.

Filaggrin, the intermediate filament associated protein, is initially synthesized as a high molecular weight precursor, profilaggrin, which contains many filaggrin repeats (Resing et al., 1989). To generate filaggrin, profilaggrin undergoes proteolytic processing and consequently resolves as multiple bands by SDS–polyacrylamide gel electrophoresis. In both wild-type and transgenic keratinocytes, profilaggrin was detected at low levels under proliferating conditions and induced in response to calcium (Fig 2A). Nonetheless, a significant difference emerged in profilaggrin levels during differentiation. Compared with wild-type cells, the transgenic keratinocytes displayed a substantial reduction in profilaggrin following calcium treatment. Thus, in contrast to its effect on K1, the inv–whn transgene caused a downregulation of profilaggrin in differentiating cells.

During differentiation in vitro, wild-type keratinocytes induce involucrin and loricin (both components of the cornified envelope), but the levels of these proteins are significantly higher in detached cells than in adherent differentiating populations (Roop et al., 1987). Thus to examine these markers, detached keratinocytes were harvested from confluent wild-type or inv–whn cultures. As shown in Fig 2B, transgenic keratinocytes exhibited a dramatic decrease in involucrin and loricin levels. At the same time, K1 was elevated in detached inv–whn cells, consistent with the transgene’s effect on adherent differentiating keratinocytes. K5, a marker of the basal epidermal layer, is not modulated in this culture system.
MAP kinase phosphorylation status in primary keratinocytes. Cell lysates were analyzed by immunoprecipitation/immunoblot as in lysates were prepared from treated or untreated cultures, and Whn levels using antibodies specific for phosphorylated p42/p44 MAPK. To calculate calcium addition (2 mM). Total soluble protein (200 μg) were prepared from wild-type cultures at the indicated times after recognize total p42/p44 MAPK (lower panel). (C) MAP kinase activity in primary keratinocytes. Wild-type cultures were treated with 2 mM calcium for the indicated times. p42/p44 MAP kinase was immunoprecipitated from 1 mg total soluble protein and assayed for activity as described in Materials and Methods. The graph expresses the phosphorylation of myelin basic protein as a percentage of the activity found under low calcium conditions, with standard deviation indicated.

Figure 3. whn expression is modulated by inhibitors of MEK1. (A) Analysis of Whn protein. Wild-type keratinocytes were treated for 6 h with 2 mM calcium, 1 μM U0126, or 50 μM PD98059. Cell lysates were prepared from treated or untreated cultures, and Whn levels were analyzed by immunoprecipitation/immunoblot as in Fig 1(A). (B) MAP kinase phosphorylation status in primary keratinocytes. Cell lysates were prepared from wild-type cultures at the indicated times after calcium addition (2 mM). Total soluble protein (200 μg) was electrophoresed on 10% SDS–polyacrylamide gels and immunoblotted using antibodies specific for phosphorylated p42/p44 MAPK. To normalize samples, membranes were reprobed with antibodies that recognize total p42/p44 MAPK (lower panel). (C) MAP kinase activity in primary keratinocytes. Wild-type cultures were treated with 2 mM calcium for the indicated times. p42/p44 MAP kinase was immunoprecipitated from 1 mg total soluble protein and assayed for activity as described in Materials and Methods. The graph expresses the phosphorylation of myelin basic protein as a percentage of the activity found under low calcium conditions, with standard deviation indicated.

(Calautti et al, 1995) and served as a loading control in Fig 2(A, B). Thus, like its effect on profilaggrin, whn overexpression led to a downregulation of the involucrin and loricrin proteins.

In all, whn overexpression resulted in higher levels of an early differentiation marker (K1) and decreased levels of three later markers (profilaggrin, loricrin, and involucrin). These marker profiles indicate that Whn promotes the early stages of differentiation while inhibiting the later stages.

Effects of the MAP kinase cascade on Whn and terminal differentiation As shown in vivo (Lee et al, 1999) and in vitro (Fig 1), Whn is induced as epithelial cells make the transition from proliferation to differentiation. Though little is known about the control of keratinocyte fate, the decision to divide or differentiate may be influenced by the p42 and p44 MAPKs. In many cell types, the p42/p44 MAPKs transduce mitogenic signals to substrates such as transcription factors and other kinases (reviewed in Seger and Krebs, 1995; Chang and Karin, 2001). The signals are received from MAP kinase kinases (MEK), which phosphorylate and thereby activate the MAPKs. In human keratinocyte cultures, epidermal growth factor stimulates the activity of p42 MAPK, and this response is blocked by calcium treatment (Medema et al, 1994). Moreover, following the introduction of a dominant-negative MEK1, human keratinocytes exhibit reductions in clonal growth and colony-forming efficiency (Zhu et al, 1999). These effects correlate with the inhibition of p42/p44 MAPK activity, suggesting that keratinocytes require MAPK signaling to maintain their proliferative potential (Zhu et al, 1999). Thus, we investigated whether the p42/p44 MAPK cascade influences whn expression.

Wild-type keratinocytes were treated with U0126 (Favata et al, 1998) or PD98059 (Alessi et al, 1995), which are specific inhibitors of MEK1 and block the activation of its substrates, the p42/p44 MAPKs. Under low calcium conditions, both inhibitors stimulated a great increase in Whn, and these Whn levels were higher than the level observed after calcium treatment (Fig 3A). Thus, the MEK1 inhibitors are the strongest inducers of Whn found to date. As Whn was stimulated by two different inhibitors, it is likely that MEK1 mediates this effect, and that MAPK signaling suppresses Whn levels in proliferating keratinocytes. These results provide the first insight into Whn regulation at the molecular level.

To date, there has been little study of how the MAP kinases affect or respond to keratinocyte decisions. For example, it is not known whether MAPK activity changes as keratinocytes lose proliferative potential or initiate terminal differentiation. Thus to correlate MAPK function with keratinocyte behavior, p42/p44 MAPK status was examined using antibodies specific for their phosphorylated forms. As shown in Fig 3B (upper panel), the p42/p44 MAPKs were phosphorylated under low calcium conditions, indicating that these kinases are active in proliferating keratinocytes. Following calcium addition, the phosphorylated forms of both proteins rapidly decreased and remained low as cells progressed through the differentiation program. This decrease resulted strictly from a change in phosphorylation status, as the total level of MAPK protein was not affected by calcium treatment (Fig 3B, lower panel). To examine MAPK activity directly, total p42/p44 MAPK was immunoprecipitated and assayed for the ability to phosphorylate myelin basic protein. Consistent with the phosphorylation pattern, p42/p44 MAPK activity declined rapidly following the addition of calcium (Fig 3C). Thus, these MAPKs become inactivated as keratinocytes commit to the terminal differentiation program.
As shown in Fig 2, inv-whn keratinocytes display an altered differentiation program, as early differentiation markers increase and later markers decrease. Since MEK1 inhibitors induce high levels of Whn (analogous to the inv-whn transgene), wild-type keratinocytes were examined for their marker profile following U0126 treatment. Under low calcium conditions, the MEK1 inhibitor stimulated an increase in K1 (Fig 4A), and this early marker, like Whn (Fig 3A), was higher in the presence of U0126 than calcium. Despite this apparent start of differentiation, the U0126-treated cells failed to induce the later marker profilaggrin (Fig 4A), instead displaying a small decrease in this protein. In conjunction with this marker profile, U0126 (like calcium) caused a substantial reduction in the incorporation of 3H-thymidine (Fig 4B), showing the arrest of cell division, another early event in the differentiation program. Thus, the inhibition of the MAPK pathway activates early, but not later, features of terminal differentiation, effects essentially identical to the overexpression of whn.

DISCUSSION

During the self-renewal of the epidermis and hair follicles, postmitotic, differentiating cells arise from less differentiated populations capable of proliferation. In this study, we identify a role for Whn in the conversion of progenitor cells to a differentiated phenotype.

In murine epidermis and hair follicles, the induction of whn expression correlates with the initiation of terminal differentiation (Lee et al, 1999). During epidermal development, whn is induced at the same time that K1 first appears (embryonic stage E15.5), and whn expression is detected principally in suprabasal cells. As the epidermis matures, whn is expressed primarily in the first suprabasal layer, which contains cells in the early stages of terminal differentiation. As epidermal keratinocytes migrate to more superficial layers, whn expression disappears, and thus keratinocytes suppress whn during later stages of differentiation (Lee et al, 1999; Prowse et al, 1999).

As shown here, primary keratinocytes also induce whn expression during the early stages of terminal differentiation. Under conditions that stimulate proliferation, wild-type cultures possess low levels of Whn, and following calcium treatment, Whn increases dramatically, peaking within 6 h. As cells proceed through the differentiation program, the protein declines, analogous to the whn expression pattern in the epidermis. In parallel with the protein, the Whn transcript also rises and falls, but the changes in Whn mRNA appear smaller than the changes in Whn protein. Consistent with the increase in Whn transcript, the Whn promoter (Schopp et al, 1997) contains potential binding sites for AP-1, a transcription factor that activates many genes associated with keratinocyte differentiation (Eckert et al, 1997). In all, the results suggest regulation of Whn at several levels. As shown in skin, whn expression is controlled, at least in part, at the level of transcription, since Whn promoter activity is induced as cells initiate terminal differentiation (Lee et al, 1999). In culture, whn may undergo similar transcriptional regulation, since the Whn transcript increases in differentiating cells. Despite this increase, Whn mRNA levels do not correspond precisely to Whn protein levels, as the protein exhibits greater changes than the transcript. Thus, in addition to transcriptional controls, the results suggest the regulation of Whn at the level of translation or protein stability.

In nude epidermis and hair follicles, the differentiating cells exhibit morphologic defects, and some differentiated structures (e.g., the hair cortex and cuticle) often fail to form entirely (Köpf-Maier et al, 1990). Consistent with this impaired differentiation, nude skin contains reduced transcript levels of several hair keratin genes (Schlake et al, 2000; Schopp et al, 2000). In culture, nude keratinocytes display an abnormal differentiation program, as early markers decrease and later markers increase (Brissette et al, 1996). Given these phenotypic effects, we proposed that Whn directly regulates genes associated with terminal differentiation (Lee et al, 1999; Prowse et al, 1999). Nonetheless, it remained possible that Whn’s effects were indirect and that the nude differentiation defects were a secondary consequence of other perturbations. As shown in this study, whn overexpression shifts the balance of markers in the differentiation program, suggesting a direct role for Whn in the regulation of differentiation. In inv-whn keratinocytes, high Whn levels induce the early marker K1, even under conditions that stimulate proliferation. Concomitantly, the exogenous Whn inhibits profilaggrin, loricrin, and involucrin, three later markers of differentiation. This marker profile is consistent with the epidermal whn expression pattern, as Whn is induced during early differentiation stages and repressed during later stages (Lee et al, 1999; Prowse et al, 1999). Taking these findings together, it is likely that Whn promotes early features of differentiation, directly activating part of the differentiation program. At the same time, Whn appears to suppress or inhibit the later features of differentiating cells. Thus, the results support a role for Whn in the stepwise or temporal regulation of terminal differentiation. That is, Whn can influence a cell’s progress through the differentiation program and ensure that events are carried out in the proper sequence.

While we do not know the mechanism by which Whn inhibits later differentiation markers, we note that Whn reduces endogenous involucrin levels without clearly affecting transgene expression, which is driven by the involucrin promoter. The transgene’s promoter is carried within a 3.7 kb genomic fragment (Carroll et al, 1993), and it is possible that this fragment lacks promoter elements mediating Whn repression. Alternatively, Whn may suppress endogenous involucrin levels through post-transcriptional regulation.

In addition to its induction during terminal differentiation, Whn is phosphorylated at multiple sites, and the phosphorylated residues consist primarily of serine. Whn belongs to the winged-helix/forkhead family of transcription factors (Kaestner et al, 2000), and in studies of other family members, phosphorylation modulated transcriptional activity or nuclear translocation (Biggs et al, 1999; Brunet et al, 1999; Kops et al, 1999). As shown by immunoblots, phosphorylation generates multiple species of Whn, which raises the possibility that different species perform different functions.

The differentiation of a cutaneous epithelial cell can be divided into two distinct components: the loss of the ability to multiply and the acquisition of specialized characteristics. In this study, we present evidence that whn expression is regulated by a cell’s ability to multiply. Whn is strongly induced by two different inhibitors of MEK1, which block activation of the p42/p44 MAPKs. The p42/p44 MAPK pathway is activated through Ras signaling and known to influence cell proliferation as well as other responses to extracellular factors (Chang and Karin, 2001). In human keratinocytes, growth potential decreases following the inhibition of MAPK activity, suggesting a role for MAPK signaling in the maintenance of progenitor populations (Zhu et al, 1999). As an extension of this work, we show that the MEK1 inhibitor U0126 arrests the proliferation of murine keratinocytes. Moreover, we demonstrate that MAPK signaling changes dramatically as keratinocytes initiate differentiation. Following calcium treatment, keratinocytes rapidly inactivate the p42 and p44 MAPKs, which implicates these kinases in the switch from a proliferative to a postmitotic state. Taking the results together, the p42/p44 MAPKs perform the related functions of promoting keratinocyte proliferation and suppressing Whn levels. As cells commit to the differentiation program, the MAPK pathway is inactivated, and Whn is induced. This increase in Whn then stimulates the acquisition of specialized characteristics. Thus, MAPK inactivation and Whn induction are part of an early series of steps that convert progenitor cells to a differentiated phenotype.

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