AKT1 Provides an Essential Survival Signal Required for Differentiation and Stratification of Primary Human Keratinocytes*

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Keratinocyte differentiation and stratification are complex processes involving multiple signaling pathways, which convert a basal proliferative cell into an inviable rigid squame. Loss of attachment to the basement membrane triggers keratinocyte differentiation, while in other epithelial cells, detachment from the extracellular matrix leads to rapid programmed cell death or anoikis. The potential role of AKT in providing a survival signal necessary for stratification and differentiation of primary human keratinocytes was investigated. AKT activity increased during keratinocyte differentiation and was attributed to the specific activation of AKT1 and AKT2. Targeted reduction of AKT1 expression, but not AKT2, by RNA interference resulted in an abnormal epidermis in organotypic skin cultures with a thin parabasal region and a pronounced but disorganized cornified layer. This abnormal stratification was due to significant cell death in the suprabasal layers and was alleviated by caspase inhibition. Normal expression patterns of both early and late markers of keratinocyte differentiation were also disrupted, producing a poorly developed stratum corneum.

The keratinocyte is the major cellular constituent of human epidermis, which through a carefully orchestrated process of growth arrest and differentiation, provides a stratified protective barrier responsible for fluid balance and evasion of infection (1). During normal differentiation, an uncommitted stem cell yields transit amplifying cells, which divide a limited number of times and, subsequently, exit the cell cycle, detach from the basement membrane, and begin expression of proteins required for production of the cornified envelope (2, 3). The fully developed epidermis has at least four histologically distinct layers: the innermost basal layer containing undifferentiated stem cells and proliferative transit amplifying cells, the suprabasal spinous layer highlighted by increased cell-to-cell contacts, a granular layer marked by the presence of granules containing proteins required for formation of the final layer, the stratum corneum. Contributions from each layer ensure the structural integrity of mature skin.

Despite detachment from the basement membrane and loss of integrin contacts, which is a potent apoptotic stimulus in most epithelial cells (4), the keratinocyte remains viable and metabolically active until reaching the interface of the granular and cornified layers, a region called the transition zone. At the transition zone, the keratinocyte undergoes a specialized form of cell death similar to apoptosis but lacking many key morphological features normally associated with programmed cell death (5). It is estimated that it may take the cell 2 weeks to reach this point, during which time the cell expresses and assembles the proteins required for cornified envelope formation (1, 2). Thus, the keratinocyte is a specialized epithelial cell that contains an innate anchorage independent survival mechanism necessary for suprabasal viability and stratification.

Normal epidermal development and homeostasis require multiple signaling pathways that act sequentially and in parallel in an “integrated” control system to govern the balance between proliferation and differentiation (6). Developmental cues stem from both the Notch and Wnt gene families, which regulate commitment (2). The protein kinase C and tyrosine kinase/Ras/Rho pathways have been shown to play a role in expression of differentiation specific proteins (6), while certain isoforms of the transcription factor p63 appear to control commitment to stratification (7). However, very little is known about survival signaling required for the stratification process.

AKT, or protein kinase B, is a predominantly cytoplasmic serine/threonine kinase, which acts as a major downstream effector of insulin and growth factor receptor signaling and a regulator of cell attachment. Upon activation of phosphatidylinositol 3-kinase (PI3K) and the subsequent generation of phosphatidylinositol 3,4,5-trisphosphate, AKT, as well as its upstream regulator phosphoinositide-dependent protein kinase 1 (PDK1), become colocalized at the membrane due to the binding of their pleckstrin homology domains to phosphatidylinositol 3,4,5-trisphosphate. Once localized to the membrane, AKT is activated through phosphorylation of two main regulatory sites located in the catalytic T-loop (Thr308) and the hydrophobic pocket (Ser473). PDK1 is responsible for phosphorylation of Thr308, while the kinase (or kinases), which phosphorylates Ser473, designated as PDK2, is still under investigation but most likely is due to the richtor-mTOR complex (8, 9). Once activated, AKT translocates to either the cytoplasm or nucleus, where it in turn activates a variety of substrates in both of these compartments. The diversity of reported AKT substrates corroborates the essential role of AKT in cellular processes as diverse as glycosgen synthesis, protein synthesis, cell growth, cell cycle regulation, cell death, and differentiation.

AKT activity has been implicated in controlling differentiation of various tissues in mice including muscle, adipose tissue, bone, and skin.

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2 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; GSK3, glycogen synthase kinase-3; HKG5, human keratinocyte growth supplement; IPK, immunoprecipitation kinase; PDK, phosphoinositide-dependent protein kinase; pBt, retinoblastoma protein; TUNEL, terminal deoxynucleotidyl deoUTP nick end labeling; EGF, epidermal growth factor; IGF, insulin-like growth factor; Z, benzyloxycarbonyl; fmk, fluoro-methyl ketone; DM, differentiation medium; BM, basal medium; AEBSF, 4-(2-aminoethyl)benzenesulfonfonyl fluoride hydrochloride; sh, small hairpin.

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(10). While the predominant function of AKT signaling in muscle differentiation appears to be the regulation of muscle-specific transcription factors, some reports indicate AKT function in the postmitotic survival of myocytes required for terminal differentiation and myotube formation (11–14). Whether a similar survival function of AKT exists in keratinocyte terminal differentiation is not known. Using RNA interference in combination with organotypic culture, the importance of AKT activity in human keratinocyte differentiation and skin formation was assessed in the work presented here.

EXPERIMENTAL PROCEDURES

Reagents—Total AKT (9272), p-473 (9271), p-473 for IFA (9277), p-308 (9275), pGSK-3 (9331), GSK3 (9332) antibodies and LY294002 and GSK-3 fusion protein were from Cell Signaling Technology. AKT1 (B-1), AKT2 (F-7), AKT3 (C-14), NF-κB p65 (F-6), TRAF-2 (C-20), actin (C-2), and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Keratin 1 and keratin 10 antibodies were from Covance, filaggrin antibodies (BT-576) were from Biomedical Technologies Inc., and caspase 14 antibodies (B-1-71) were from Upstate Biotechnology. EGF, insulin-like growth factor-1 (IGF-1), and a control fluoromethyl ketone (fmk)-derivitized peptide against cathepsins B and L (Z-FA-fmk) were from R&D Systems. The general caspase inhibitor Z-VAD-fmk was from Bachem.

Keratinocyte Cell Culture and Differentiation—Primary human keratinocytes were isolated from neonatal foreskins and passaged in Epilife Medium (Cascade Biologics) with human keratinocyte growth supplement (HKGS). Keratinocytes from at least three donors were pooled for each experiment and used up to the sixth passage. For calcium-mediated differentiation, freshly confluent keratinocytes were fed Epilife Medium without HKGS (basal medium (BM)) for 12 h and were then treated with 1.5 mM calcium in Epilife Medium without HKGS (differentiation medium (DM)) for the indicated times. For suspension-induced differentiation, subconfluent keratinocytes were fed Epilife Medium without HKGS for 12 h, trypsinized, and suspended in medium containing 1.5% methylcellulose (Sigma).

In vitro skin reconstitution (organotypic culture) was performed using keratinocytes cultured in serum with a fibroblast feeder layer (15). Keratinocytes were seeded with mitomycin C (Sigma)-treated 3T3-J2 cells (ATCC CCL-92) and fed with E-medium (DMEM: Ham’s 3:1, 5 μg/ml insulin, 5 μg/ml transferrin, 0.5 μg/ml hydrocortisone, 1.8 x 10^-4 adenine, 2 x 10^-11 triiodothyronine, 10% fetal bovine serum, 10^-10 M chola toxin, and 10 ng/ml EGF). Keratinocytes were then seeded on a collagen matrix (rat tail Type I, BD Biosciences) containing 3.75 x 10^-3 3T3 cells/ml, submerged in E-medium. Once cells formed a confluent monolayer, the matrix was raised to the air:medium interface and the organotypic culture was fed from below with E-medium containing 3.75 μg/ml insulin, 2 μg/ml hydrocortisone, 10% fetal bovine serum, 10^-4 adenine, 2 x 10^-11 triiodothyronine, 10% fetal bovine serum, 10^-10 M cholera toxin, and 10 ng/ml EGF). Keratinocytes were then seeded on a collagen matrix (rat tail Type I, BD Biosciences) containing 3.75 x 10^-3 3T3 cells/ml, submerged in E-medium. Once cells formed a confluent monolayer, the matrix was raised to the air:medium interface and the organotypic culture was fed from below with E-medium containing 3.75 μg/ml insulin, 2 μg/ml hydrocortisone, 10% fetal bovine serum, 10^-4 adenine, 2 x 10^-11 triiodothyronine, 10% fetal bovine serum, 10^-10 M cholera toxin, and 10 ng/ml EGF). Keratinocytes were then seeded on a collagen matrix (rat tail Type I, BD Biosciences) containing 3.75 x 10^-3 3T3 cells/ml, submerged in E-medium. Once cells formed a confluent monolayer, the matrix was raised to the air:medium interface and the organotypic culture was fed from below with E-medium containing 3.75 μg/ml insulin, 2 μg/ml hydrocortisone, 10% fetal bovine serum, 10^-4 adenine, 2 x 10^-11 triiodothyronine, 10% fetal bovine serum, 10^-10 M cholera toxin, and 10 ng/ml EGF). Keratinocytes were then seeded on a collagen matrix (rat tail Type I, BD Biosciences) containing 3.75 x 10^-3 3T3 cells/ml, submerged in E-medium. Once cells formed a confluent monolayer, the matrix was raised to the air:medium interface and the organotypic culture was fed from below with E-medium containing 3.75 μg/ml insulin, 2 μg/ml hydrocortisone, 10% fetal bovine serum, 10^-4 adenine, 2 x 10^-11 triiodothyronine, 10% fetal bovine serum, 10^-10 M cholera toxin, and 10 ng/ml EGF).

For caspase inhibition experiments, 100 μM Z-VAD-fmk or Z-FA-fmk were added to the culture medium at the time the cultures were raised to the air:medium interface and included at the same concentration at each refeeding.

Immunoblotting and Immunoprecipitation Kinase (IPK) Assay—For immunoblot analysis of endogenous proteins, cells were lysed in 8 M urea, 50 mM Tris (pH 7.5), 1 mM β-mercaptoethanol, supplemented with AEBSF and aprotinin. Typically 50 μg of total protein was added per lane of a SDS-PAGE gel. Lysates for IPK assays were prepared with 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO3 with protease and phosphatase inhibitors (Sigma). Lysates containing 500 μg of protein were immunoprecipitated with 1 μg of total AKT or isofrom specific AKT antibody and protein G-Sepharose (Amersham Biosciences). The washed precipitate was resuspended in kinase buffer containing 25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na2VO3 and 10 mM MgCl2 with 200 μM ATP and 100 μM GSK3 fusion protein as a substrate in a volume of 40 μl. Kinase reactions were carried out at 30°C for 1 h and terminated by addition of 2X SDS-PAGE sample buffer. Phospho-GSK3 was detected by immuno blot.

RNA Interference—For stable RNA interference, the following target sequences were used to produce pSUPER.puro retroviral vectors (OligoEngine) according to the manufacturer’s instructions: shAKT1, 5’-ggacgggccacattaagatctt-3’; shAKT2, 5’-gaagtgccgcatcggcaagc-3’; shSCRAM, a non-coding sequence generated by OligoEngine software. The vectors were cotransfected with pHCMV-VSV-G into phoenix-gp cells by the calcium phosphate method (BD Biosciences). AKT1/AKT2 dual knockdown was accomplished by cotransfecting shAKT1 and shAKT2 into phoenix-gp cells. After 48 h, supernatants were concentrated by centrifuging for 90 min at 12,000 x g, 4°C. First passage primary human keratinocyte cultures were infected for 6 h in a humidified 37°C incubator, 5% CO2, in the presence of 8 μg/ml polybrene. After 18 h of recovery, cells were selected with 1.25 μg/ml puromycin for 2 days and allowed to recover for an additional 2 days. Transduction efficiency was typically 30–50% as assessed by pBabe-GFP control infection.

For transient RNA interference, the following target sequences were produced to produce pBS/U6 constructs: pBS/U6/AKT1, 5’-ggccgcaagcttggaacggcc-3’; pBS/U6/AKT2, 5’-ggccgcaagcttggaacggcc-3’; pBS/U6/luc, 5’-acacgacttgactgca-3’ (targeting luciferase as a control). The vectors were transfected into early passage primary human keratinocytes using FuGENE (Roche Applied Science) at a transfection reagent: DNA ratio of 3:1.

Microscopy—Tissue embedding, hematoxylin and eosin and Ki-67 staining were performed by the University of Rochester Medical Center Department of Pathology and Laboratory Medicine. Five-micrometer-thick paraffin-embedded sections were deparaffinized/rehydrated and treated with boiling citrate buffer for 10 min. Indirect immunofluorescence staining was performed at ambient temperature using the indicated primary antibodies and Alexa-488-conjugated secondary reagents (Molecular Probes) in phosphate-buffered saline with 0.1% Triton X-100 and 3% bovine serum albumin. Sections were mounted with Pro-Long Gold antifade reagent (Molecular Probes). Images were captured using a Leica DM IRB microscope equipped with either a 10X (0.3 NA) or 20X (0.4 NA) Plan-Fluor objective and a PCO Sensicam QE CCD camera controlled with Image ProPlus 5.0 software. In addition, an RGB tunable imaging filter (Cambridge Research & Instrumentation, Inc.) was used to collect bright field color images. Exposure times were kept constant within an experiment, images were psuedocolored, and no additional image modification was performed.

Fluorescein-cadaverine Uptake—For transglutaminase assay experiments, 100 μM fluorescein-cadaverine or unmodified fluorescein (Molecular Probes) was added to the medium of 13.75 day old organotypic cultures. The cultures were harvested 6 h after addition as suggested by Mack et al. (16). Deparaffinized sections were rehydrated and mounted with 4’,6-diamidino-2-phenylindole-supplemented ProLong Gold antifade reagent.

TUNEL Assays—The in situ cell death detection kit (fluorescein) from Roche Applied Science was used essentially as described by the manufacturer’s instructions. For TUNEL analysis by flow cytometry, cells were fixed in 4% paraformaldehyde for 10 min at ambient temperature with shaking. Cells were permeabilized with 0.1% Triton X-100,
AKT Activity Increases during Keratinocyte Differentiation

AKT is a serine/threonine kinase that is activated by phosphorylation at serine 473 and threonine 308. It is involved in various cellular processes such as cell growth, survival, and metabolism. In keratinocytes, AKT activity is required for differentiation.

**RESULTS**

**AKT Activity Increases during Keratinocyte Differentiation**—Differentiation-specific activation of AKT has been detected in various cell types including myocytes and adipocytes (13, 17). To examine AKT activity in differentiating primary human keratinocytes, two different in vitro methods of stimulating differentiation were used and included calcium treatment and suspension culture. Following growth to confluence, addition of calcium-containing DM induced differentiation of keratinocytes as assessed by immunoblotting for keratin 1, an early marker of differentiation (Fig. 1A). While AKT protein level remained constant throughout 36 h of differentiation, the level of activated AKT increased significantly, as assessed by immunoblot for phosphorylated serine 473 (p-473). The level of phosphorylation at threonine 308 (p-308) remained constant, indicating that the putative PDK2, not PDK1, is regulated by keratinocyte differentiation. Further examination of AKT activity during calcium-stimulated keratinocyte differentiation was accessed by IPK assay using purified GSK3 fusion protein as a substrate. Following 24 h of treatment in DM, AKT activity increased, while cells in BM maintained low AKT kinase activity (Fig. 1B) indicating that the increase in AKT activity is specific to calcium-induced differentiation.

As an alternative means of inducing differentiation, keratinocytes were subjected to suspension culture in 1.5% methylcellulose, and AKT activity was assessed by IPK assay. Despite a modest reduction in AKT expression over 32 h of suspension culture, AKT activity increased significantly (Fig. 1C). Increased AKT activity has been observed previously after suspension-induced differentiation of keratinocytes in a similar time frame (18). Thus, AKT activity increases as a result of two mechanistically independent methods of keratinocyte differentiation suggesting AKT activation is a general requirement for this process.

To determine whether the increased AKT activity seen in vitro correlates with what occurs in vivo, we performed indirect immunofluorescent staining for active AKT (p-473) on paraffin embedded sections of human skin and organotypic cultures (described below). Active AKT was present in the basal and suprabasal layers (Fig. 1D) similar to results reported for murine and human reconstituted skin (18, 19).

AKT exists in at least three isoforms in mammalian cells, however, the expression of each varies depending on the tissue (20). While each isoform has been shown to equally respond to agonists such as platelet-derived growth factor (PDGF) and EGF, specific physiological roles have been attributed to individual isoforms (21, 22). Thus, isoform-specific immunoprecipitations were performed to assess the activation of each in keratinocyte differentiation. Primary human keratinocytes express all three isoforms as detected by isoform-specific immunoprecipitation followed by immunoblot with a pan-AKT antibody (total AKT; Fig. 2A). Two alternatively spliced forms of AKT3 were detected by immunoprecipitation as reported previously (23); however, four times the amount of lysate was needed to produce the levels shown here. Therefore, AKT3 levels were low and were not examined further. After treatment of confluent keratinocytes with calcium to induce differentiation, expression levels of AKT1 and AKT2 were determined by immunoblot. A modest increase in AKT2 24 h after induction was observed, while AKT1 expression remained constant throughout the course of the experiment (Fig. 2B). Differentiation-specific transcriptional events may lead to induction of AKT2 expression similar to what has been observed in muscle (12, 24).

To determine which isoform contributed to the increased AKT activity observed during differentiation, isoform-specific IPK assays were performed. As a positive control, keratinocytes were treated with EGF and IGF-1, resulting in a detectable increase in activity of each isoform (Fig. 2C). Both AKT1 and AKT2 activities were consistently elevated after 24 h of calcium treatment (Fig. 2D) suggesting both isoforms become active during keratinocyte differentiation.
AKT Is Required for Keratinocyte Stratification

**FIGURE 2.** Isoform-specific activation of AKT during keratinocyte differentiation. A, whole cell lysate from proliferating keratinocytes (500 μg of total protein for AKT1 and AKT2; 2000 μg for AKT3) was immunoprecipitated with isoform-specific AKT antibodies, and the washed precipitates were immunoblotted with a pan AKT antibody (total AKT). B, keratinocytes were grown to confluence and then treated with calcium to induce differentiation and then analyzed for AKT1 and AKT2 by immunoblot using isoform-specific antibodies. C, to demonstrate detection of isoform specific kinase activity, quiescent keratinocytes were treated with (+) or without (−) 100 ng/ml IGF-1 and 20 ng/ml EGF for 15 min. Lysates were immunoprecipitated with isoform-specific antibodies, and the washed precipitates were used for kinase assays. D, Akt isoform-specific IP assay from confluent (0 h) and calcium-differentiated (24 h) keratinocytes.

AKT Inhibition by RNA Interference Results in Altered Epithelial Stratification—While both calcium treatment and suspension culture initiate aspects of the keratinocyte transcriptional differentiation program, completely mimics the spatio-temporal cell biological changes observed during stratification in vivo. Organotypic culture of human keratinocytes provides a unique opportunity to perform genetic analysis of human epithelial stratification using both normal and engineered keratinocytes (15, 25). Stable RNA interference technology, in combination with organotypic culture, was used to study AKT loss-of-function in a more physiological differentiation system. Small hairpin RNAs targeting either AKT1 or AKT2 were designed and cloned into the pSUPER retroviral vector producing shAKT1 or shAKT2 constructs, respectively. Following infection of primary keratinocytes with virus containing either shAKT1 or shAKT2 alone, or in combination, cells were assessed for AKT1 and AKT2 expression. Densitometric analysis of immunoblots indicated that infection with the shAKT1 construct resulted in an 80–90% reduction in AKT1 expression levels, while 50–75% reduction was achieved with the shAKT2 molecule. Expression of a nonspecific small-hairpin RNA, shSCRAM, was used as a control (Fig. 3A). Equivalent reduction in expression for each was observed with the combination (Fig. 3A) and could be maintained for at least six passages after selection. To show dysfunction of AKT pathway signaling in knockdown cells, phosphorylation of endogenous GSK3 after stimulation with EGF and IGF-1 was assessed. While levels of phospho-GSK3 in proliferating keratinocytes were comparable in shSCRAM and shAKT1/2 cells, the latter failed to respond to growth factor stimulation (Fig. 3B), demonstrating impaired AKT signaling in the knockdown cells.

These cells were subjected to organotypic culture, where each cell type was seeded onto a collagen I matrix embedded with fibroblasts and allowed to grow to confluence. Subsequently, the monolayer was raised to the air-medium interface, which stimulates the stratification process. Targeted disruption of AKT1 (shAKT1) resulted in a severe defect in stratification with a thin parabasal region but a thickened stratum corneum, or hyperkeratosis, when compared with control (shSCRAM; Fig. 4A), while knockdown of AKT2 (shAKT2) resulted in a normal appearing epithelium. The effect of combined AKT1 and AKT2 knockdown (shAKT1/2) appeared different from that of targeting AKT1 alone. Lamination of the upper squamous layer as observed in control cultures was absent in shAKT1 cultures, suggesting disruption of aspects of late differentiation. This is consistent with previous studies showing that AKT/Pi3K is required for late differentiation (18, 19). Furthermore, the presence of a granular layer was not apparent in the shAKT1 cultures and together these data demonstrate that reduced AKT1 expression disrupts homeostatic stratification.

Since the aberrant appearance of AKT1-deficient reconstituted skin might have been caused by a hyperproliferative phenotype, the proliferative rate of cells in shSCRAM and shAKT1 organotypic cultures was assessed by immunostaining. Sparse proliferative cells in the basal layer of both cultures were detected by Ki-67 staining, suggesting that the thickened upper layer was not due to hyperproliferation (Fig. 4B). In addition, there was no significant difference in growth rate of shAKT1 cells in monolayer culture when compared with control.

In addition to the structural differences seen in shAKT1 cultures, early and late marker expressions were also disorganized. Expression of keratin 10 is normally restricted to the suprabasal spino-granular region, and immunofluorescent staining revealed a tight band of expression in shSCRAM and shAKT2 cultures (shSCRAM; Fig. 5A, upper panel). In contrast, shAKT1 cultures displayed keratin 10 staining throughout the suprabasal and well into the cornified region with intensely stained individual cells still apparent in the upper regions. A similar disruption was noted in filaggrin staining, a marker of mid to late stage differentiation, where shAKT1 cultures displayed non-uniform expression when compared with control (Fig. 5A, lower panels). These

3 B. R. Thrash and D. J. McCance, unpublished data.
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FIGURE 4. AKT deficiency results in abnormal epidermal stratification. A, hematoxylin and eosin staining of sections from organotypic cultures produced from the indicated knockdown cells. Indicated are: basal layer (b), spinous layer (s); granular layer (g), and cornified layer (c). B, cell proliferation in organotypic cultures was assessed by Ki-67 immunohistochemistry and were counterstained using a light hematoxylin stain. C, a higher power magnification of the suprabasal layers in shSCRAM and shAKT1 cultures are shown to demonstrate the presence of condensed (pyknotic) nuclei in shAKT1 cultures. Results shown are representative of three independent experiments using two different donor pools. Scale bar, 50 μm.

FIGURE 5. AKT1 deficiency disrupts normal keratinocyte differentiation. A, organotypic cultures from shSCRAM and shAKT1 keratinocytes were immunostained for early (keratin 10; upper panels) and late (filaggrin; lower panels) markers of keratinocyte differentiation (both in green). The sections were counterstained with Hoechst (blue). Scale bar, 50 μm. B, the media of organotypic cultures from shSCRAM and shAKT1 cultures were supplemented with fluorescein-cadaverine (Fl-Cad) or unmodified fluorescein (Fl) as a control 6 h before harvest. The presence of covalently linked fluorescein was detected by immunofluorescence (green), and nuclei were detected using 4',6-diamidino-2-phenylindole staining (blue).

Data suggest that AKT1 deficiency results in either aberrant transcriptional regulation of keratin 10 and filaggrin or abnormal assembly of these proteins into the cornified layer or both.

We also assessed transglutaminase activity in shSCRAM and shAKT1 using a fluorescein-cadaverine uptake assay as described by Mack et al. (16). The presence of transglutaminase activity covalently links fluorescein-cadaverine to cornified envelope proteins. We find that in contrast to the normal pattern of transglutaminase activity restricted to the transition zone, shAKT1 cultures had widespread and disorganized activity. The combined results of Fig. 5 further suggest that late aspects of terminal differentiation are disrupted and fail to occur in an orderly fashion.

Targeted Disruption of AKT Causes Increased Cell Death in Differentiating Keratinocytes—While untreated primary keratinocytes subjected to suspension-induced differentiation are resistant to anoikis, preliminary experiments showed that inhibition of PI3K, the upstream effector of AKT, by the addition of LY294002 during suspension resulted in rapid onset of cell death as assessed by TUNEL assay (Fig. 6A). Transient knockdown of AKT1/2 using RNA interference constructs, which targeted different regions of AKT1 and AKT2 than the pSUPER retroviral vectors, produced a similar effect on survival (Fig. 6B). These results confirm AKT as the primary downstream effector of PI3K-mediated survival signaling in keratinocyte suspension. In addition, pyknotic or condensed nuclei, a histological marker of apoptosis, were observed in suprabasal cells of shAKT1 cultures but not in shSCRAM or shAKT2 (Fig. 4C). Previous reports have shown TUNEL-positive cells in the transition zone of both normal skin and organotypic cultures (5, 26), which suggests cell death occurs in this region. Similar results were observed in organotypic cultures of shSCRAM and shAKT2 keratinocytes with sparse TUNEL-positive cells that extended from the suprabasal layer throughout the cornified layer. Closer inspection revealed TUNEL-positive cells immediately adjacent to the basal layer, and these combined data suggested rapid onset of cell death once cells are detached from the basement membrane (Fig. 6B, lower panels).

Transition zone keratinocytes die by a form of programmed cell death that is distinct from classic apoptosis and may involve caspase 14 activity (27, 28). Therefore, caspase 14 expression was determined by immunostaining. A suprabasal expression pattern of caspase 14 was observed in shSCRAM cultures, consistent with previous reports (27, 29). Similar results were observed for shAKT2. However, caspase 14 expression was deregulated in shAKT1 cultures with extensive staining throughout all suprabasal layers (Fig. 6C). Active caspase was not detected by immunofluorescence in any of the organotypic cultures nor in immunoblots of extracts from these cells (5). These data suggest that normal differentiation induced cell death is enhanced in the absence of AKT1.
AKT1 Is Required for Keratinocyte Stratification

Caspase Inhibition Alleviates Effects on Stratification Caused by AKT1 Deficiency—To confirm that AKT1-deficient keratinocytes do not stratify properly due to enhanced cell death, we subjected organotypic cultures to caspase inhibition. shSCRAM or shAKT1 cultures were treated with the general caspase inhibitor Z-VAD-fmk, or an inhibitor of cathepsins B and L as a control (Z-FA-fmk), for the duration of the culture. Caspase inhibition of shAKT1 cultures (shAKT1 + Z-VAD-fmk) prevented the formation of the abnormal cornified layer seen without caspase inhibition (shAKT1 + Z-FA-fmk) and reduced cell death, as detected by TUNEL assay, to levels similar to control (Fig. 7, upper and middle panels). Furthermore, the suprabasal region appeared structurally similar to control cultures including the presentation of a granular layer and normal keratin 1 expression (Fig. 7, upper and lower panels). These data suggest AKT1 functions primarily as a survival factor required for normal epithelial stratification.

DISCUSSION

Keratinocyte differentiation and stratification are complex processes involving multiple signaling pathways whereby an uncommitted basal cell is converted into an inviable rigid squame that serves as an organism’s primary defense against the environment (for review, see Refs. 1, 2, and 6). The data presented here support AKT as key signaling component in this process as its activity increases during two mechanistically different methods of keratinocyte differentiation. This increase was attributed to the specific activation of both AKT1 and AKT2, and using organotypic skin reconstitution culture in combination with RNA interference, the requirement for each isoform in human skin formation was examined. Surprisingly, AKT1 deficiency alone resulted in a severe defect in stratification and late aspects of differentiation. In AKT1-deficient reconstituted skin, widespread cell death was observed in suprabasal keratinocytes immediately adjacent to the basal layer; this is in contrast to control skin where death was observed only in the transition zone as reported previously (5, 26). This is the first study to directly show the role of AKT in keratinocyte survival during stratification.

Despite loss of integrin-mediated attachment to the basement membrane, suprabasal keratinocytes remain viable but eventually succumb to a specialized form of programmed cell death at the transition zone (30). While the role of caspase 3 in this process remains controversial, increased expression of caspase 14 in the transition zone suggests its importance in differentiation-induced cell death (27, 31). Although the extent of cell death seen in shAKT1 skin was more pronounced than in control, the mode appears to be consistent with normal differentiation as caspase 14 expression but not active caspase 3,3 was up-regulated. Regardless of which caspase is involved, general inhibition of caspases rescued defects caused by AKT1 deficiency.

AKT1 deficiency also translated to a severe defect in epidermal structure and abnormal differentiation marker expression. While control cultures produced the expected pattern of keratin 10 and filaggrin expression in the lower and upper suprabasal layers, respectively, AKT1 knockdown produced a thickened suprabasal compartment with disrupted marker expression and without the laminated feature of a normal stratum corneum. Furthermore, widespread transglutaminase activity was present throughout all suprabasal layers of shAKT1 cultures in contrast with control cultures where the activity was restricted.

FIGURE 6. Early onset of cell death after detachment of AKT1-deficient keratinocytes from the basement membrane. A, keratinocytes were place in suspension for 12 h in the presence (+LY) or absence (−LY) of the PI3K inhibitor LY294002 (50 µM). Cell death was detected by TUNEL assay using flow cytometry. The results shown are the average of three independent experiments + S.D., each using a different pool of donors. B, keratinocytes transfected with pBS/U6 RNA interference constructs targeting either AKT1/2, or luciferase (luc) as control, were placed in suspension for 12 h. In addition, control cells were treated with 50 µM LY294002 (luc + LY). Cell death was detected by TUNEL assay using flow cytometry. The results shown are the average of two independent experiments, each using a different donor pool. C, cell death in organotypic cultures from shSCRAM, shAKT1, or shAKT2 cells was analyzed by TUNEL assay. The upper panel images were taken at ×100 magnification; the lower panel images were taken at ×200 magnification. The results are representative of three independent experiments. Scale bar, 50 µm. D, expression of caspase 14 in organotypic cultures from shSCRAM, shAKT1, or shAKT2 was detected by immunostaining. The dotted line indicates the basement membrane. Scale bar, 50 µm.
to the transition zone. These data suggest AKT plays a functional role in construction of the suprabasal layers. Aside from effects on survival, AKT loss could be triggering differentiation independently, as has been observed by PI3K inhibition (32). However, this would not exclude affects on survival. In our view, the role of survival is more likely due to the presence of pyknotic and TUNEL positive nuclei in the suprabasal layers and due to the fact that caspase inhibition alleviated phenotypic effects of AKT loss. Furthermore, spontaneous marker expression in proliferating cells were not detected nor was any difference in the rate of expression observed once cells were stimulated to differentiate by suspension.

Circumstantial evidence, derived from studies focused on AKT pathway members, suggested the importance of AKT in regulating epidermal homeostasis. Increased Akt activity either by targeted expression of IGF-1 or deletion of Pten in mouse skin both lead to hyperproliferation with differentiation defects including hypergranulosis and hyperkeratosis (33, 34). In contrast, others have reported that blockade of PI3K/AKT signaling using the chemical inhibitor LY294002 prevents some aspects of differentiation that we have confirmed (18, 35). More direct evidence has been shown by the presence of active AKT in both mouse and human reconstituted epidermis (18, 36, 37). Recently, Peng et al. (10) reported a severe skin defect in mice lacking both Akt1 and Akt2. These mice presented with a thin, translucent stratified epithelium, such as survival status, was not presented.

In mice, both Akt1 and Akt2 loss are required for an aberrant skin phenotype, as knock-out of either isoform individually appears normal (21, 22), while we observe a dramatic effect with AKT1 deficiency alone. The difference is likely due to compensation of Akt1 by Akt2 after chronic loss following germline knock-out, as opposed to acute knock-down using RNA interference. This has been observed previously for the retinoblastoma protein (pRb), where acute knockdown of pRb in normal fibroblasts allows escape from quiescence, while cells derived from pRb null embryos remained arrested due to up-regulation of the pRb homolog, p107 (38). Another explanation for the difference of our results compared with those from null mice may be due to the contribution from the mesenchymal compartment in the two systems, as paracrine stimulation of keratinocytes by mesenchymal cells controls many aspects of epidermal development (39). The signals provided by Akt null mesenchymal cells in the knock-out mouse may confer a different phenotype than the wildtype cells used in our skin reconstitution cultures. The experiments presented here would more likely represent spontaneous Akt inactivation in the skin than germline deletion.

While AKT controls multiple pathways that either directly or indirectly control cell survival, including NF-κB, BAD, mdm-2, and forkhead transcription factors (40–42), the downstream effectors responsible for AKT-mediated survival signaling during keratinocyte differentiation are not known. Sufficient evidence would support the role of the NF-κB pathway as late stage IKKα/β regulated anti-apoptotic factors TRAF-2 and c-IAP1. Preliminary results from our laboratory have shown reduced TRAF-2 expression in AKT-deficient cultures supporting the role of the NF-κB pathway in AKT-mediated survival signaling.

A number of questions remained to be answered regarding the involvement of AKT in keratinocyte differentiation, one of which pertains to the nature and origin of the signal which activates AKT during differentiation. While keratinocytes lose integrin-mediated contacts as they progress into the suprabasal layers, they gain E-cadherin-mediated cell-to-cell contacts concentrated in adherens junctions (2). Since E-cadherin ligation has been shown to stimulate the AKT pathway in epithelial cells, it is possible that a similar activation mechanism occurs in suprabasal keratinocytes (44–46). Recent work from Calautti et al. (19) would suggest both E-cadherin and tyrosine kinase activation are required for differentiation specific AKT activation in murine keratinocytes.

Keratinocytes are unique epithelial cells that are resistant to cell death after detachment from the basement membrane, allowing for stratifica-
AKT1 Is Required for Keratinocyte Stratification

tion and formation of the multilayered epidermis. The data presented here confirm AKT as a key signaling component in this process and show that acute AKT1 loss in human skin organotypic culture results in a defect in stratification. Furthermore, we extend previous studies reporting skin defects in an AKT knock-out mouse by showing that AKT functions as a survival factor in suprabasal keratinocytes. We suggest the NF-κB pathway may be an important downstream effector for survival signaling.

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