AKT-dependent HspB1 (Hsp27) Activity in Epidermal Differentiation*

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AKT activity has been reported in the epidermis associated with keratinocyte survival and differentiation. We show in developing skin that Akt activity associates first with post-proliferative, para-basal keratinocytes and later with terminally differentiated keratinocytes that are forming the fetal stratum corneum. In adult epidermis the dominant Akt activity is in these highly differentiated granular keratinocytes, involved in stratum corneum assembly. Stratum corneum is crucial for protective barrier activity, and its formation involves complex and poorly understood processes such as nuclear dissolution, keratin filament aggregation, and assembly of a multiprotein cell cornified envelope. A key protein in these processes is filaggrin.

We show that one target of Akt in granular keratinocytes is HspB1 (heat shock protein 27). Loss of epidermal HspB1 caused hyperkeratinization and misprocessing of filaggrin. Akt-mediated HspB1 phosphorylation promotes a transient interaction with filaggrin and intracellular redistribution of HspB1. This is the first demonstration of a specific interaction between HspB1 and a stratum corneum protein and indicates that HspB1 has chaperone activity during stratum corneum formation. This work demonstrates a new role for Akt in epidermis.

The epidermis is the primary environmental barrier, protecting from infection, allergens, and damage from UV radiation. The major constituent of this epidermal barrier is the terminally differentiated, anuclear keratinocyte. This structure is bounded by a cornified envelope, an elaborate, cross-linked protein structure covalently bound to hydrophobic lipid externally and aggregated keratin internally (1). Formation of this structure is poorly understood.

The complexity of keratinocyte terminal differentiation and the importance of precisely timed and compartmentalized processing is illustrated by the maturation of the stratum corneum protein filaggrin. Filaggrin is synthesized as a high molecular mass precursor comprising multiple subunits that are sequentially processed and modified in temporally and spatially regulated steps by diverse proteases and enzymes to produce mature filaggrin subunits. Mature filaggrin is thought to be important in the aggregation and collapse of the keratin network leading to flattening of the keratinocyte and the destruction of the nucleus in granular layer (terminally differentiating) keratinocytes (2). Filaggrin is also incorporated into the cornified envelope (2). Premature or aberrant filaggrin processing can be catastrophic, leading to disruption of cornified envelope integrity and skin barrier function (3–5) and is far more damaging than reduced filaggrin levels that, in contrast, lead only to mild skin defects (6).

Possible regulators of protein processing and trafficking during terminal differentiation are heat shock proteins (Hsps).2 Hsps have diverse roles as cellular chaperones, anti-apoptotic factors, stress-protective proteins, and cytoskeletal stabilizers (7, 8). Human HspB1 (Hsp27) (9–11) and the mouse HspB1 homologue Hsp25 (12) are expressed in the upper epidermal strata. The function of epidermal HspB1 has not yet been determined, but HspB1 colocalizes histologically with cornified envelope constituents, including filaggrin (13). HspB1 can bind a large number of proteins including Akt.

AKT is a Ser/Thr kinase and downstream mediator of the phosphatidylinositol 3-kinase pathway regulating cell survival, differentiation, and growth factor responsiveness (14). Mice doubly null for the Akt1 and 2 isoforms lack stratum corneum and die neonatally, probably from barrier defects (15), and Akt has been shown to have a role in keratinocyte survival and differentiation (16–18). HspB1 is a well established Akt substrate (19, 20).

While examining Akt activity during epidermal development, we discovered specific Akt activity associating with highly terminally differentiated keratinocytes. This activity is the dominant Akt activity in adult, barrier-forming epidermis. We propose that this activity is regulating the complex processes associated with keratinocyte late terminal differentiation. One way of doing this could be through modulating chaperone activities of HspB1, for example by modulating its

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‡§1 The abbreviations used are: Hsp, heat shock protein; REK, rat epidermal keratinocyte; En, embryonic day(s); siRNA, small interfering RNA; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MAPKAPK2, MAPK-activated protein kinase 2.

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interaction with keratinocyte terminal differentiation proteins, such as filaggrin. This would define a new, epidermal-specific function for Akt signaling.

EXPERIMENTAL PROCEDURES

**Immunohistochemical and Immunofluorescent Analysis**—Immunohistochemistry on paraffin and frozen sections was by standard techniques. Keratinocytes were fixed for 10 min at 4 °C in 4% paraformaldehyde in phosphate-buffered saline and then permeabilized in 0.2% Triton X-100 (Sigma) for 30 min. The antibodies were used as follows; anti-phosphorylated Akt substrate and pAKT (serine 473), 1:50; anti-AKT1, 1:50; anti-AKT3, 1:25 (all Cell Signaling Technology); chicken anti-AKT2, 1:20 (Abcam); anti-loricrin, 1:1000 (Covance); anti-mouse filaggrin, 1:500 (Zymed Laboratories Inc.); anti-human filaggrin, 1:500 (Oncogene); anti-mouse pHsp25 (Ser86), 1:50, 1:50 (Zymed Laboratories Inc.); anti-mouse Hsp25, 1:100 (Abcam); and anti-human and rat pHsp27, 1:100 (Upstate Biotechnology Inc.). Antibody detection was by either the Elite Avidin-Biotin-Complex system and 3,3′-diaminobenzidine (Vector Laboratories) or Alexa 488-conjugated (green) and 536-conjugated (red) secondary antibodies (Molecular Probes). Counterstaining was with hematoxilin for immunohistochemistry and 4′,6-diamidino-2-phenylindole for immuno fluorescence. The images were taken with a Nikon Eclipse E600 microscope with either ×20 (NA 0.4) or ×60 Oil Immersion (NA 1.40) objectives, using a SPOT digital camera (Diagnostic Instruments Inc.) with Spot RT Software v3.0.

**Preparation of Protein Lysates and Western Blots**—The protein lysates were prepared from adult mouse skin by boiling minced skin in 10 mM Tris-HCl, pH 7.5, 5% SDS, and 20% β-mercaptoethanol for 10 min. Keratinocytes were lysed by incubation on ice for 10 min in standard radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Nonidet-40).

For immunoprecipitation, the lysates were incubated overnight at 4 °C with 2.5 μg of agarose immobilized goat anti-Hsp27 (Santa Cruz Biotechnology). The complexed antibody conjugate was pelleted and washed five times in cold radioimmunoprecipitation buffer prior to loading on a gel. The lysates were separated on 7.5–10% SDS/polyacrylamide gels and transferred onto nitrocellulose filters (Hybond C+; Amersham Biosciences). Primary antibodies and concentrations were: anti-pAKT (Ser473), 1:100; total AKT, 1:1000; AKT2, 1:500 (all Cell Signaling Technologies); anti-loricrin, 1:5000 (Covance); anti-mouse filaggrin, 1:1000 (Zymed Laboratories Inc.); mouse anti-actin, 1:2000 (clone AC13; Sigma-Aldrich); anti-mouse keratin 14, 1:4000 (Covance); pHsp25 (Ser86), 1:1000 (Zymed Laboratories Inc.); and Hsp25, 1:200 (Abcam). Primary antibody incubations were in TBST (100 mM Tris-HCl, 0.2 M NaCl, 0.1% Tween 20 (v/v) containing 5% bovine serum albumin (Sigma)) either overnight at 4 °C or for 1–2 h at room temperature, whereas secondary antibody incubations were in 5% skimmed milk powder for 1 h at room temperature. The following concentrations were used: goat anti-rabbit horseradish peroxidase (Jackson Immunoresearch), 1:5000; and rabbit anti-mouse horseradish peroxidase (DakoCytomation), 1:2000. The protein was visualized using an ECL kit (Amersham Biosciences).

**Constructs, Transfections, Cell and Organotypic Culture**—The SureSilencing shRNA plasmid to rat HspB1 (insert sequence: GAAGAAAGGCAGGATGAACAT in pSuperNeo) was from Tebu-bio. Full-length rat HspB1 was cloned from rat epidermal keratinocyte (REK; Ref. 22) cDNA into pcDNA-HA-Tag (Invitrogen), and mutagenesis of serine 86 to alanine was performed using the QuikChange kit (Stratagene) with the following primers: (Ser86 → AlaF, GCGCTCAACCGGCAACTCACCAGGTGTTGTCAGAGATC; Ser86 → AlaR, ATCTCTGACACACCCTGCGAGTTGGCGGTAGCGGCG; Ser86 → AspF, GCGCTCAACCCGAACTCGACA-GCGGTGTTGTAGAGATC; and Ser86 → AspR, GATCTCTGACACACCCTGCAGTTGGCGGTAGCGGCG). A 4-hydroxytamoxifen-inducible, constitutively active (myristylated) Akt1 expression construct under the cytomegalovirus promoter (myrAkt1-ER) was from S. Basu (21). REKs (22) were transfected with Lipofectamine Plus (Invitrogen). Selection in 100 μM G418 (Invitrogen) was for 2 weeks. REK cells were passaged in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 4-Hydroxytamoxifen in ethanol was added at 10 μM. Keratinocyte organotypic cultures were generated as described (22) and treated with 4-hydroxytamoxifen or vehicle (ethanol) for 24 h.

**Cornified Envelope Extraction**—Cornified envelopes were extracted from whole adult skin by boiling for 10 min. in (50 mM Tris-HCl, pH 7.5, 2% SDS, 5 mM EDTA). Next cornified envelopes were pelleted by centrifugation and washed in cornified envelope washing buffer (10 mM Tris-HCl in 0.1% SDS).

**RESULTS**

**Phosphorylated Akt and HspB1 Show Distinct Expression Pattern during Epidermal Barrier Development**—During late murine fetal development, the epidermis changes from an embryonic, poorly differentiated form at E15.5 to a tissue that expresses correctly ordered differentiation markers at E16.5 and an immature stratum corneum at E17.5, with continued stratum corneum maturation through to the neonate. Skin acquires its protective barrier function between E16 and E17 (23), and filaggrin, a key stratum corneum protein that associates with barrier formation, is detected at E16.5 (Fig. 1A, and is processed to its active monomer form by E17 when barrier formation, is detected at E16.5 (Fig. 1A, and then from E17.5 in the upper granular layer. Lower pSerAkt (Ser473) expression was monitored over this critical period—A pattern during Epidermal Barrier Development (24). The SureSilencing shRNA plasmid to rat HspB1 (insert sequence: GAAGAAAGGCAGGATGAACAT in pSuperNeo) was from Tebu-bio. Full-length rat HspB1 was cloned from rat epidermal keratinocyte (REK; Ref. 22) cDNA into pcDNA-HA-Tag (Invitrogen), and mutagenesis of serine 86 to alanine was performed using the QuikChange kit (Stratagene) with the following primers: (Ser86 → AlaF, GCGCTCAACCGGCAACTCACCAGGTGTTGTCAGAGATC; Ser86 → AlaR, ATCTCTGACACACCCTGCGAGTTGGCGGTAGCGGCG; Ser86 → AspF, GCGCTCAACCCGAACTCGACA-GCGGTGTTGTAGAGATC; and Ser86 → AspR, GATCTCTGACACACCCTGCAGTTGGCGGTAGCGGCG). A 4-hydroxytamoxifen-inducible, constitutively active (myristylated) Akt1 expression construct under the cytomegalovirus promoter (myrAkt1-ER) was from S. Basu (21). REKs (22) were transfected with Lipofectamine Plus (Invitrogen). Selection in 100 μM G418 (Invitrogen) was for 2 weeks. REK cells were passaged in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 4-Hydroxytamoxifen in ethanol was added at 10 μM. Keratinocyte organotypic cultures were generated as described (22) and treated with 4-hydroxytamoxifen or vehicle (ethanol) for 24 h.

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Following recent reports of Akt involvement with keratinocyte differentiation (16–18) Ser473 phosphorylated Akt (pSerAkt) expression was monitored over this critical period because it has been shown to accurately indicate keratinocyte Akt activity (18). pSerAkt was expressed in the lower epidermal strata at E16.5 (parabasal, post-mitotic cells; Fig. 1A, pSerAkt) and then from E17.5 in the upper granular layer. Lower pSerAkt is down-regulated from E18.5 (Fig. 1A) and although still detectable in neonatal epidermis is down-regulated in adult mature skin. Lower Akt activity has been noted in keratinocyte culture models (23–25); however, this lower activity is substantially reduced or undetectable in most postnatal skin (Fig. 2A).
It is the upper activity that persists and is the dominant Akt activity in most adult skin (Fig. 2A). Hence, Akt signaling in adult skin differs from keratinocyte culture models.

Akt1 and 2 are expressed and active in human keratinocytes (17, 18). Akt1 expression is most prominent in the murine granular layer from E17.5 (Fig. 1A), coexpressing temporally and spatially with pSerAkt, suggesting that upper Akt contains mostly Akt1. In contrast Akt2 was present in all epidermal layers by E15.5 onwards and was expressed ubiquitously (Fig. 1A). This implies that the lower, embryonic phosphorylated Akt is largely Akt2, with probable Akt2 contribution to upper pSerAkt. Akt3 was never detected in the epidermis in this study, although it was readily detected in other tissues (e.g. small intestine; data not shown). Akt3 activity has been reported in post-mitotic cultured keratinocytes (17), although levels are extremely low (18).

Multiple authors have shown that HspB1 is an Akt substrate, phosphorylated on Ser\(^{82}\) (Ser\(^{86}\) in mouse) (19, 20). Although unphosphorylated HspB1 is widely expressed in fetal skin (Fig. 1C), the Ser\(^{86}\) phosphorylated form (pSerHspB1) has a changing expression pattern during the fetal barrier formation period. There is multistrata expression from E15.5, most prominent by E16–17.5 (Fig. 1A). However, by E18.5 pSerHspB1 expression associates more strongly with the terminally differentiating cells in upper epidermis and in the granular layer cells in adult (see Fig. 4). In addition, by E17.5 and E18.5 expression is less prominent in the nucleus (Fig. 1A) and appears to be associating with the forming granules of the granular layer (later confirmed in adult skin; see Fig. 4). Hence, both Akt and its phosphorylated HspB1 substrate coexpress in the granular layer of epidermis by late development and in adult skin.

Western analysis of total HspB1 expression during development reveals the 25-kDa monomer form (Fig. 1C, closed arrowhead) and a series of covalently linked high molecular mass forms (Fig. 1C, asterisks, open arrowhead). Heat shock proteins are known to aggregate, and these higher molecular mass forms must represent either aggregated HspB1 (possible trimeric 75 kDa and tetrameric 100 kDa; Fig. 1C) or HspB1 covalently cross-linked to additional proteins. pSerHspB1 is present as monomer (Fig. 1C, closed arrowhead) and 75-kDa forms (Fig. 1C, asterisk). Developmentally the pSerHspB1 monomer downregulates to be replaced in mouse by the phosphorylated 75-kDa forms.

**HspB1 Phosphorylation at Ser\(^{86}\) in Upper Epidermis Correlates with Adult Akt Expression**—Upper granular layer pSerAkt expression predominates in adult skin (Fig. 2A, dorsal skin). One exception is the highly specialized and thickened palmar/plantar (footpad) epidermis. Here the parabasal, lower Akt activity is retained, and there is much reduced upper Akt activity. Akt1 is undetectable in footpad (Fig. 2B), although Akt2 is maintained (Fig. 2C). This finding strengthens the proposal...
that upper pSerAkt is largely Akt1, whereas lower pSerAkt is largely Akt2. In addition, it suggests that upper pSerAkt may regulate the distinct patterns of terminal differentiation leading to physiologically different stratum corneums.

If upper pSerAkt phosphorylates HspB1, then in footpad pSerHspB1 should be reduced in upper layers. pSerHspB1 is concentrated in the granular layer in dorsal adult skin (Fig. 2D), whereas in footpad weak expression commenced in the lower strata but is reduced in the granular layer (Fig. 2D).

Akt1 and Akt2 Null Mice Have Reduced Phosphorylation of HspB1 at Ser186 and Reduced Filaggrin—Although the double Akt1 and 2 null mouse dies soon after birth with a major stratum corneum defect (15), single Akt1 and 2 null mice survive and appear phenotypically normal (Refs. 24–26 and Fig. 3A), although the late terminal differentiation marker filaggrin is reduced (Fig. 3A). To demonstrate that epidermal Akt isoforms regulate epidermal HspB1, we examined dorsal skin from Akt1 and Akt2 single null mice. Total HspB1 levels did not vary in the wild type and null mice (Fig. 3B, closed arrowhead). In adult wild type murine epidermis, the higher molecular mass 75-kDa pSerHspB1 (Fig. 3B, asterisk) predominates, consistent with the demonstration that as epidermis develops the phosphorylated lower form is down-regulated (Fig. 1C). There was much reduced phosphorylation of HspB1 at Ser186 in both the Akt1 and Akt2 null mice (Fig. 3B). These data show that both Akt isoforms regulate phosphorylation of HspB1 at Ser186 in adult skin.

Although the single Akt1 and 2 null mice survive and have apparently phenotypically normal skin, the reduction in phosphorylated HspB1 led us to question whether there were subtle...
stratum corneum defects. We assay cornified envelope integrity by resistance to sonication (27). We show that isolated cornified envelopes from the Akt1 and 2 null mice are more susceptible to sonication (i.e., more fragile) than wild type (Fig. 3C), with the Akt1 null cornified envelopes showing significantly (p = 0.0086) more fragility.

pSerHspB1 and Filaggrin Colocalize to Epidermal Keratohyalin Granules—Because we have shown that Akt regulates phosphorylation of HspB1 at Ser86 in epidermis and the phosphorylated form appears to associate with granular structures as development progresses (Fig. 1A), we examined subcellular location by immunoelectron microscopy in adult mouse skin. Filaggrin localizes to specialized keratohyalin granules (Fig. 4, B and G), which accumulate in transitional keratinocytes, (i.e., cells about to cornify or undergo keratinocyte-specific programmed cell death), just under the stratum corneum (Fig. 4, A and B). Sequestration probably prevents premature action (nuclear degradation, filament aggregation to form the matrix of cornified cells) by filaggrin. pSerHspB1 also concentrates in these keratohyalin granules (Fig. 4C). In addition, pSerHspB1 localizes to the keratin filaments in transitional cells (Fig. 4D), which is intriguing because these will be the substrates of filaggrin action during cornification. Secondary antibody alone did not produce any specific labeling (Fig. 4E), and nor did pSerHspB1 associate specifically with keratin filaments in lower strata (Fig. 4F). These data suggest that Akt-phosphorylated HspB1 associates with filaggrin in epidermis.

Loss of HspB1 Leads to a Thickened Cornified Layer and Alterations in Filaggrin Processing—To determine whether HspB1 affects epidermal terminal differentiation and filaggrin processing, we used siRNA to reduce HspB1 expression in cultured keratinocytes (REKs; Ref. 22) and a REK-derived keratinocyte organotypic culture model that mimics the three-dimensional structure of skin and undergoes authentic terminal differentiation and barrier formation (Ref. 22 and Fig. 5). In differentiated cell culture the siRNA-containing cells expressed more filaggrin than the scrambled oligonucleotide controls, but the filaggrin was incorrectly processed because there was a reduction in the expression of the mature subunit of filaggrin (Fig. 5A, asterisk). Expression of keratin 10 was unchanged, suggesting that early epidermal differentiation was unaffected. Expression of loricrin, an unrelated cornified envelope protein, was increased in the siRNA-expressing culture. In the REK keratinocyte organotypic model, reduction in HspB1 expression led to production of an abnormal thickened stratum corneum (Fig. 5B). The overexpression of loricrin was confirmed by immunohistochemistry. These data suggest that HspB1 plays an important role in controlling late terminal differentiation processes involving filaggrin and additional stratum corneum proteins.
Phosphorylation of HspB1 at Serine 86 Leads to Colocalization of Filaggrin and HspB1—The immunoelectron microscopy (Fig. 4) revealed that filaggrin and pSerHspB1 were coexpressed in the keratohyalin granules in the uppermost epidermis. To investigate further the interaction between filaggrin and HspB1, we transfected REK cells with hemagglutinin (HA)-tagged constructs containing wild type HspB1, an HspB1Ser86 → Asp mutant mimicking phosphorylation by Akt, and an HspB1Ser86 → Ala preventing phosphorylation at this site (Fig. 6). Immunofluorescence of highly differentiated cells with decomposing nuclei (Fig. 6, arrowheads) showed partial colocalization of filaggrin and HA in the cells expressing wild type HspB1 (Fig. 6). There was complete colocalization of the Ser → Asp mutant with filaggrin, whereas the alanine mutant showed complete segregation between the HA tag and filaggrin. This shows that phosphorylation of HspB1 at Ser86 by Akt is required for the interaction between HspB1 and filaggrin.

HspB1 Binds Filaggrin in an AKT-dependent Manner, and the Interaction Is Transitory—To find whether there was an interaction between HspB1 and filaggrin, we expressed a tamoxifen-inducible, myristylated Akt1 (21) in the REK model. These cells show increased pSerAkt expression upon tamoxifen treatment (Fig. 7A). Induction of Akt1 signaling led to increased filaggrin expression (Fig. 7A), consistent with the data from the Akt null mice and possibly reflecting increased Akt-mediated terminal differentiation (17).

Immunoprecipitation with anti-HspB1 antibody revealed that, compared with vehicle-treated cells, inducing Akt1 activity resulted in a rapid association between filaggrin and HspB1 (Fig. 7B). This association was strongest at 2 h, followed by reduced association at 8 h (the apparent increased association at 24 h also occurs in the control). Note that the pattern of filaggrin intermediates associating with HspB1 differs from the pattern unassociated with HspB1 (Fig. 7, compare A and B), suggesting an HspB1-specific association with a subset of filaggrin intermediates. Akt1 activation was marked by the rapid appearance of high molecular mass pSerHspB1 (Fig. 7C), confirming that epidermal Akt pSerHspB1 at the equivalent serine (Ser17 in rat).

Akt induced HspB1-filaggrin interaction appears down-regulated 8 h after experimental Akt activation (Fig. 7B), suggesting that the interaction is transitory. In the REK cell line, filaggrin can be detected associating with the nuclei of late terminally differentiating cells in monolayer culture, probably reflecting its in vivo role in nuclear degradation (28). Immunofluorescence experiments showed that Akt-induced filaggrin and phosphorylated HspB1 express in both the cytoplasm and nucleus 2 h after Akt induction, permissive for the physical binding revealed by immunoprecipitation (Fig. 7E). However, by 8 h filaggrin and phosphorylated HspB1 are segregating (Fig. 7F) in many of the cells. In these cells nuclear filaggrin is non-homogenous, and pSerHspB1 is excluded from the nucleus. These data confirm that the Akt-induced-SerHspB1 interaction with filaggrin is transitory, at least in the nuclei, and that following the interaction the two proteins segregate.

**AKT Phosphorylation of HspB1 Is Followed by Intracellular Redistribution—**Developmentally, pSerHspB1 appears to move from nucleus to cytoplasm or keratohyalin granules during the barrier formation period (Fig. 1A). In addition, there have been previous reports of keratinocyte HspB1 shuttling between the cytoplasm and nucleus in response to stress (29–31). The Akt-inducible monolayer culture model above (Fig. 7), we show nuclear-cytoplasmic redistribution of HspB1, which follows Ser phosphorylation and filaggrin interaction. To explore possible intracellular movement in a more authentic skin model and find whether it was linked to Akt activation, tamoxifen-inducible, myristylated Akt (constitutively active) was induced for 24 h in a the REK keratinocyte organotypic culture (22). Note that the addition of 4-hydroxytamoxifen to the organotypic culture without the Akt construct caused no change in either the expression of Akt or phosphorylation of Akt (data not shown).

Akt activation results in hyperkeratinization in the organotypic model, as previously reported (Refs. 16 and 32 and Fig. 8A) and increased and inappropriate expression of filaggrin (Fig. 8A). This culture system is a closer mimic of fetal/neonatal skin than mature adult skin, with pSerAkt expressed in both the parabasal cells and granular layers in control and induced cultures (Fig. 8A) and pSerHspB1 expressed throughout the epidermis as in fetal skin (Fig. 8B; cf. Fig. 1A). Induction of constitutively activated Akt in this system produces dramatic change in pSerHspB1 subcellular location with depletion of pSerHspB1 from the nuclei and a greater concentration of protein in the cytoplasm compared with vehicle-treated controls (Fig. 8B). This suggests that Akt activity is altering the balance of nuclear versus cytoplasmic pSerHspB1 in favor of concentration in the cytoplasm. Because this culture model is a closer analogue to fetal/neonatal than adult skin, these data suggest that the developmental movement of pSerHspB1 from nuclei to cytoplasm/granules could be driven by the newly induced fetal Akt activities.

**DISCUSSION**

In this work we show that epidermal Akt expression in vivo is more complex than suggested by previous culture-based
We demonstrate a mainly fetal, parabasal Akt activity, probably comprising mostly Akt2. This fetal form is down-regulated in most mature adult epidermis, with the exception of specialized epidermis such as footpad. In most mature adult epidermis, Akt signaling associates with the terminally differentiating granular layer cells subjacent to the stratum corneum and comprises largely Akt1, with probable minority Akt2. Hence, fetal/neonatal parabasal serine-phosphorylated Akt provides one of the first known molecular markers for late fetal/neonatal epidermis. We also show that Akt1 and Akt2 null mice, previously thought to lack epidermal phenotype, have reduced filaggrin levels, and the Akt1 null mouse has significant cornified envelope defects.

We find that upper granular layer pSerAkt is reduced in footpad, which is a specialized epidermis with a much thickened stratum corneum. pSerHspB1 in the upper layers is correlative reduced in the granular layer. Stratum corneum quality varies around the body surface and in response to outside influences such as mechanical pressure and UV (see below). We speculate that regulation of stratum corneum quality is being mediated by granular Akt.

We show that epidermal Akt activity leads to phosphorylation of HspB1 at Ser86, demonstrated both in Akt null mice and by overexpression. Phosphorylation of HspB1 induces rapid, reversible binding of HspB1 to filaggrin. Akt-induced HspB1 phosphorylation also changes the equilibrium between cytoplasmic and nuclear pSerHspB1, leading to greater concentration in the cytoplasm. Although HspB1 has been proposed as a chaperone of late terminal differentiation proteins (10, 33), this is the first example of direct, reversible binding to a cornified envelope protein. We also demonstrate that reduction in HspB1 leads to keratinization abnormality and changes not only to filaggrin processing but also to levels of an unrelated keratinization protein loricrin, underlying the probable pleiotropic effect of HspB1 on terminal differentiation. The demonstration that epidermal Akt is affecting the binding behavior and intracellular location of this important epidermal chaperone in cells undergoing cornification implies a new role for Akt.

**Akt and Hsp27 in Epidermis**

During late development in mouse (E16.5–17.5) (23) and at −24–28 weeks of human gestation (34), skin rapidly acquires barrier activity. We demonstrate complex and changing Akt activities over this period. We infer that the fetal, parabasal pulse of Akt activity is equivalent to that reported in cultured keratinocytes because (i) it reappears in organotypic cultures (this work; Ref. 16) and (ii) it associates with early differentiation in cultured keratinocytes (17, 18) where it has been linked to keratinocyte differentiation and survival. In adult skin it is likely that this lower form is still present, although the lower levels are usually below or just bordering the limits of detection histologically. It may be this lower Akt activity that is reactivate during wound healing (35).

We show that the HspB1 intracellular location changes during this crucial developmental period from a cytoplasmic/nuclear location to a nuclear location, corresponding to the induction of terminal differentiation. We find that upper granular layer pSerAkt is reduced in footpad, which is a specialized epidermis with a much thickened stratum corneum. pSerHspB1 in the upper layers is correlative reduced in the granular layer. Stratum corneum quality varies around the body surface and in response to outside influences such as mechanical pressure and UV (see below). We speculate that regulation of stratum corneum quality is being mediated by granular Akt.

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FIGURE 8. Akt-induced redistribution of phosphorylated HspB1. A, histology of vehicle- and tamoxifen-treated (Tam) keratinocyte organotypic cultures expressing myrAkt1-ER. Note the thickened cornified layer in the tamoxifen treated raft. Immunohistochemistry shows an increase in both pSerHspB1 expression and expression of filaggrin in all layers of the epidermis in response to tamoxifen treatment. The dotted line indicates the dermo-epidermal boundary. Bars, 25 μm (Histology and pSerAkt) and 15 μm (Filaggrin). B, HspB1 is shuttled to the cell periphery in response to Akt signaling. Expression of pSerHspB1 in vehicle and tamoxifen-treated rafts. Coimmunofluorescence staining clearly indicates nuclear staining of pSerHspB1 in the vehicle-treated keratinocyte organotypic cultures (arrowheads), whereas pSerHspB1 translocates to the cell periphery in response to tamoxifen (asterisks). Bar, 15 μm.

clear pattern to a cytoplasmic/granular pattern. Because experimental Akt activation in a skin organotypic model mimicking fetal skin results in similar change, it is tempting to infer that Akt activation is prerequisite for, or is driving, HspB1 intracellular relocation. HspB1 transport to the keratohyalin granules and its association with keratin filaments is probably necessary for processing of critical stratum corneum proteins like filaggrin and correct stratum corneum formation during development.

During this critical barrier forming period, HspB1 monomer is converted to a higher molecular mass form that persists in adults. HspB1 proteins commonly aggregate noncovalently and dissociate upon phosphorylation (36). However, because the higher molecular mass forms detected here are present after denaturing electrophoresis, they must be formed by covalent cross-linking of multiple monomers to themselves (the molecular mass of the major forms in mouse suggests a trimer) or other proteins. In amyloid plaques, HspB1 is cross-linked into large multi-species protein aggregates by tissue transglutaminases (37). HspB1 has multiple glutamine and lysine residues, making it a potential substrate for the abundant transglutaminases in the upper epidermis that become active developmentally when HspB1 monomer is lost. The molecular nature of these higher molecular mass forms and their formation during development is a subject for future investigation.

The Significance of AKT-dependent Interaction between HspB1 and Filaggrin—We show Akt-dependent interaction of HspB1 with filaggrin, although we predict interaction with multiple stratum corneum proteins. Filaggrin is the best characterized member of the fused type S100 multi-protein family, including hornerin and trichohyalin. These proteins are produced as high molecular mass, insoluble precursors consisting of repeated monomer units with an N-terminal S100 homology region. Filaggrin was originally described as involved in keratin aggregation (38) but later shown to have an additional role in nuclear destruction (2). Therefore, free processed filaggrin is a dangerous protein, and sequestration in keratohyalin granules is thought to prevent premature action. Our data suggest that an important role for HspB1 in the epidermis is to bind filaggrin and probably other fused group proteins to regulate correct temporal and spatial processing.

Akt, HspB1, and Environmental Stress—Epidermal stratum corneum is the primary barrier against environmental insults such as UV light, in particular genotoxic UVB. Although keratinocytes from the lower strata undergo apoptosis in response to UV-induced DNA damage, such a response in granular layer keratinocytes needed for stratum corneum assembly would be catastrophic, leaving underlying cells without protection (39). In addition, it may not be necessary to remove cells harboring UV-induced DNA damage if they are soon to be “cornified” and shed into the environment. Our demonstration of granular layer Akt and HspB1 is significant because these are known anti-apoptotic proteins (14, 40), and others have proposed that keratinocyte Akt has an antiapoptotic role (17, 18). In addition to their role in the regulation of normal cornification demonstrated here, granular Akt and HspB1 may play an apoptotic protective role for granular layer keratinocytes.

We show that upper epidermal phosphorylation at Ser\(^{36}\) in mouse is regulated by Akt1 and Akt2. However, HspB1 is also a substrate of the stress-induced p38 MAPK/MAPKAPK2 pathway, resulting in phosphorylation at the same serine residue in human and mouse, as well as additional serine residues (36). Additional kinases are also known to regulate HspB1 (reviewed in Ref. 41), and we suspect that these kinases are active developmentally and in adult epidermis. The p38 MAPK pathway resulting in HspB1 phosphorylation is active in keratinocytes subject to stress (31, 42). In neither the Akt1 null or Akt2 null mouse is there complete dephosphorylation of HspB1 at Ser\(^{36}\) (Fig. 3B), probably because of redundant action from the other Akt isoforms, although possibly by phosphorylation via MAPKAPK2 or other epidermal kinases.

We demonstrate here redistribution of pSerHspB1 from nucleus to the cytoplasm as a response to Akt signaling and interpret this as evidence for a chaperone role for Akt-phosphorylated HspB1 during normal differentiation. Phosphorylation and redistribution of HspB1 from the cytoplasm to the nucleus after heat shock (30) and UVB exposure (29, 31) has been demonstrated in cultured keratinocytes, with UVB-medi-
ated cytoplasmic to nuclear shutting downstream of p38 MAPK signaling (31). Hence, the stress-responsive phosphorylation of HspB1 and Akt-mediated phosphorylation produce opposite cellular localization effects.

Roles of Akt Isoforms in Skin Barrier—Although the Akt1 and 2 double null mouse fails to form stratum corneum and has an apparent major barrier defect (15), the single isoform null mice (Akt1, 2 and 3) (24–26, 43, 44) survive postnatally with no apparent major barrier defect (15), the single isoform null mice. Nevertheless, the Akt1 null animals fail to thrive (25, 26), which is a feature of human patients with subtle barrier defects (45). Knock-out of many key stratum corneum proteins has failed to produce significant barrier defect (45). We believe that there is compensation by other Akt isoforms or pathways that prevent severe pathology in the Akt single null animals. Nevertheless, the Akt1 null animals fail to thrive (25, 26), which is a feature of human patients with subtle barrier defects (46). This suggests that knock-out of single Akt isoforms may affect epidermal physiology.

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