The AKT family and other animals – Protein kinases and the epidermal barrier

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Abstract

Formation of a stratified epidermis is required for the formation of the skin barrier, an essential skin function; to act as an outside-in barrier against the access of microorganisms and other external factors, to prevent loss of water and solutes via inside-out barrier functions and withstand mechanical stresses. Epidermal barrier function is initiated during embryonic development and is then maintained throughout life and restored after injury. Interrelated processes occur that comprise the formation of the essential epidermal barrier. In this review we specifically focus on the roles that protein kinases play in these processes.

A brief overview of processes in epidermal barrier formation

A number of interrelated processes occur in endstage keratinocyte terminal differentiation, which are initiated during embryogenesis. The formation of the stratified epithelium originates from a single layer of proliferating ectoderm cells above a layer of mesenchyme[1–3]. After commitment to stratification at embryonic day 9.5 the periderm forms; a specialised embryonic structure, which prevents the formation of inappropriate contacts of developing epithelia before formation of the stratified epidermis[4]. Initially the cells of the basal layer divide asymmetrically to form daughter cells that are not attached to the basement membrane and mesenchyme but localise above the basal cells in a suprabasal layer, the spinous layer. These daughter cells differentiate further and form the cells of the granular layer, establishing the skin barrier through the development of tight junctions, before completing the final differentiation stage into corneocytes; keratinocytes that are devoid of internal organelles and surrounded by layers of lipid lamellae[5,6]. Formation of the epidermal barrier coincides with removal of the periderm in developing epidermis[2]. In adult epidermis continued corneocyte differentiation causes these cells to be lost from the cell surface in a process known as desquamation.

This terminal differentiation process has been described as a controlled cell death program which culminates in the formation of corneocytes; differentiated keratinocytes which are do not contain any internal structures, are flattened in morphology, develop a cornified envelope and are surrounded by highly organised lipid layers[7]. Formation of the four keratinocyte layers and the skin barrier involves

drastic alterations in keratinocyte morphology and function and requires the interplay of several interlinked processes including tight junction formation, filament aggregation and cellular flattening, nuclear and organelle degradation, protein-protein crosslinking, lipid extrusion and ultimately the desquamation of corneocytes from the skin surface.

The greatest alteration in keratinocyte morphology occurs in the upper layers of the granular layer, at the transition between the granular and cornified layers. Keratohyalin granules, frequently observed in the granular layer and which give the granular layer its name, disappear at the transition between the granular and cornified layers. These granules are comprised of keratin filaments and keratin binding proteins such as profilaggrin, and their removal is suggested to be through the processing of profilaggrin to filaggrin monomers, which aggregate keratin filaments into bundles or “rods” forming the internal structure of the corneocytes, and promoting the flattened morphology[8]. Additionally granular cells also remove their nucleus and intracellular organelles when undergoing differentiation, requiring autophagy and nucleophagy, although these processes are not yet completely defined[9,10].

Corneocytes, devoid of internal structures, not only maintain their morphology through the aggregated keratin filaments but also through a surrounding structure, known as the cornified envelope. This structure forms a hydrophobic barrier and connects the corneocytes to the intracellular lipid lamellae of the cornified layer. It is primarily proteinaceous[11] containing loricrin, involucrin, envoplakin, periplakin and small proline-rich proteins, which are cross-linked to a “scaffold” of ω-hydroxyceramides with very long fatty acid chains, by transglutaminase enzymes, which also function to organise the surrounding lipid layers[12,13].

The lipid lamellae of the cornified layer are composed of lipids: ceramide, free fatty acids and cholesterol[14] and the formation and homeostasis of the corneocytes and lipid layers is heavily dependent on the extrusion of lamellar body contents from keratinocytes of the granular layer[15]. Lamellar bodies are lysosome-related organelles[16] produced by keratinocytes of the spinous and granular layers which extrude their contents at the junction between the granular and cornified layers. A

large proportion of their interior consists of lipid lamellae organised into disc-like arrays[17], which once secreted are further processed to form the lipid lamellae of the cornified layer. They also contain a wide variety of proteins and other molecules including proteases, protease inhibitors, structural proteins, lipid processing enzymes, glycosidases and antimicrobial peptides. This combination of components is essential for the formation of the lipid lamellae, for corneocyte formation, for protection against pathogens and for desquamation[18], where corneodesmosomes between corneocytes are degraded and corneocytes are lost from the skin surface[19].

Kinase expression in the upper epidermis

With the exception of single phosphorylated kinase immunohistochemical or western blot analysis, it is relatively difficult to identify active kinases in the upper epidermis. Gene expression data of differentiating epidermis and more recently single cell sequencing of mouse epidermis reveals a number of kinases that are specifically expressed in the granular layer of the epidermis[20,21], Table 1. In total 26 kinases are expressed specifically in murine granular layer cells, a number of which are interrelated based on Analysis in STRING (Figure 1), with AKT1 at the centre of an interconnected network of kinases.

AKT kinases

The AKT kinase family comprises three highly homologous isoforms whose molecular targets are cell-type specific but all contain the canonical Akt consensus motif RXRXXS/T[22]. This comprises a large number of potential keratinocyte protein targets, as approximately 6000 proteins have this sequence, however, only around 100 targets have been identified suggesting that the effects of AKT in the epidermis are likely pleiotropic. In the epidermis, AKT activity is bimodal, activity in lower suprabasal cells is associated with wound healing, remodelling and cancer, and activity in upper suprabasal cells is associated with terminal differentiation[23–26]. Loss of all AKT activity leads to severe epidermal defects and perinatal death[27], while reduced AKT1 activity leads to upper epidermal defects including defects in corneocyte integrity and filaggrin expression and reduced nuclear degradation and clearance[24,28]. In contrast, loss of AKT2 in murine models, although inducing diabetes mellitus and insulin resistance, does not have any identifiable or reported epidermal defects[29].

The mammalian target of rapamycin (mTOR) is a multi-protein serine/threonine kinase controlling metabolism, protein synthesis, and protein kinase phosphorylation, including phosphorylation of AKT kinases. There are two mTOR complexes, mTORC1 which controls protein metabolism[30], and mTORC2 which activates AKT signalling[31]. We and others have shown that mTORC1 and 2 activity are present in the upper epidermis[32–34] with mTORC1 activity, measured by S6 phosphorylation, is concentrated in the upper epidermis[32,33].

mTORC1 and mTORC2 complexes contain the subunits Raptor and Rictor respectively[35] and selective deficiency of these subunits allows manipulation of mTORC1 and mTORC2 activity to dissect their roles in the epidermis. These pathways converge on AKT signalling in the epidermis with different effects[32,36,37]. Rapamycin-mediated inhibition of Raptor, and mTORC1, increases the activity of AKT1 kinase through inhibition of a negative feedback loop, indicating the inhibition of AKT activity by the mTORC1 complex[32]. Conversely, increased Raptor expression decreases AKT1 activity through decreased mTORC2 activity, by competition for mTOR subunits, highlighting that mTORC2 activity promotes AKT1 activity via the direct phosphorylation[36].

ERK/MAPK/JNK kinases

The canonical ERK-MAPK signalling cascade transduces extracellular signals from various cell surface receptors to regulation of Ras GTPase activity. Active Ras (Ras-GTP) initiates signalling through a sequential kinase cascade; through Raf-1, a mitogen activated protein kinase kinase kinase (MAPKKK), MEK-1/2 a mitogen activated protein kinase kinase (MAPKK) and then to phosphorylation of ERK-1/2 a mitogen activated protein kinase (MAPK) that affects the phosphorylation and activity of a wide range of cellular effectors[38].

Several MAP kinases, Mapk3, Mapk13 and Mapk14 are specifically expressed in the upper epidermis on the mRNA level[21] and analysis of knockouts of some of these proteins display barrier defects, implicating them in epidermal terminal differentiation[39–41]. Additionally, the ERK1/2 effector c-Jun is phosphorylated in the granular layer, suggesting that ERK1/2 is active in this region[42–44]. More recent work

indicates a gradient of MAPK and ERK activation across the differentiating epidermis, correlating to the calcium gradient, with ERK1/2 active in the nucleus of granular layer keratinocytes further suggesting a role for this signalling pathway in keratinocyte differentiation[38].

LIM kinases

The LIM kinase family contains two members: LIMK1 and LIMK2[45]. These are dual specificity serine/threonine and tyrosine kinases which have the same downstream targets but appear to be differentially regulated by expression, cellular localisation and upstream signalling[45–47]. LIMK2 is expressed in the basal layer of the epidermis and reportedly promotes adhesion to the basement membrane and inhibits keratinocyte differentiation through the phosphorylation and inhibition of cofilins; actin-binding proteins which destabilise actin filaments[48]. Conversely, LIMK1 is expressed in upper cells of the granular layer where phosphorylation and inhibition of cofilin promotes granular layer compaction[48].

Periderm and epidermal development

Although the molecular mechanisms underlying the formation of the periderm are not well understood, several phosphorylation events have been proposed in periderm development. Mutations in the kinase domain of RIPK4, a serine/threonine kinase, prevent periderm formation; leading to epidermal fusions and Bartsocas Papas syndrome in humans[49,50]. One phosphorylation target of RIPK4 is IRF6, expressed in the periderm and required for periderm formation, and whose function requires phosphorylation for translocation to the nucleus[4,51,52]. Mutations in IRF6 also cause epidermal fusions and Van der Woude syndrome[53]. Although the kinase IKKα is also required for periderm formation, and mutations cause cocoon syndrome with severe malformations[54], the kinase domain is not required for epidermal differentiation[55]. Stratifin, 14-3-3σ, which modulates cellular pathways by binding target proteins through recognition of phospho-serine/threonine motifs, also localises to the periderm and deficiency leads to lack of periderm formation[4]. Suggesting, protein modulation by phosphorylation and recognition of phosphorylation motifs may be involved in periderm function. Additionally, stratifin is known to bind to keratin 17, an intracellular marker of the periderm, and through this interaction activate AKT/mTOR

signalling and affect cell growth[56]. Functional epidermal barrier formation is required before the periderm is sloughed off[57].

Tight junction formation

Tight junctions form the initial epidermal barrier in the periderm[1,2] and in addition to the cornified layer, a network of tight junctions throughout the granular layer perform the barrier function of the epidermis, preventing water and solute loss[58,59]. In epidermal development the barrier forms in a 'wave' of tight junction formation that coincides with Akt activity and requires dephosphorylation of c-Jun by protein phosphatase 2a [1,2,24,25,42]. In the adult epidermis tight junctions are dynamic structures that require return of junctional components to the plasma membrane and similar pathways may be operating in the stratified epidermis[60].

Nuclear degradation

As keratinocytes transition from granular layer cells to the cornified layer they also undergo controlled removal of all intracellular organelles including the nucleus[9,10]. The process of nuclear degradation is as yet only partially understood but Akt activity has also been implicated in this process; AKT1-dependent phosphorylation of Lamin A/C is required for degradation of the nuclear lamina and the removal of nuclear contents from terminally differentiated keratinocytes[28,33,36].

Filaggrin processing and compaction

Filaggrin is an intermediate filament organising protein which is expressed as profilaggrin, a large protein consisting of several filaggrin repeats. In the granular layer profilaggrin is highly phosphorylated and localises to the frequent keratohyalin granules which give the granular layer its name[61,62]. In the cornification process, profilaggrin is dephosphorylated and undergoes proteolytic cleavage into filaggrin monomers which bind to and align the keratin intermediate filaments throughout the cytoplasm[63–65]. This causes collapse of the keratinocyte cytoskeletal network, promoting cell compaction and providing a scaffold for the formation of the cornified envelope[66,67]. Controlled expression and processing of filaggrin is not only important for corneocyte compaction but is also required for cornified layer acidification and hydration, mediated through the breakdown of filaggrin into individual amino acids[68,69]. Additionally alterations in filaggrin expression and processing alter lamellar

body formation and nuclear degradation, indicating filaggrin processing may be a central step in keratinocyte differentiation[70,71]. AKT1-dependent phosphorylation of HspB1, a protein chaperone, has been shown to be required for filaggrin processing and function[25]. LIM kinases also phosphorylate cofilin, an actin organising protein, in the granular layer of the epidermis, inhibiting cofilin activity and leading to stabilisation of actin filaments[47,48,72]. Consistent with this, expression of constitutively active cofilin prevents cell compaction reinforcing that filament organisation is an important step in cell compaction[48].

Protein crosslinking

In addition to the filaggrin-keratin aggregates of the cornified layer, proteins including loricrin, involucrin, envoplakin, periplakin and small proline-rich proteins are crosslinked to each other and to ω-hydroxyceramides of the plasma membrane by transglutaminase enzymes, through covalent bonds between glutamine residues and lysine residues or ω-hydroxyl groups which also function to organise the surrounding lipid layers[13]. These crosslinking reactions create the cornified envelope and are also linked to the filaggrin-keratin network of the corneocytes. Expression of the cornified envelope proteins are known to be regulated through kinase signalling pathways such as TNFα-dependent JNK kinase activation in the control of filaggrin and loricrin expression[73]. Cross-talk of transglutaminase activity with other kinase signalling pathways has been described in other tissues and may also be important in desquamation[74,75].

Lipid synthesis and extrusion

Lamellar body synthesis commences in the spinous layer and lamellar bodies accumulate in granular layers cells where they extrude their contents at the apical junction of granular layer cells at the junction with the cornified layer. Regulation of lamellar body biogenesis and secretion is an as yet partly understood process, but likely involves extracellular signal transduction through intracellular kinases for regulation of these processes. Several kinases were identified in lamellar body enriched fractions of human epidermis[76], protein kinase C (PKC) transduction of intracellular calcium concentrations modulates barrier function with alterations in lamellar body production and function[77,78], and a lamellar body cargo,

corneodesmosin, is highly phosphorylated[79] which may indicate a role for phosphorylation in corneodesmosin function.

Desquamation

After formation of the corneocytes, they continue to mature chemically[7] becoming larger, more rigid and hydrophobic[80] before undergoing desquamation. This process requires proteases of the Kallikrein family for cleavage of the corneocyte specific junctions: corneodesmosomes. Kallikreins are known to be regulated transcriptionally through hormone-dependent kinase signalling pathways[81,82] and also specifically thorough Akt phosphorylation in breast cancer cells lines[83]. This may indicate that kinase dependent signalling cascades are required for desquamation, although this has yet to be determined.

Kinases and barrier pathology

The essential role of kinases in epidermal barrier formation is evident from the severe pathologies which result from alterations in their function. Defects in RIPK4 kinase function, lack of the RIPK4 substrate IRF6 and mutations in the phospho-motif binding protein stratifin cause epidermal fusions[4,49–53]. These arise from an inability to form the periderm, an essential layer formed at the initiation of epidermal development, required to separate adjacent developing epidermal surfaces[84]. This illustrates the importance of kinase function in epidermal development.

LIMK1 kinase downregulation has been linked to the hyperproliferative disease psoriasis[48,72] and alterations in Akt kinase activity also cause severe hyperproliferative skin disorders. Whilst, AKT1 hyperactivation causes hyperplasia and hyperkeratosis in PTEN-deficient mice[85], AKT1 and AKT2-deficient mice lack a stratum corneum and die perinatally[27]. This indicates that not only is kinase activity important for epidermal homeostasis but the balance of this activity is essential.

A commonality to these pathologies a defective epidermal barrier, demonstrating the importance of kinases in the regulation of epidermal barrier formation and maintenance.

Conclusions

Kinase dependent signalling is important in a number of pathways that regulate formation of the epidermal barrier, emphasized by the pathologies that arise from defects in these pathways. This defective barrier contributes to further symptoms in these pathologies such as severe inflammatory symptoms associated with increased infiltration of external microbes and molecules. The kinase dependent control of processes that drive barrier maintenance may indicate the importance of treating the barrier dysfunction in common skin diseases such as eczema and psoriasis in preference to the immunosuppressant treatment of the inflammatory symptoms and further understanding these pathways will inform future treatments.

Author contribution statement

CR and RO wrote the review

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	Protein	RNA	Description	Barrier Related Functions
ADRBK1			adrenergic, beta, receptor kinase 1	None reported
AKT1			Akt1	Filaggrin processing, nuclear lamin degradation, nucleophagy[25,28,36,86]
CSNK1E			casein kinase 1, epsilon	None reported
CSNK2A1			casein kinase 2, alpha 1 polypeptide	None reported
CSNK2A2			casein kinase 2, alpha prime polypeptide	None reported
GAK			cyclin G associated kinase	Regulates cathepsin maturation, cathepsins are important in filaggrin processing; GAK knockout mice are perinatal lethal with severe skin barrier defect[87–89]
GRK5			G protein-coupled receptor kinase 5	Endothelial barrier[90]
GSK3B			glycogen synthase kinase 3 beta	Generally induces differentiation by degrading β-catenin[91,92]
HSPB8			heat shock 22kDa protein 8	Stress induced, fusion of autophagosome and lysosome, promotes autophagy[93,94]
MAP2K3			mitogen-activated protein kinase kinase 3	MEK3, mediated keratinocyte differentiation[95]
MAP3K8			mitogen-activated protein kinase kinase kinase 8	As above, Tpl2
MAP3K9			mitogen-activated protein kinase kinase kinase 9	None reported
MAPK13			mitogen-activated protein kinase 13	Induces p21 and epidermal terminal differentiation[40]
MAPK14			mitogen-activated protein kinase 14	Controls epidermal barrier function via phospholipase Cδ1[41]
MAPK3			mitogen-activated protein kinase 3	Controls epidermal differentiation via β-catenin[39]
PAK3			p21 (CDKN1A)-activated kinase 3	Controls actin and myosin during wound healing in *Drosophila melanogaster*[96]
PIM1			pim-1 oncogene	Increased in psoriatic skin; loss of Pim-1 exacerbates lung epithelium barrier disruption by house dust mites[97,98]
PINK1			PTEN induced putative kinase 1	Mitophagy in skin, protection from UV damage[99]
PLK3			polo-like kinase 3 (Drosophila)	Hyperosmotic stress induced Jun phosphorylation; induced by hypoxic stresses[100,101]
RPS6KA6			ribosomal protein S6 kinase, 90kDa, polypeptide 6	None reported/ target of mTOR
SRPK1			SFRS protein kinase 1	None reported
STK10			serine/threonine kinase 10	None reported
STK4			serine/threonine kinase 4	Hippo; Involved in growth advantage in epidermal stem cells; maturation of vascular barrier[102,103]
TAOK1			TAO kinase 1	None reported
WNK1			WNK lysine	None reported

			deficient protein kinase 1	
YSK4			yeast Sps1/Ste20-related kinase 4 (S. cerevisiae)	Tpl2; Knockout keratinocytes are invasive; in airway phosphorylated in response to pathogens[104,105]

Table 1 – Kinases in the granular layer of the epidermis. List of all genes with are identified by gene ontology as protein kinases that are expressed in the granular layer Protein; known to be expressed in the granular layer of the epidermis on the protein level, or displayed granular layer expression in the protein atlas (http://www.proteinatlas.org). RNA, expressed in the IFE II compartment of the mouse epidermis according to the single cell RNAseq analysis of Joost et al[21]**.** Description; Full gene name; Barrier related functions; known barrier related functions either in skin or other epithelia based on previous publications.

Figure 1

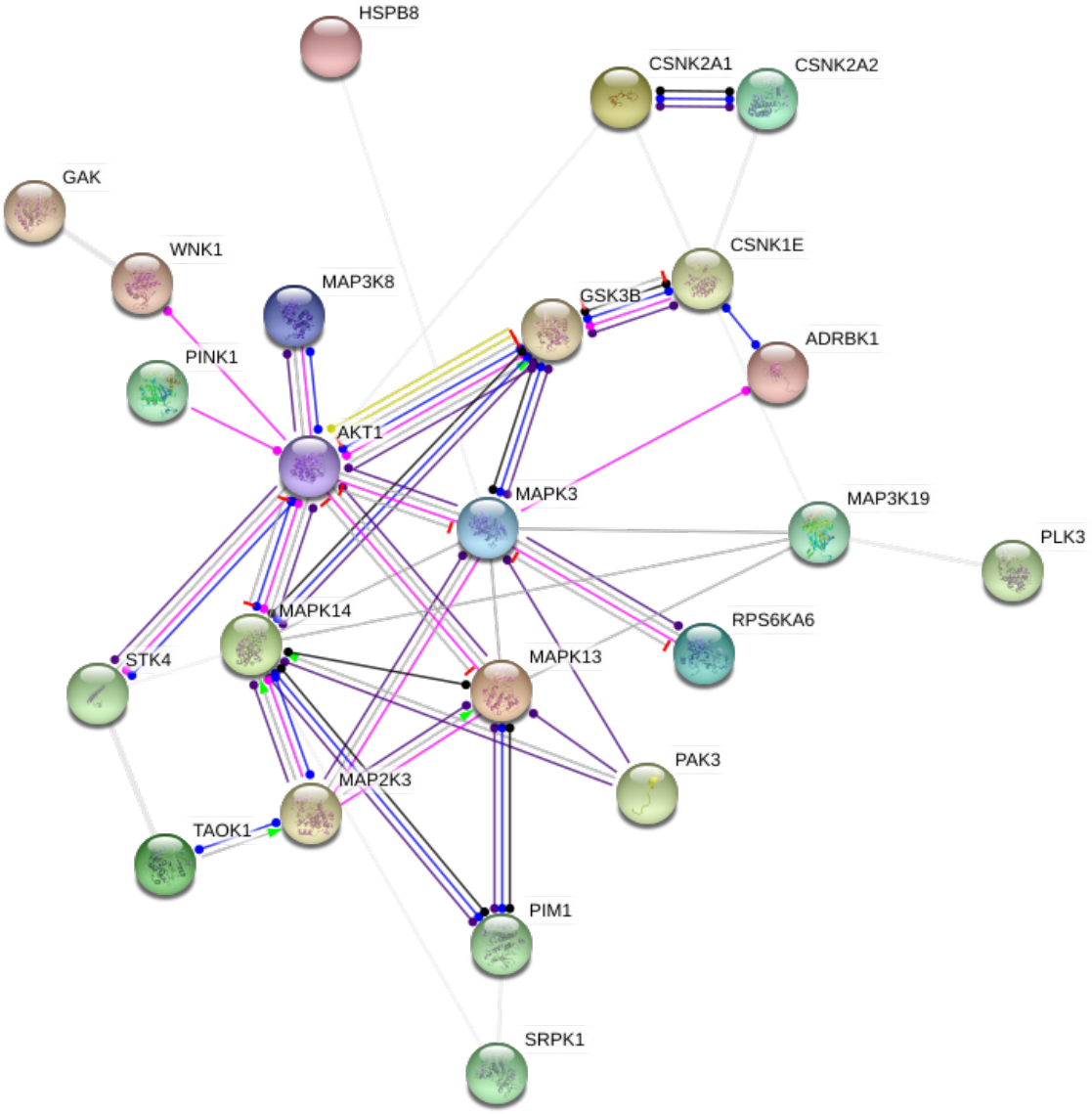

Figure 1 – Network analysis of functional interactions between proteins kinases expressed in the granular layer of the epidermis generated in STRING (https://string-db.org), form the lists of Kinases expressed in the granular layer from Table 1.