ORIGINAL ARTICLE

See related commentary on pg 742

Role of aPKC Isoforms and Their Binding Partners Par3 and Par6 in Epidermal Barrier Formation

Iris Helfrich[1], Annika Schmitz[1], Paola Zigrino[2], Christian Michels[1], Ingo Haase[1,2], André le Bivic[3], Michael Leitges[4] and Carien M. Niessen[1]

The skin water barrier, essential for terrestrial life, is formed by a multilayered stratifying epithelium, which shows a polarized distribution of both differentiation and intercellular junction markers. Recently, several reports showed the crucial importance of tight junctions for the *in vivo* water barrier function of the skin. In simple epithelial cells, intercellular junction formation is closely coupled to the establishment of polarity. However, if and how polarity proteins contribute to epidermal differentiation and junction formation is not yet known. Here, we have characterized the localization and isoform expression of the polarity protein atypical PKC (aPKC) and its binding partners Par3 and Par6 in epidermis and primary keratinocytes of mice. Their distribution is only partially overlapping in the granular layer, the site of functional tight junctions, suggesting that next to a common Par3/Par6/aPKC function they also may have functions independent of each other. Both aPKCζ and aPKCι/λ, are expressed in the epidermis but only aPKCι/λ showed a strong enrichment in the junctions, suggesting that this aPKC isoform is important for epidermal tight junction function. Indeed, inhibition of aPKC function showed that endogenous aPKC is crucial for *in vitro* barrier function and this required the presence of both the Par3 and Par6 binding sites.

Journal of Investigative Dermatology (2007) **127,** 782–791. doi:10.1038/sj.jid.5700621; published online 16 November 2006

INTRODUCTION

The outer layer of the skin, the epidermis, forms the most direct contact of the organism with the outside world. As such, it supplies the organism with a barrier against detrimental influences from the outside, such as adverse chemicals or pathogens. It also provides a barrier to the inside by preventing the unnecessary loss of water and small nutrients, thus protecting the organism from dehydration. The epidermis is a stratifying, multilayered epithelium, in which each layer has obtained specialized properties that ultimately contribute to the formation of such a two-way barrier. Importantly, the barrier is not static but requires continuous renewal to maintain proper protection. This is achieved by a tightly controlled balance between proliferation and differentiation. The basal layer contains the proliferating cells from which the differentiated suprabasal layers are derived (Watt, 2001; Fuchs and Raghavan, 2002). In many skin diseases disturbances in the barrier are often associated with alterations in this balance and with inflammation, although it is unclear what is cause and consequence.

Cell polarity is a fundamental property of cells and is crucial for the establishment of anterior–posterior embryonal axis set-up, morphogenesis, differentiation, and cell identity by regulating asymmetric cell division, directed migration, axonal polarity, control of proliferation, and apical-basolateral polarity in simple epithelia (Nelson, 2003). The atypical PKC (aPKC) is a key player in the generation of almost all forms of polarity (Henrique and Schweisguth, 2003). aPKC forms a complex with Par6 and Par3, proteins initially identified in the set-up of anterior/posterior polarity in *Caenorhabditis elegans* (Kemphues *et al.*, 1988). This complex is essential for the formation of tight junctions in simple epithelial cells (Ohno, 2001; Macara, 2004).

Both Par6 and Par3 belong to the PDZ family of adaptor proteins. Par6 appears to be necessary for activation of aPKC, by connecting aPKC to the GTP bound form of the small GTPases Rac and Cdc42 (Joberty *et al.*, 2000; Lin *et al.*, 2000). This results in a conformational change in Par6, allowing phosphorylation and subsequent activation of aPKC (Garrard *et al.*, 2003). Par3 is thought to be a scaffolding protein necessary for the proper recruitment of the Par6/aPKC to sites where its activity is required. For example, Par3 binds directly to junctional adhesion molecules (JAMs), and this recruits it to the tight junctions in simple epithelial cells (Ebnet *et al.*, 2001). In addition, Par6 may also allow for the placement of the complex to membranes by interaction with

[1]*Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany;* [2]*Department of Dermatology, University of Cologne, Cologne, Germany;* [3]*Laboratoire de Neurogenese et Morphogenese au cours du Developpement et chez l'Adulte, Unite Mixte Recherche 6156 Centre National de la Recherche Scientifique, Universite de la Mediterranee, Marseille, France and* [4]*Department of Nephrology, Hannover Medical School, Hannover, Germany*

Correspondence: Dr Carien M. Niessen, Center For Molecular Medicine Cologne, University of Cologne, Joseph Stelzmannstrasse 9, 50931 Cologne, Germany. E-mail: carien.niessen@uni-koeln.de

Abbreviations: aPKC, atypical PKC; Crb, Crumbs; eGFP, enhanced green fluorescent protein; JAM, junctional adhesion molecule; RT-PCR, reverse transcription PCR; TER, transepithelial resistance

Received 10 March 2006; accepted 19 September 2006; published online 16 November 2006

Pals1, which connects it to the Crumbs (Crb) transmembrane proteins (Hurd *et al.*, 2003), or by direct interaction with Crb3 (Lemmers *et al.*, 2004).

Next to its role in polarity aPKC may also play a role in inflammatory responses by regulating NF-κB (Moscat *et al.*, 2006) and in metabolic regulation of tissues because insulin receptor activation stimulates aPKC kinase activity (Farese, 2002).

Two types of aPKC exist, aPKCzeta (aPKCζ) and aPKCiota/lambda (aPKCι/λ), which are highly related but encoded by separate genes (Suzuki *et al.*, 2003). At present it is unclear if both aPKCs are functionally redundant and thus can mediate the various aPKC functions or if different roles are performed by either one of the aPKCs.

The epidermis is not a classical polarized epithelium, such as intestinal epithelium, which has a clear apical and basolateral membrane domain separated by the apical junctional complex. Nevertheless, the epidermis shows polarization in a broader sense, such that specific layers of the epidermis not only express a unique set of differentiation but also junctional markers (Watt, 2001; Getsios *et al.*, 2004). Recently, it was shown that asymmetric cell division does occur in the epidermis, thus contributing to stem cell maintenance and the balance between basal proliferation and differentiation (Lechler and Fuchs, 2005). In cells undergoing asymmetric cell division, aPKC is targeted to the apical membrane. However, presently it is unknown how polarity is achieved in stratifying, multilayered epithelia and if central players in other forms of polarity, like for example, aPKC and its binding partners Par3 and Par6, contribute to the formation, maintenance, and function of stratifying epithelia.

To obtain further insight into the role of the aPKC/Par3/Par6 complex in stratifying epithelia we have characterized its tissue distribution and aPKC isoform expression in mouse epidermis under normal homeostasis and wound healing. In addition, we have characterized their *in vitro* expression pattern in mouse keratinocytes under undifferentiated and differentiated conditions. Finally we show that *in vitro* inhibition of aPKC in keratinocytes results in impaired barrier formation.

RESULTS

Very little is known about the role of the aPKC/Par3/Par6 polarity complex in multilayered epithelia. To characterize this complex in squamous differentiating epithelia the distribution of aPKC, Par3, and Par6 was examined in newborn mouse skin. The predominant localization of aPKC was found in basal keratinocyte at the dermal/epidermal junction, using an antibody that recognizes both isoforms of aPKC, aPKCζ, and aPKCι/λ (Figure 1a). This antibody also showed a faint positive signal in the nuclei of the basal cells. Using an antibody that potentially recognizes all three Par3 isoforms (180, 150, and 100 kDa, which are generated by alternative splicing), Par3 was mostly found in the cytoplasm of basal cells, whereas in suprabasal layers it was predominantly localized to sites of cell–cell contact (Figure 1a, (Tunggal *et al.*, 2005). A somewhat different pattern was observed using a Par3 antiserum that recognizes predominantly the smallest, 100 kDa, isoform (Figure 1a).

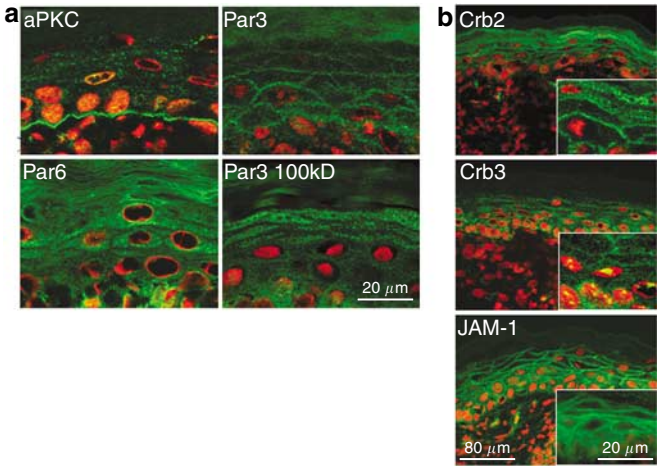

Figure 1. Localization of polarity proteins in the skin of newborn mice. (**a**) Localization of components of the Par3/Par6/aPKC complex. (**b**) Localization of the transmembrane proteins Crb2, Crb3, and JAM-1. Cryosections of newborn skin were fixed with 4% paraformaldehyde, incubated using the appropriate antibodies and stained with Alexa 488-conjugated secondary antibodies (green). Nuclei were counterstained with propidium iodide (red). Insets in (**b**) show higher magnification.

This showed positive staining in the granular layer, where it seemed to be mostly localized in cytoplasmic granules and, although weakly, on the cell membrane. Interestingly, antibodies to Par6 showed a relatively similar distribution as the Par3 100 kDa isoform. It was predominantly localized to the granular layer, where it was enriched in the cytoplasm.

In simple epithelial cells the Par3/Par6/aPKC complex is localized to tight junctions, and it can be recruited to these structures either by the interaction of Par3 with JAM-1 (Ebnet *et al.*, 2001), or by binding of Par6 to the Crb complex (Hurd *et al.*, 2003; Lemmers *et al.*, 2004). We therefore also examined the distribution of the transmembrane molecules JAM-1 and Crb2 and Crb3. Both JAM-1 and Crb2 are enriched on the cell membrane in suprabasal layers (Figure 1b). In basal cells JAM-1 appears mostly diffuse whereas Crb2 staining is mostly negative. Crb3 showed a strong diffuse staining in basal cells with some enrichment at the basal side of these cells. In the stratum spinosum, Crb3 localization was predominantly perinuclear whereas in the stratum granulosum faint membrane localization could be observed (Figure 1b).

Regulation of expression by calcium-mediated differentiation

The differential distribution observed for the Par3/Par6/aPKC proteins suggested that protein expression might be affected by differentiation of the keratinocytes. To examine if differentiation indeed affects protein expression, mouse keratinocytes were switched from low calcium containing medium (<50 μM) to high Ca^{2+} (1.8 mM) containing medium and incubated for different time points. High calcium does not only induce differentiation of keratinocytes but also permits the formation of intercellular junctions. Keratinocytes express all three Par3 isoforms (Lin *et al.*, 2000), albeit it that the 180 and 100 kDa are more predominant than the 150 kDa

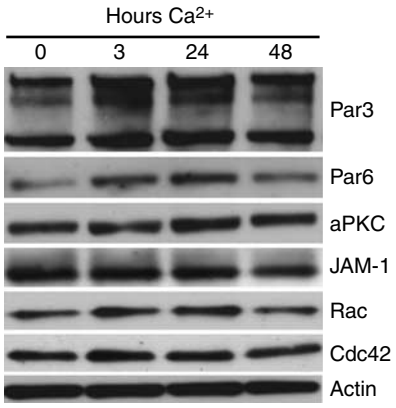

Figure 2. Expression of Par3/Par6/aPKC complex components and associated proteins in keratinocytes upon differentiation. Primary mouse keratinocytes were differentiated with 1.8 mM Ca^{2+} for the indicated time points after which cells were lysed. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted for the indicated proteins.

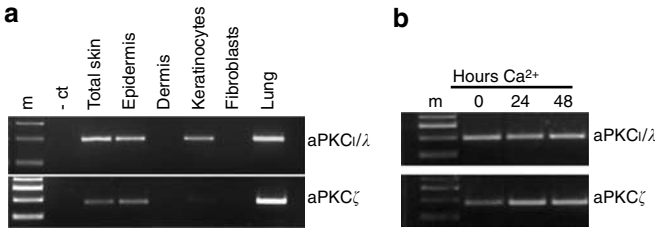

Figure 3. RNA expression of aPKC isotypes in skin and primary keratinocytes. (**a**) RT-PCR analysis for aPKCζ and aPKCι/λ in murine skin or cells. Lung RNA was used as a positive control. (**b**) RT-PCR analysis for aPKCζ and aPKCι/λ in non-differentiated (0 hour) and differentiated keratinocytes. Cells were differentiated with 1.8 mM Ca^{2+} for the indicated times. PCR was carried out using primers specific for either aPKCζ or aPKCι/λ. -ct: without reverse transcriptase.

isoform (Figure 2). Both Par3 and aPKC are already present in substantial amounts in undifferentiated keratinocytes (Figure 2, 0 hour Ca^{2+}), even though junctions are not formed under these conditions. Importantly, switching to high levels of Ca^{2+} did not substantially upregulate overall protein levels, indicating that regulation of Par3 and aPKC expression is largely independent of junction formation and/or differentiation (Figure 2). However, differentiation may affect relative levels of Par3 isoform expression as the 150 kDa isoform is upregulated after 3 and 24 hours of high calcium treatment (Figure 2). Differentiation did not significantly alter expression levels of the small GTPases Cdc42 and Rac, which can both activate the Par3/Par6/aPKC complex (Joberty *et al.*, 2000; Lin *et al.*, 2000), and the Par3-binding molecule JAM-1 (Figure 2).

The situation was different for Par6. Although some Par6 was present in non-differentiated keratinocytes, expression was increased after 3 and 24 hours of Ca^{2+}-induced differentiation (Figure 2). Surprisingly, after 48 hours of calcium treatment Par6 levels decreased to a level comparable to that of undifferentiated keratinocytes.

Both aPKCζ and aPKCι/λ are expressed in skin

At present it is unclear if the two isotypes of aPKC, aPKCζ, and aPKCι/λ, have separate or overlapping function. To examine which aPKCs are expressed in skin, reverse transcription PCR (RT-PCR) was performed. Both aPKCζ and aPKCι/λ RNA were found in total skin (Figure 3a). Upon separation of the epidermis and dermis, expression of both forms was found in the epidermis whereas only a very faint signal could be detected in dermis. A similar result was found *in vitro* using RNA isolated from either keratinocytes or fibroblasts. Keratinocytes express both aPKC isotypes whereas fibroblasts showed little to no signal (Figure 3a). We next examined if RNA expression of the two isotypes is sensitive to Ca^{2+}-induced differentiation. For both aPKC types no discernable regulation of expression levels was observed (Figure 3b).

To examine if there are differences in protein expression of the two different aPKCs, antibodies were generated using antigens that are specific for either aPKCζ or aPKCι/λ. Western blot analysis revealed that the aPKCι/λ antiserum specifically recognized a protein at the expected molecular weight of 70 kDa of aPKCι/λ (Figure 4a), in addition to a smaller molecular weight protein of around 50 kDa, the identity of which is not known. Similar to the RNA data, Ca^{2+}-induced differentiation did not affect the steady-state levels of aPKCι/λ. The anti-aPKCζ antibodies also recognized a protein of the expected molecular weight, the levels of which were slightly upregulated upon differentiation (Figure 4a).

Using the specific antibodies we examined the distribution of aPKCζ and aPKCι/λ in the epidermis. An intense staining was seen for aPKCι/λ in the cytoplasm of basal cells. These cells also showed faint nuclear localization. However, in the suprabasal cells localization at the membrane was the predominant pattern for aPKCι/λ (Figure 4b). Interestingly, in the granular layer a highly concentrated staining was observed at lateral intercellular borders, which is reminiscent of the distribution of several tight junctional markers in the skin (Tunggal *et al.*, 2005). The pattern was different for aPKCζ. A strong cytosolic signal, in addition to weak nuclear staining, was observed in basal cells, whereas the suprabasal layers were only weakly positive (Figure 4b).

Localization of the Par3/par6/aPKC complex in differentiated keratinocytes

The distribution of aPKCι/λ and Par3 on cell membranes of the suprabasal epidermal layers suggested that they are incorporated into intercellular junctions. To examine the time frame after which the Par3/Par6/aPKC complex is recruited to intercellular junctions, mouse keratinocytes were differentiated with medium containing high Ca^{2+} concentration (1.8 mM) for different time points. Staining was predominantly cytosolic under non-differentiating conditions or after 3 hours of calcium-induced differentiation (results not shown), a time point at which a marker for tight junctions (occludin, Figure 5b) is already recruited to the membrane and found at sites of initial contacts. After 24 hours of Ca^{2+}-induced

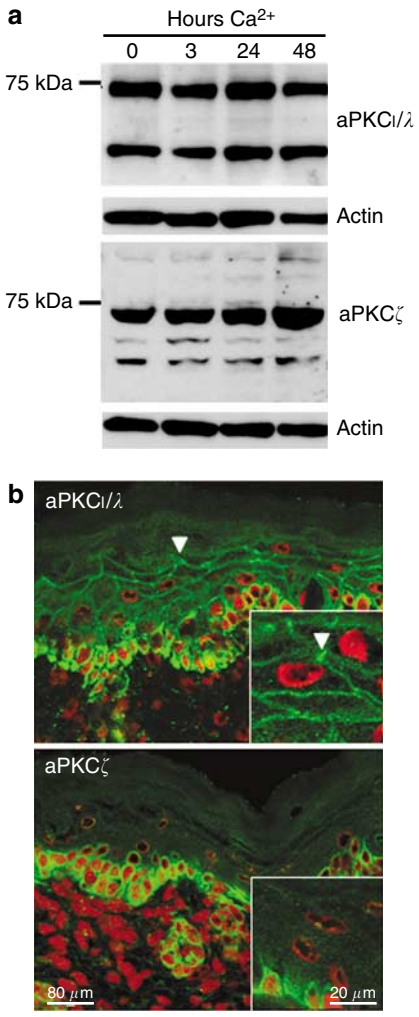

Figure 4. Expression and localization of aPKC isotypes in newborn skin and primary keratinocytes. (**a**) aPKCζ or aPKCι/λ protein expression in non-differentiated keratinocytes (0 h) and keratinocytes differentiated with Ca^{2+} for the indicated time points. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with antibodies specific for aPKC ζ or aPKCι/λ. (**b**) Expression of aPKCι/λ or aPKCζ in newborn mouse skin. Cryosections were stained with the specific aPKCζ or aPKCι/λ antibodies using Alexa 488-labeled secondary antibodies. Nuclei were counterstained with propidium iodide. The arrowhead points out aPKCζ expression at the membranes in the granular layer. Insets show areas with higher magnification.

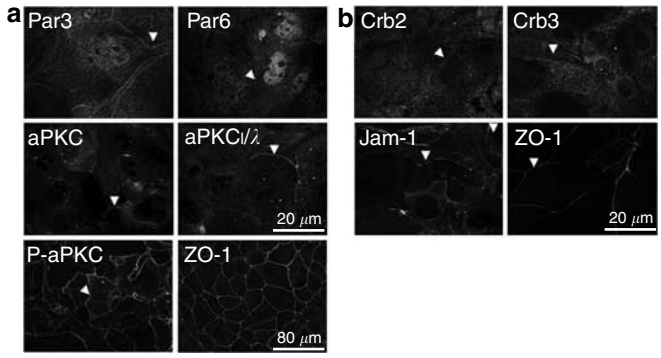

Figure 5. Localization and recruitment of polarity proteins to junctions in differentiated primary keratinocytes. Immunohistochemistry for (**a**) Par3, Par6, aPKC, aPKCι/λ, phospho-aPKC (P-aPKC), and ZO-1 in primary keratinocytes after differentiation with Ca^{2+} for 48 hours and (**b**) Crb2, Crb3, JAM-1, and ZO-1 in primary keratinocytes 72 hours after Ca^{2+} induction. Cells were fixed with ice-cold methanol for 10 minutes at 4°C, stained with the appropriate antibodies and secondary antibodies labeled with Alexa 488. Arrowheads: expression at cell–cell contacts.

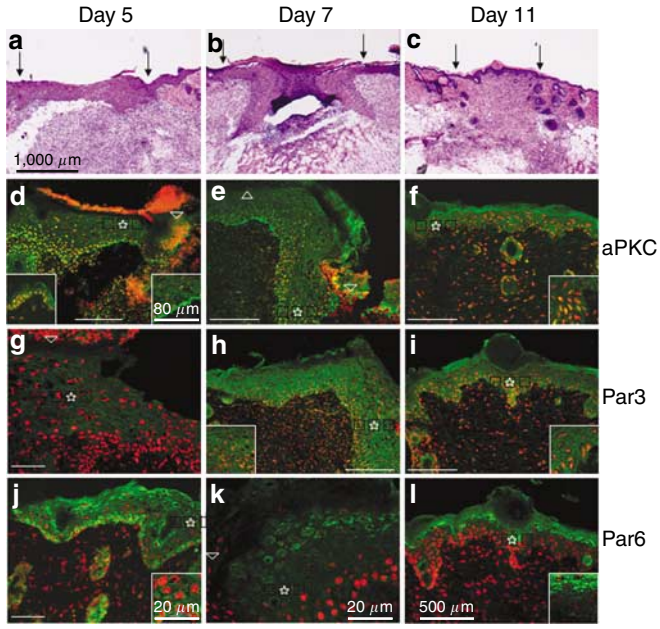

Figure 6. Localization of Par3/Par6/aPKC complex components at different stages during wound healing. (**a–c**) Wounds stained for hematoxylin/eosin, (**d–f**) aPKC, (**g–i**) Par3 and (**j–l**) Par6 at days 5, 7, and 11 after wounding. Cryosections were stained with the specific antibodies using Alexa 488-labeled secondary antibodies. Nuclei were counterstained with propidium iodide. Left inset in (**d**) shows staining for aPKC in normal adult skin. Other insets are higher magnification. Bar in inset of (**d**) also applies to insets in (**f**, **h**, **i**, and **l**). Δ and ↓: wound edge, ☆: new epithelial sheet, ∇: wound scab.

differentiation, occludin (Figure 7b), and ZO-1(not shown) were predominantly found at junctional structures. At this time, in some cells, Par3 was recruited to these ZO-1 containing junctions whereas no Par6 or aPKC was found at the membrane (results not shown). This suggests that Par3 is recruited to junctions before the other components. Indeed, Par3 was found at the membrane in all cells after 48 hours (Figure 5a), where it colocalized with ZO-1. Nevertheless, cytosolic staining was still observed, indicating that not all Par3 is recruited to the junctions. Some Par6 and aPKC was recruited to the membrane in some cells but only after prolonged differentiation (48–72 hours) (Figure 5a), even though robust tight junctions are formed at those points, as judged by ZO-1 (Figure 5) and occludin staining (Figure 7b). Membrane association was more obvious when we used the aPKCι/λ-specific antibody in comparison to staining with the aPKC antibody that recognizes both isotypes (Figure 5a). Interestingly, strong membrane staining was seen when using an antibody that recognizes phosphorylated

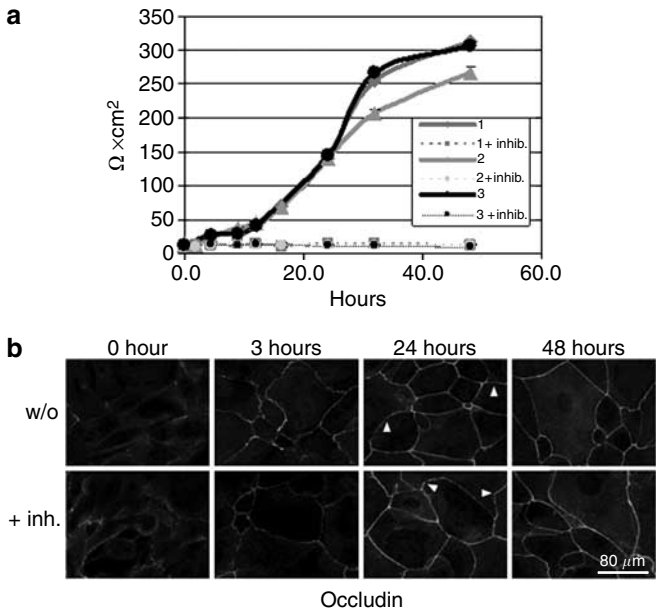

Figure 7. Inhibition of aPKC activity in primary keratinocytes. (**a**) TER measurement of three independently isolated primary keratinocyte cultures (passage 5–10). Primary mouse keratinocytes were differentiated with 1.8 mM Ca^{2+} for the indicated time points in the absence or presence of 100 μM aPKCζ pseudosubstrate inhibitor. All measurements were carried out in triplicates. (**b**) Immunohistochemistry for occludin in primary mouse keratinocytes differentiated with 1.8 mM Ca^{2+} for the indicated time points in the presence or absence of 100 μM of aPKCζ pseudosubstrate inhibitor. Secondary antibodies were labeled with Alexa488. Nuclei were stained using propidium iodide. White arrowheads in 24 hours: sites where occludin is not completely continuous at the membrane and shows a more zig-zag pattern.

forms of both aPKCs, indicative of the active form (Figure 5a). Most staining for Par6 remained cytoplasmic after 48 hours (Figure 5a).

A similar result was found for both Crb2 and Crb3. Although weak membrane association was observed for both Crb2 and Crb3, most of their localization was cytoplasmic 48 or 72 hours after the calcium switch (Figure 5b). In contrast, JAM-1 was mostly found at the membrane (Figure 5b), where it colocalized with ZO-1.

Distribution in wound healing

As polarity is altered or locally lost during wound healing, we were interested in assessing the distribution of the Par3/Par6/aPKC complex during different stages of wounding. In adult murine skin the distribution of aPKC, using an antibody that recognizes both isoforms, is mostly cytosolic and nuclear (left inset in Figure 6d) but after 5 days, aPKC accumulated at the basal side of the basal cells in the hyperproliferative area (Figure 6d, right inset), similar to its distribution in newborn skin (Figure 1a). This epidermal–dermal distribution in basal cells was observed in the wound margins but disappeared in basal cells of the newly re-epithelialized skin. In addition, a strong nuclear staining was also observed. A similar staining pattern was observed in day 7 old wounds (Figure 6e). However, after 11 days the basal localization of aPKC in basal cells had completely disappeared coinciding with reduced hyperproliferation (Figure 6f). Instead, its distribution resembled that seen in adult murine skin.

Par3 is found at the cell membrane in suprabasal layers (Figure 6g), similar to its localization in newborn skin. This distribution did not significantly change upon wounding (Figure 6h and i). Par3 was still associated with the cell membrane, both in the wound margin and in the newly formed epidermis (Figure 6) after 5 and 7 days of wounding.

In contrast, Par6 was redistributed after 5 days of wounding. The vesicle like staining in the granular layer found in normal skin (Figure 1) was largely absent and Par6 appeared to have a more cytosolic localization in the most upper layers (Figure 6j). In addition, an intense cytoplasmic staining was found in the basal layer both of the hyperproliferative domain and the new epithelial sheet. This basal Par6 localization was largely gone after 7 days of wounding. At this point the vesicle like staining in the granular layer was again the most predominant staining (Figure 6k). This pattern remained unaltered at day 11 after wounding, when re-epithelialization was complete (Figure 6l).

aPKC contributes to *in vitro* barrier formation

To test if aPKC is important for epidermal barrier function, a non-invasive *in vitro* barrier formation assay was established for primary keratinocytes, which was adapted from trans-epithelial resistance (TER) measurements, often used for simple epithelial cells (Matter and Balda, 2003). This assay indirectly assesses tight junction formation and function. Keratinocytes are switched to high calcium medium (1.8 mM) to induce differentiation and the formation of intercellular junctions and TER is measured over time. An increase in TER indicates the formation of an epithelial barrier. Indeed upon induction of differentiation, primary mouse keratinocytes increased their trans epithelial resistance over time with high TER at 48 hours of Ca^{2+} treatment (Figure 7a). This increase coincides with translocation of occludin (Figure 7b) ZO-1 (Figure 6) and claudin-1 (not shown) to sites of cell–cell contacts. At 24 hours of Ca^{2+} treatment, occludin was found in junctional structures at the membrane (Figure 7b), corresponding with a TER of around 150 Ohm × cm^2 (Figure 7a). After 48 hours of Ca^{2+} treatment occludin is more continuous at the membrane of differentiated, flattened cells and this correlated with an even higher TER ($+/-300\,\Omega \times cm^2$). Thus, *in vitro* keratinocytes are able to form a functional barrier, the formation of which can be followed over time.

To examine the role of aPKC in *in vitro* epidermal barrier formation, keratinocytes were differentiated in the presence of an aPKC-specific myristoylated pseudo-substrate inhibitor, which blocks both aPKCζ and aPKCι/λ activity. Whereas TER increased over time in control keratinocytes, little to no increase in TER was observed in the presence of the aPKC inhibitor, using three independently isolated keratinoctye cultures (Figure 7a). Surprisingly, no discernable difference was osberved in the recruitment of occludin (Figure 7b), ZO-1, and claudin-1 (results not shown) to intercellular junctions in the presence of the inhibitor, either in extent or in its timing.

The kinase activity and Par3 and Par6 binding domains of aPKC are necessary for barrier formation

If inhibition of aPKC results in lower trans epithelial resistance in differentiated keratinocytes than increasing aPKC function should enhance and accelerate TER build up. Keratinocytes were transfected with enhanced green fluorescent protein (eGFP)-tagged wt aPKCζ and expression was detected with antibodies to either GFP or aPKC using western blot analysis after 48 hours of Ca^{2+}-induced differentiation (Figure 8c). Indeed, overexpression of either wt aPKCζ (Figure 8a) or wt aPKCι/λ (not shown) increased TER and accelerated the formation of a barrier when compared to control keratinocytes.

To assess if kinase activity is indeed important for the role of aPKC in *in vitro* epidermal barrier formation assays, a kinase dead mutant (aPKCζK281M) was expressed in keratinocytes (Figure 8c). This abrogated the increase in TER observed in control keratinocytes upon differentiation (Figure 8a).

Next we wanted to assess if other domains are essential for aPKC function in keratinocyte barrier formation. Overexpression of the aPKCζ kinase domain alone, which can bind Par3, also inhibited barrier formation upon keratinocyte differentiation, as measured by TER. The N-terminal region, containing both the Par6 binding domain and the pseudosubstrate domain, also blocked to a large extent the increase in trans epithelial resistance (Figure 8b).

None of the expressed proteins significantly affected endogenous aPKC or Par3 expression levels either under low or high calcium conditions (Figure 8c and d). In addition, using the aPKCι/λ antibodies no cross reactivity was found with the ectopically expressed aPKCζ proteins (Figure 8c), showing the specificity of this antibody for the aPKCι/ζ isotype.

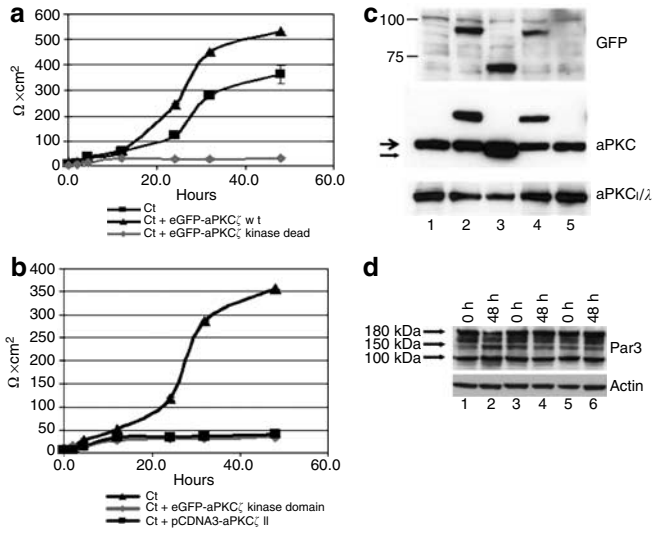

Figure 8. Functional requirement of aPKC in keratinocyte barrier formation. TER measurement of (**a**) control (ct) and transfected primary mouse keratinocytes using the kinase dead mutant eGFP-aPKCζ(K281M), the eGFP-aPKCζwt construct, and (**b**) control (ct), eGFP-aPKCζkinase domain and myc-aPKCζIIpCDNA3.1 transfected keratinocytes. After transfection and plating on filters primary keratinocytes were differentiated with 1.8 mM Ca^{2+} for the indicated time points. All measurements were made in duplicates. (**c**) Detection of eGFP, aPKC, and aPKCι/λ in primary keratinocytes transfected with different aPKCζ mutants and subsequent differentiation with 1.8 mM Ca^{2+} for 48 hours. (1) untransfected, (2) eGFP-aPKCζwt, (3) eGFP-PKCζkinase domain, (4) eGFP-aPKCζ(K281M) kinase dead, (5) pcDNA3.1myc-aPKCζII. (**d**) Expression of Par3 and aPKC in control (1, 2) and keratinocytes transfected with eGFP-aPKCζwt (3, 4), eGFP-aPKCζkinase domain (5, 6) undifferentiated (0 hour) or differentiated with 1.8 mM Ca^{2+} for 48 hours. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted for Par3. Actin was used as a loading control. Open arrow: endogenous aPKC, closed arrow: eGFP-aPKCζkinase domain only.

DISCUSSION

The epidermis is not considered a classical polarized epithelial tissue, such as simple epithelia. However, it does show an overall polarization with each layer having specific differentiation and junctional markers. How stratifying epithelia establish polarity and if the classical players, the polarity complex proteins, play a role in the polarization and differentiation of these tissues are at present not known. In this study, we have characterized the localization and distribution of the Par3/Par6/aPKC polarity complex and of transmembrane proteins that have the potential to recruit the complex to junctions in murine skin. Furthermore, we have shown that endogenous aPKC activity is important for *in vitro* barrier formation and thus functional junction assembly.

The partially overlapping distribution of the individual components in newborn skin and during wound healing suggests that Par3, Par6, and aPKC are not only incorporated in a tertiary complex in the skin but are also localized independently of each other. Thus, these proteins may have shared but also individual functions in skin. Indeed, distinct functions have been observed for these proteins in other cell systems. For example, Par6 and aPKC cooperate in astrocyte migration (Etienne-Manneville and Hall, 2001) and simple epithelial apico-basolateral membrane determination (Plant *et al.*, 2003; Yamanaka *et al.*, 2003), both processes that do not seem to require Par3 function. In addition, regulation of NFκB by aPKC appears to be independent of Par3 and Par6 (Moscat *et al.*, 2006). Similarly, our data suggest that Par6 and aPKC might cooperate during wound healing because they both show dynamic localization whereas Par3 is unaffected by wound repair (Figure 7).

Another layer of complexity to the formation and function of different Par3/Par6/aPKC complexes in cells is formed by the existence of different isoforms of all three proteins. Par6 consists of three members that are encoded by different genes, Par6A, B, and C. All three isoforms can interact with aPKC and GTP-bound Rac, or Cdc42. However, differential effects on tight junction formation have been observed upon overexpression of either Par6A and Par6B and this may be due to a differential interaction with Pals1, which forms part of the Crb/Patj complex (Gao and Macara, 2004). Three Par3 variants are generated by alternative splicing, the 180, 150, and 100 kDa forms, the latter of which lacks the aPKC binding domain (Lin *et al.*, 2000). Keratinocytes express all three isoforms (Figure 2), albeit that the 180 and 100 kDa variants are more strongly expressed. Interestingly, we found

a differential distribution in the different layers between an antibody that can recognize all three isoforms (Figure 1a) and an antibody recognizing predominantly the 100 kDa isoform. Thus, although these proteins can form a ternary complex, they may also function independently of each other, depending on the isotype expressed and on different compositions with other interaction partners (Moscat *et al.*, 2006). The existence of different isoforms and different complexes in the epidermis and cultured keratinocytes will therefore require further analysis.

The aPKC family has two isoforms: aPKCζ and aPKCι/λ, which are both expressed in epidermis and in cultured keratinocytes (Figure 4). At present it is not clear if both aPKCs are functional significant for polarity establishment and maintenance. Both isoforms can interact with Par3 and Par6, and are able to rescue tight junction formation. Moreover, dominant negative aPKCζ and aPKCι/λ are both able to interfere with polarity and junction formation (Suzuki *et al.*, 2001; Mertens *et al.*, 2005). Indeed, we also found that overexpression of either isoform can enhance barrier formation in keratinocytes. However, aPKCζ total knockout mice are viable (Leitges *et al.*, 2001) whereas aPKCι/λ inactivation results in an early embryonic lethal phenotype (Michael Leitges, unpublished results, Soloff *et al.*, 2004). Interestingly, staining with isotype-specific antibodies showed close association of aPKCι/λ but not aPKCζ with the tight junctions in the granular layer of the epidermis (Figure 4b), suggesting that this aPKC isotype may be the most significant for endogenous epidermal tight junction formation.

An antibody directed against the C-terminus of aPKC, which recognizes both isotypes of aPKC recognized the epidermal–dermal junction in basal cells (Figure 1, Tunggal *et al.*, 2005). This enrichment was not observed with both specific aPKC antibodies. The latter are raised against the regulatory N-terminus and the difference may therefore be due to masking of certain epitopes. Nuclear staining was also observed for aPKC in the epidermis, as has been described previously (Perander *et al.*, 2001; White *et al.*, 2002), where it may regulate downstream targets in mitogenic or metabolic pathways.

How do Par3 and aPKC and, to a much lesser extent, Par6 get recruited to sites of cell contact within the epidermis? A likely candidate is JAM-1, a binding partner for Par3 (Ebnet *et al.*, 2001), as its localization both *in vivo* and *in vitro* was quite similar to that of Par3. aPKC may then be recruited by direct binding to Par3. Other candidates are Crb2 and Crb3, which can recruit Par6 directly or by associating with the Par6 binding molecule Pals (Hurd *et al.*, 2003; Lemmers *et al.*, 2004). In simple epithelial Crb3 has been implicated in tight junction formation. This may be different in newborn murine epidermis as Crb3 localization is mostly cytoplasmic, with some enrichment at membrane in the granular layer. Comparatively, Crb2 is strongly enriched at sites of cell–cell contact in the granular layer, and may be more important for proper tight junction formation in the epidermis and recruitment of Par6 to the cell membrane.

To measure *in vitro* barrier function over time in primary keratinocytes we adapted an assay often used for simple epithelial cells, which measures TER. To our knowledge this is the first non-invasive assay for primary keratinocytes in which *in vitro* differentiation of keratinocytes can be directly coupled to barrier formation. Using this assay as a read-out, endogenous aPKC activity was indeed found to be crucial for barrier build up in primary mouse keratinocytes (Figures 7 and 8). Therefore, similar to simple epithelia (Suzuki *et al.*, 2001), aPKC is also crucial for the formation of functional tight junctions in stratifying epithelia. A similar result was recently shown for T-lymphoma invasion and metastasis (TIAM)-1, an exchange factor for the small GTPase Rac (Mertens *et al.*, 2005). Nevertheless, aPKC activity in keratinocytes did not appear to be crucial for the recruitment of tight junctional markers to sites of cell–cell contact (Figure 7b). This is in agreement with the *in vivo* situation, in which both ZO-1, claudin-1, but also phospho-aPKC, are enriched at the membrane already in the spinous layers, where there are no functional tight junctions (Furuse *et al.*, 2002; Tunggal *et al.*, 2005). This situation may thus be analogous to simple epithelia where aPKC was found to be one of the last components recruited to junctions and important for the transition of spot-like junctions to belt-like junctions (Suzuki *et al.*, 2001).

How than does aPKC regulate the formation of functional tight junctions? One possibility is that it may regulate the formation of functional tight junctional strands by claudins and ZO-1 (Umeda *et al.*, 2006), by direct or indirect phosphorylation of tight junctional components by aPKC. It has already been shown that phosphorylation of tight junctional components can regulate permeability (Yamauchi et al., 2004). In the epidermis such an activation step must only occur in the stratum granulosum of the epidermis and must be regulated by as yet unknown mechanisms as active aPKC is already recruited to the membrane in lower layers (Tunggal *et al.*, 2005).

Overexpression of the N-terminal domain of aPKCζ interfered with barrier formation (Figure 7b), similar to what was found for the N-terminal variant of aPKCζ in simple epithelial cells (Parkinson *et al.*, 2004). Next to the pseudosubstrate domain, this protein also contains the Par6 binding region. As such, it could inhibit both endogenous aPKCζ and aPKCι/λ activity by either inhibiting their kinase activity or by recruiting endogenous Par6 away from the complexes necessary for tight junction formation.

Surprisingly, the C-terminus containing only the kinase domain and the Par3 binding region also interfered with keratinocyte barrier formation. A similar aPKCζ isoform, aPKMζ, exists *in vivo*, owing to an alternative brain-specific promotor located in an intron (Marshall *et al.*, 2000). This protein has constitutive kinase activity owing to the lack of the pseudosubstrate domain and can be recruited to the membrane via binding to Par3, potentially making it a dominant active form of aPKC. However, our observation suggests that regulation of aPKC kinase activity is necessary for tight junction formation. In addition, aPKC may also

require interactions with other proteins next to Par3, such as for example Par6, for functional activity.

Overall, our results show that the polarity Par3/Par6/aPKC complex components have distinct localization patterns, both *in vivo* and *in vitro*. In addition to a possible common function, these results suggest that they may also have distinct roles in epidermal morphogenesis and homeostasis. We also find that aPKC function is essential for tight junction formation and barrier formation in keratinocytes and this may require its interaction with Par3 and Par6.

MATERIALS AND METHODS

Keratinocyte isolation, culture, and transfection

Primary keratinocytes were isolated from the skin of newborn mice as described (Pasparakis *et al.*, 2002) and cultured on collagen type-1 (Biochrom, Berlin, Germany) coated dishes in F12, adenine, DMEM (FAD) medium containing 50 μM Ca^{2+} in the absence or presence of mitomycin C-treated 3T3 fibroblasts (4 μg/ml) as feeders. For induction of differentiation and cell contact formation, keratinocytes were transferred to FAD medium containing 1.8 mM Ca^{2+} (calcium switch) and cultured for the indicated time points. Primary mouse keratinocytes were transfected using the basic primary endothelial nucleofactor kit (Amaxa, Cologne, Germany) in combination with program T-27 of the Nucleofector system (Amaxa). Using this method a transfection efficiency of 40–60% was achieved. The following aPKCζ constructs were used in transfection: eGFP-aPKCζwt, eGFP-aPKCζK281M (kinase dead, mutation in ATP binding site), eGFPaPKCζ kinase domain, myc-aPKCζIIpcDNA3.1 and eGFP-aPKCι/λwt have all been described (Perander *et al.*, 2001; Mostafavi-Pour *et al.*, 2003; Parkinson *et al.*, 2004).

RT-PCR analysis

RNA was isolated from murine newborn skin biopsies, epidermis, dermis, and isolated murine primary fibroblasts and keratinocytes using Trifast solution (Peqlab, Erlangen, Germany). The epidermis was separated from the dermis after skin of newborns was floated for 20 minutes at 4°C on 0.5 M ammonium thiocyanate in phosphate buffer (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4 pH 6.8). First strand cDNA synthesis was performed using superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) and oligo(dT) nucleotides according to the manual. RT-PCR was carried out as described (Tunggal *et al.*, 2005) using the following primers: mouse PKCι/λ (acc. no. NM008850) 5′-CACTTTGAGCCTTCCATCTCC-3′ (203–223 bp, coding sequence) and 5′-GTGACCAGCTTGTGGCACTTC-3′ (605–625 bp, coding sequence); mouse PKCζ (acc. no. NM008860) 5′-GCGTGGA TGCCATGACAACAT-3′ (112–132 bp, coding sequence); and 5′-GG CTCTTGGGAAGGCATGACA-3′ (581–601 bp, coding sequence).

Wound healing experiments

C57/Bl6 (6–8 weeks old) mice were anesthetized with a single intraperitoneal injection of ketamine/xylazine. The back of the animals was shaved and the skin cleaned with 70% ethanol. Two full-thickness excisional wounds (4 mm diameter) were generated on the back of each animal by excising skin and *panniculus carnosum* as described (Werner *et al.*, 1994). The wounds were allowed to dry to form a scab. Animals were killed at different time points post-wounding and the complete wounds including the epithelial margins were isolated, embedded in OCT (Shandon, Pittsburg, PA), and stored at −80°C. Sections taken from the center of the wound were used in immunohistochemistry and for hematoxylin/eosin (Shandon) staining. Two independent sets of wound healing were analyzed. All animal protocols were approved by the local veterinary authorities.

Antibodies

Antibodies specific for aPKCζ or aPKCι/λ were raised in rabbits by injection of the following specific peptides: aPKCζ amino acid 185–244: MDSVMPSQEPPVDGKNDGVDLPSEETDGIAYISSSRKHD-NIKDDSE DLKPVIDGVDGIKISQGLGL and aPKCι/λ amino acid 184–234: SLPPEPMMPMDQTMHPDHTQTVIPYNPSSHESLDQVGEE-KEAMNTRESGKASSSLGL.

The following antibodies were used rabbit polyclonal antibodies against all isoforms of Par3 (Upstate, Hamburg, Germany), Par3 (recognizing preferentially the 100 kDa form, (Vogelmann and Nelson, 2005)), aPKC (Santa-Cruz, Heidelberg, Germany), phospho-aPKC (Cell Signaling, Frankfurt, Germany, it should be noted that later batches of this antibody failed to show membrane staining either in immunohistochemistry or immunofluorescence and could therefore not be used in subsequent analysis), Crb2 and Crb3 (Lemmers *et al.*, 2004) and JAM-1 (a gift of Dr Klaus Ebnet), and goat antibodies to Par6 (N-18, Santa-Cruz) or JAM-1 (R&D system, Minneapolis, MN), rat monoclonal anti-ZO-1 R26.4 (Anderson *et al.*, 1988, DSHB, University of Iowa), and mouse mAbs against actin (ICN, Heidelberg, Germany), Rac (Sigma, St Louis, MO), or 3E1-GFP (Invitrogen).

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed on cryosections (6 μm) and were fixed with 4% paraformaldehyde or methanol. For immunofluorescence keratinocytes were cultured on collagen coated coverslips before fixation with methanol. Cells and tissues were blocked in PBS^{++} containing 1% BSA, 5% normal goat serum, and 0.02% Tween-20, after which cells were incubated with the appropriate primary antibody in blocking buffer followed by secondary antibodies coupled to Alexa 488 or Alexa 594 (Invitrogen). For cryosections nuclei were counterstained using propidium idodide (Sigma-Aldrich, Taufkirchen, Germany). Photos were taken with a NIKON Eclipse E800 microscope equipped with a NIKON DMZ1200 camera or a LEICA confocal microscope.

Western blot analysis

Primary keratinocytes of newborn mice (passage 3–9) were washed with ice-cold PBS containing 1 mM orthovanadate and were lysed at 4°C in 1% Nonidet P-40 buffer (10 mM Tris/HCl (pH7.5), 140 mM NaCl, 1 mM orthovanadate, 2 mM phenylmethylsulphonyl fluoride, 5 mM EDTA, 50 mM NaF, and protease inhibitor cocktail (Sigma)). After 15 minutes at 4°C, cells were scraped off and nuclei pelleted by centrifugation. Protein levels were determined using Bradford reagent (Biorad, Munich, Germany). Equal amounts of total protein were separated by SDS-PAGE using NuPage 4–12% gradient Bis–Tris Gels (Invitrogen), transferred to nitrocellulose (G&E, Freiburg, Germany), and probed with the appropriate antibodies. Experiments assessing the regulation of expression by Ca^{2+}-concentrations were performed at least 3x using different batches of primary keratinocytes.

TER measurement

Mouse primary keratinocytes, varying between passage 2–8, were plated directly or after transfection in a confluent manner onto polycarbonate Transwell filters (0.4 μm pore size, 12 mm diameter, Millipore). TER was measured at different time points with a Millicell−ERS Voltometer (Millipore, Eschborn, Germany) after calcium switch and shortly before ($T=0$). TER values were corrected by subtracting the blank value (no cells), and were expressed in ohm $\times$ cm^2. Transfection experiments were repeated at least twice for each construct using independently isolated primary mouse keratinocyte cultures.

Inhibition of PKCζ activity

For inhibition of aPKC activity, primary murine keratinocytes were transferred to FAD medium containing 1.8 mM Ca^{2+} and 100 nM myristoylated PKCζ pseudosubstrate inhibitor (Merck Biosciences, Bad Soolen, Germany) and grown for the indicated time points. The medium with fresh inhibitor was changed every 12 hours to ensure activity of the inhibitor. TER was measured at the indicated time points during incubation whereas immunohistochemistry was performed as described after treatment for the indicated time. Inhibition experiments were repeated four times using independently isolated primary keratinocyte cultures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Angelika Arora and Hady Ibrahim for excellent technical assistance. We thank Drs Klaus Ebnet, James Nelson, Gernot Herrmann, Peter Parker, and Terje Johansen for antibodies and constructs and members of the lab for constructive discussion. This project was financed by SFB589 P7 and Köln Fortune/Maria Pesch Stiftung.

REFERENCES

Ebnet K, Suzuki A, Horikoshi Y, Hirose T, Meyer Zu, Brickwedde MK *et al.* (2001) The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J* 20:3738–48

Etienne-Manneville S, Hall A (2001) Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* 106:489–98

Farese RV (2002) Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states. *Am J Physiol Endocrinol Metab* 283:E1–11

Fuchs E, Raghavan S (2002) Getting under the skin of epidermal morphogenesis. *Nat Rev Genet* 3:199–209

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y et al. (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 156:1099–111

Gao L, Macara IG (2004) Isoforms of the polarity protein par6 have distinct functions. *J Biol Chem* 279:41557–62

Garrard SM, Capaldo CT, Gao L, Rosen MK, Macara IG, Tomchick DR (2003) Structure of Cdc42 in a complex with the GTPase-binding domain of the cell polarity protein, Par6. *EMBO J* 22:1125–33

Getsios S, Huen AC, Green KJ (2004) Working out the strength and flexibility of desmosomes. *Nat Rev Mol Cell Biol* 5:271–81

Henrique D, Schweisguth F (2003) Cell polarity: the ups and downs of the Par6/aPKC complex. *Curr Opin Genet Dev* 13:341–50

Hurd TW, Gao L, Roh MH, Macara IG, Margolis B (2003) Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat Cell Biol* 5:137–42

Joberty G, Petersen C, Gao L, Macara IG (2000) The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat Cell Biol* 2:531–9

Kemphues KJ, Priess JR, Morton DG, Cheng NS (1988) Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* 52:311–20

Lechler T, Fuchs E (2005) Asymmetric cell divisions promote stratification and differentiation of mammalian skin. *Nature* 437:275–80

Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF *et al.* (2001) Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Mol Cell* 8:771–80

Lemmers C, Michel D, Lane-Guermonprez L, Delgrossi MH, Medina E, Arsanto JP *et al.* (2004) CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. *Mol Biol Cell* 15:1324–33

Lin D, Edwards AS, Fawcett JP, Mbamalu G, Scott JD, Pawson T (2000) A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol* 2:540–7

Macara IG (2004) Par proteins: partners in polarization. *Curr Biol* 14:R160–162

Marshall BS, Price G, Powell CT (2000) Rat protein kinase c zeta gene contains alternative promoters for generation of dual transcripts with 5′-end heterogeneity. *DNA Cell Biol* 19:707–19

Matter K, Balda MS (2003) Functional analysis of tight junctions. *Methods* 30:228–34

Mertens AE, Rygiel TP, Olivo C, van der Kammen R, Collard JG (2005) The Rac activator Tiam1 controls tight junction biogenesis in keratinocytes through binding to and activation of the Par polarity complex. *J Cell Biol* 170:1029–37

Moscat J, Rennert P, Diaz-Meco MT (2006) PKCzeta at the crossroad of NF-kappaB and Jak1/Stat6 signaling pathways. *Cell Death Differ* 13:702–11

Mostafavi-Pour Z, Askari JA, Parkinson SJ, Parker PJ, Ng TT, Humphries MJ (2003) Integrin-specific signaling pathways controlling focal adhesion formation and cell migration. *J Cell Biol* 161:155–67

Nelson WJ (2003) Adaptation of core mechanisms to generate cell polarity. *Nature* 422:766–74

Ohno S (2001) Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. *Curr Opin Cell Biol* 13:641–8

Parkinson SJ, Le Good JA, Whelan RD, Whitehead P, Parker PJ (2004) Identification of PKCzetaII: an endogenous inhibitor of cell polarity. *EMBO J* 23:77–88

Pasparakis M, Courtois G, Hafner M, Schmidt-Supprian M, Nenci A, Toksoy A *et al.* (2002) TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417:861–6

Perander M, Bjorkoy G, Johansen T (2001) Nuclear import and export signals enable rapid nucleocytoplasmic shuttling of the atypical protein kinase C lambda. *J Biol Chem* 276:13015–24

Plant PJ, Fawcett JP, Lin DC, Holdorf AD, Binns K, Kulkarni S *et al.* (2003) A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. *Nat Cell Biol* 5:301–8

Soloff RS, Katayama C, Lin MY, Feramisco JR, Hedrick SM (2004) Targeted deletion of protein kinase C lambda reveals a distribution of functions between the two atypical protein kinase C isoforms. *J Immunol* 173:3250–60

Suzuki A, Akimoto K, Ohno S (2003) Protein kinase C lambda/iota (PKClambda/iota): a PKC isotype essential for the development of multicellular organisms. *J Biochem (Tokyo)* 133:9–16

Suzuki A, Yamanaka T, Hirose T, Manabe N, Mizuno K, Shimizu M *et al.* (2001) Atypical protein kinase C is involved in the evolutionarily conserved par protein complex and plays a critical role in establishing epithelia-specific junctional structures. *J Cell Biol* 152:1183–96

Tunggal JA, Helfrich I, Schmitz A, Schwarz H, Gunzel D, Fromm M *et al.* (2005) E-cadherin is essential for *in vivo* epidermal barrier function by regulating tight junctions. *EMBO J* 24:1146–56

Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M *et al.* (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. *Cell* 126:741–54

Vogelmann R, Nelson WJ (2005) Fractionation of the epithelial apical junctional complex: reassessment of protein distributions in different substructures. *Mol Biol Cell* 16:701–16

Watt FM (2001) Stem cell fate and patterning in mammalian epidermis. *Curr Opin Genet Dev* 11:410–7

Werner S, Breeden M, Hubner G, Greenhalgh DG, Longaker MT (1994) Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse. *J Invest Dermatol* 103:469–73

White WO, Seibenhener ML, Wooten MW (2002) Phosphorylation of tyrosine 256 facilitates nuclear import of atypical protein kinase C. *J Cell Biochem* 85:42–53

Yamanaka T, Horikoshi Y, Sugiyama Y, Ishiyama C, Suzuki A, Hirose T *et al.* (2003) Mammalian Lgl forms a protein complex with PAR-6 and aPKC independently of PAR-3 to regulate epithelial cell polarity. *Curr Biol* 13:734–43

Yamauchi K, Rai T, Kobayashi K, Sohara E, Suzuki T, Itoh T *et al.* (2004) Disease-causing mutant WNK4 increases paracellular chloride permeability and phosphorylates claudins. *Proc Natl Acad Sci USA* 101:4690–4