aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization

Atsushi Suzuki[1,*], Chikako Ishiyama[1,*], Katsutaka Hashiba[1], Miki Shimizu[1], Klaus Ebnet[2] and Shigeo Ohno[1,‡]

[1]Department of Molecular Biology, Yokohama City University School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan
[2]Institute of Cell Biology, ZMBE, University of Muenster, D-48149 Muenster, Germany

*These authors contributed equally to this work
‡Author for correspondence (e-mail: ohnos@med.yokohama-cu.ac.jp)

Accepted 24 June 2002
Journal of Cell Science 115, 3565-3573 © 2002 The Company of Biologists Ltd
doi:10.1242/jcs.00032

Summary

We have previously shown that aPKC interacts with cell polarity proteins PAR-3 and PAR-6 and plays an indispensable role in cell polarization in the *C. elegans* one-cell embryo as well as in mammalian epithelial cells. Here, to clarify the molecular basis underlying this aPKC function in mammalian epithelial cells, we analyzed the localization of aPKC and PAR-3 during the cell repolarization process accompanied by wound healing of MTD1-A epithelial cells. Immunofluorescence analysis revealed that PAR-3 and aPKCλ translocate to cell-cell contact regions later than the formation of the primordial spot-like adherens junctions (AJs) containing E-cadherin and ZO-1. Comparison with three tight junction (TJ) membrane proteins, JAM, occludin and claudin-1, further indicates that aPKCλ is one of the last TJ components to be recruited. Consistently, the expression of a dominant-negative mutant of aPKCλ (aPKCλkn) in wound healing cells does not inhibit the formation of the spot-like AJs; rather, it blocks their development into belt-like AJs. These persistent spot-like AJs in aPKCλ-expressing cells contain all TJ membrane proteins and PAR-3, indicating that aPKC kinase activity is not required for their translocation to these premature junctional complexes but is indispensable for their further differentiation into belt-like AJs and TJs. Cortical bundle formation is also blocked at the intermediate step where fine actin bundles emanating from premature cortical bundles link the persistent spot-like AJs at apical tips of columnar cells. These results suggest that aPKC contributes to the establishment of epithelial cell polarity by promoting the transition of fibroblastic junctional structures into epithelia-specific asymmetric ones.

Key words: aPKC, PAR proteins, Epithelial cell polarity, Cell-cell junction, Wound healing

Introduction

In response to an external cue, cells autonomously generate an asymmetric distribution of intracellular components, thereby producing functional asymmetry within themselves both during development and as an adult. The establishment and maintenance of this 'cell polarity' is an essential feature of all eukaryotic cells and is critical for the integrity of the organism. Recent studies have made great progress in revealing the molecular basis of this cell polarity by identifying many sets of polarity proteins and their interactions (Bilder, 2001; Ohno, 2001). One of the most essential sets of polarity proteins is an evolutionarily conserved molecular complex, the aPKC-PAR system, which plays a critical role in the establishment of cell polarity in different organisms and cell types (Rose and Kemphues, 1998; Suzuki et al., 2001; Wodarz et al., 2000).

PAR (partitioning-defective) proteins, PAR-1 to PAR-6, were first identified as proteins, mutations of which led to loss of the anterior-posterior cell polarity of the *C. elegans* one-cell embryo (Guo and Kemphues, 1996). The finding that mammalian PAR-3 (ASIP) specifically binds to atypical PKC (aPKC) leads us to further reveal that *C. elegans* aPKC (PKC-3) interacts with PAR-3 and also plays an indispensable role in cell polarization of the *C. elegans* one-cell embryo (Izumi et al., 1998; Tabuse et al., 1998). We demonstrated recently that in mammalian epithelial cells, aPKC interacts with not only PAR-3 but also PAR-6 and plays a critical role in the formation of the apical-basal polarity (Suzuki et al., 2001; Yamanaka et al., 2001). In those studies, we overexpressed a dominant-negative mutant of one of the mammalian aPKC isoforms, aPKCλ , in MDCK epithelial cells and found that the mutant inhibits the development of the tight junction (TJ) structures as well as the establishment of asymmetric distribution of membrane proteins only when cell-cell junctions are reset by calcium switch treatment. These results indicate that aPKC activity is required for the development but not the maintenance of epithelial polarity, although the molecular targets of aPKC are still unknown.

One of the interesting features of the cell polarity proteins revealed so far is that they themselves asymmetrically localize underneath the restricted regions of the plasma membrane, and this asymmetric submembranous localization is critical for their function (Bilder, 2001; Ohno, 2001; Rose and Kemphues, 1998). For example, the localization of aPKC, PAR-3 and PAR-6 is gradually restricted to the anterior periphery of the *C.*

elegans one-cell embryo in response to sperm entry, whereas PAR-1 and PAR-2 are restricted to the posterior periphery during the development of cell polarity (Rose and Kemphues, 1998). Defects in one of the three proteins, aPKC, PAR-3 or PAR-6, result in the disruption of the asymmetric localization of all other PAR proteins. On the other hand, during the asymmetric division of neuroblasts in the *Drosophila* embryo, basal crescent localization of neuronal determinants Miranda and Prospero is directed by the apically localized Inscuteable (Doe and Bowerman, 2001). Interestingly, recent findings indicate that the correct localization of Inscuteable depends on the apical localization of the *Drosophila* aPKC-PAR complex, which is inherited from the epithelium from which neuroblast delaminates (Ohno, 2001; Wodarz et al., 1999). Considering that all of these polarity proteins are not membrane proteins, these results suggest that the establishment of asymmetric submembranous structures to which these determinants anchor is essential for cell polarity. However, the molecular basis of this putative submembrane structure is not known for the *C. elegans* or *Drosophila* embryonic cells. In this sense, the results from mammalian epithelial cells provided very important clues to address this issue: in these cells, aPKC, PAR-3 and PAR-6 are asymmetrically localized to TJ, the specialized cell-cell junctional structure in the most apical region of the basolateral membrane (Dodane and Kachar, 1996; Izumi et al., 1998). Furthermore, the cytoplasmic tail of a TJ membrane protein, JAM, interacts with the first PDZ domain of PAR-3, suggesting that JAM is a strong candidate for the anchoring partner of the aPKC-PAR complex at TJ (Ebnet et al., 2001; Itoh et al., 2001). Our recent results on the inhibitory effect of the dominant-negative mutant of aPKCλ (aPKCλkn) further indicated that the suppression of aPKC activity resulted in the disruption of the submembranous structure, that is, TJ to which the aPKC-PAR complex itself is asymmetrically localized (Suzuki et al., 2001). Considering that the formation of the epithelia-specific junctional structures including TJ, adherens junctions (AJs) and desmosomes, which tightly couple with the development of asymmetric cytoskeletal structures, represents the development of submembranous asymmetric structures in epithelial cells (Denker and Nigam, 1998; Yeaman et al., 1999), these results suggest an intriguing possibility that aPKC primarily regulates the development of the epithelia-specific junctional structures of epithelial cells and thus contributes to the development of the apico-basal polarity.

TJ biogenesis has been suggested to be induced by cell-cell adhesion mediated by E-cadherin (Gumbiner et al., 1988), but the molecular mechanism underlying this process is still unclear. However, by analyzing the wound healing process of a mouse epithelial cell line, MTD1-A cells, Tsukita and co-workers have provided evidence that epithelial junctional formation can be dissected into multiple steps proceeding in a sequential manner during the cell polarization process (Ando-Akatsuka et al., 1999; Yonemura et al., 1995). On the basis of immunofluorescence analysis, they demonstrated that at the initial phase of cell polarization, fibroblastic spot-like AJs containing E-cadherin as well as ZO-1 are formed as a nascent junctional complex (Vasioukhin et al., 2000; Yonemura et al., 1995). Thereafter, as epithelial polarization progresses, ZO-1 dissociates from E-cadherin, which separately forms the epithelia-specific belt-like AJ, and gradually colocalizes with occludin at cell-cell contact sites to form TJs (Ando-Akatsuka et al., 1999). Here, by combining the same experimental system and the dominant-negative mutant of aPKCλ used previously (Suzuki et al., 2001), we attempted to clarify how aPKCλkn inhibits TJ formation during epithelial cell polarization. Our results indicate that aPKC is recruited after the establishment of the initial spot-like AJ complex to which not only E-cadherin-catenins but also several TJ components such as JAM, occludin and claudin-1 are transiently recruited and contributes to the further development of this premature junctional complex into epithelia-specific structures in which belt-like AJs, TJs, are asymmetrically segregated.

Materials and Methods

Cell culture and wounding

Mouse epithelial cells, MTD1-A cells (Enami et al., 1984), were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum. For the wound healing assay, the cells were plated on coverslips at a density of 2.5×10^5 cells/cm^2, and the next day, the resultant confluent monolayers were scratched manually with an 18G needle as described previously (Ando-Akatsuka et al., 1999). Wounded regions were allowed to heal for 6-30 hours prior to immunofluorescence analysis.

Antibodies

The following monoclonal and polyclonal antibodies (mAb and pAb, respectively) were used: rabbit anti-PAR-3/ASIP pAb, C2-3 (Izumi et al., 1998); rabbit anti-aPKCλ pAb, λ1 (Akimoto et al., 1994); rabbit anti-ZO-1 pAb, mouse anti-ZO-1 mAb, rabbit anti-occludin pAb and rabbit anti-claudin-1 pAb (Zymed); mouse anti-E-cadherin mAb (Transduction Laboratory); mouse anti-α-catenin mAb (Takara); rat anti-nectin mAb (Takahashi et al., 1999); and rat anti-JAM mAb (Ebnet et al., 2001).

Adenovirus infection

The adenovirus vectors carrying cDNA encoding LacZ or aPKCλkn have been described previously (Suzuki et al., 2001; Yamanaka et al., 2001). For adenovirus infection, MTD1-A cells were seeded on coverslips in 24-well plates at a density of 1.25×10^5 cells/cm^2 1 day before infection, as described previously (Suzuki et al., 2001). After 2 hours of preincubation in low calcium medium containing 5% FCS and 3 μM Ca^{2+} (Stuart et al., 1994), the cells were incubated for 2 hours with 150 μl of the appropriate virus solution diluted to 3×10^8 pfu/ml in LC medium. Cells were washed two times with PBS and further cultured in normal growing medium overnight before wounding.

Confocal immunofluorescence microscopy

At an appropriate time after wounding, cells were fixed with 2% formaldehyde in PBS for 15 minutes at room temperature. After washing twice with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS for 10 minutes at room temperature. The cells were then washed and soaked in blocking solution (PBS containing 10% calf serum) overnight at 4°C. Antibody incubations were performed at 37°C for 45 minutes in buffer containing 10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.01% (v/v) Tween 20 and 0.1% (w/v) BSA. The secondary antibodies used were Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR), Cy3-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG or Cy3-conjugated goat anti-rat IgG (Amersham Corp., Arlington Heights, IL). To stain F-actin, rhodamine-phalloidin (Molecular Probes) was used in place of the secondary antibodies. Coverslips were mounted using Vectashield (Vector Laboratories,

Burlingame, CA) and examined under a fluorescence microscope equipped with a confocal system (μRadiance, Bio-Rad Laboratories, Hercules, CA). An oil-immersion objective lens (Plan APOCHROMAT ×63, NA 1.40) (Nikon), and argon and red diode lasers were used. Conventional images were composed of 512×512 pixels. Usually, about 30 optical sections covering the basal to the apical region of cells were taken with an interval of 0.4 μm, and all images were projected unless indicated otherwise.

Results

aPKC and PAR-3 are recruited into cell-cell contact regions after establishment of spot-like AJ during the wound healing process of MTD-1A cells

MTD1-A cells derived from a mouse mammary tumor (Enami et al., 1984) are useful for analyzing the epithelial cell polarization process, because they show (1) less frequent overriding of the cell peripheries during the establishment of cell polarity, and (2) highly synchronous formation of cell-cell contact regions (Yonemura et al., 1995). The combination of this cell line and the wound-healing assay provides good resolution for analyzing the epithelial junctional formation process step by step (Ando-Akatsuka et al., 1999). We first attempted to clarify at which steps aPKCλ and PAR-3 are recruited into the cell-cell junctional region. When a confluent monolayer of MTD-1A cells was manually scratched with a needle, nascent cell-cell contacts appeared around 6-8 hours after wounding. At initial stages of wound closure, encountering cells formed many spot-like AJs positive for E-cadherin as well as ZO-1 at the tip of very thin membrane protrusions (Ando-Akatsuka et al., 1999; Yonemura et al., 1995). Immunofluorescence analysis using anti-PAR-3 or anti-aPKCλ antibody revealed that most of these spot-like AJs identified with the anti-ZO-1 antibody were negative for PAR-3 and aPKCλ (small arrows in Fig. 1A,B). Even at later stages when ZO-1-positive structures had gradually fused and became continuous short fragments, both proteins showed retarded recruitment into these junctions (small arrows in Fig. 1A,B, middle and right panels). Indirect comparison of the timing of the recruitment of PAR-3 and aPKCλ into cell-contact regions relative to that of ZO-1 suggested that aPKCλ was recruited later than PAR-3, although the lack of available antibodies did not allow us to confirm it directly. Nevertheless, these results indicate that PAR-3 and aPKCλ are recruited into the junctional complex after the formation of the initial spot-like AJs containing E-cadherin and ZO-1.

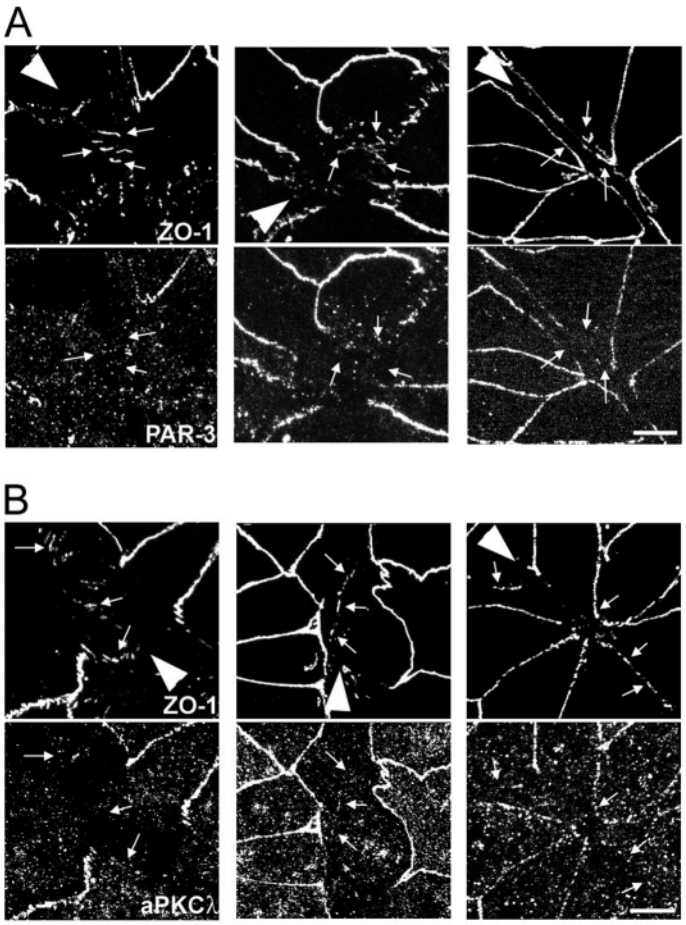

Fig. 1. aPKCλ and PAR-3 translocate to cell-cell junctional regions after the establishment of spot-like AJs. (A,B) Confluent MTD1-A monolayers were scratched with a needle, cultured for 6 hours (left panels), 9 hours (middle panels) and 12 hours (right panels) and then doubly stained with PAR-3 pAb and ZO-1 mAb (A) or aPKCλ pAb and ZO-1 mAb (B), as indicated. Each panel represents a projected view of confocal optical sections (0.4 μm) collected from the apical to the basal region of the cells, although colocalization of the proteins was confirmed in a single section. (The panels in the following figures are similarly obtained unless otherwise mentioned.) Large arrowheads indicate the direction of the wounds. Note that many ZO-1-positive spot-like AJs are negative for PAR-3 as well as aPKCλ staining. In contrast with PAR-3, aPKCλ signal is often lacking on the ZO-1-positive continuous junctions. Bars, 10 μm.

aPKCλ is recruited last to TJs

The TJ membrane proteins occludin and JAM colocalize with ZO-1 and E-cadherin in the spot-like AJs prior to forming continuous TJ structures (Ando-Akatsuka et al., 1999; Ebnet et al., 2001). Therefore, the results in Fig. 1 suggest that aPKC and PAR-3 are recruited into cell-contact regions later than these TJ membrane proteins. In fact, we demonstrated by double staining analysis that many JAM-positive spot-like AJs observed in intermediate stages of the wound healing of MTD1-A cells were negative for PAR-3 (Ebnet et al., 2001). Again, owing to the lack of appropriate antibodies, we could not perform similar experiments for the other TJ membrane proteins, occludin as well as claudin-1. However, during indirect comparison between these TJ proteins and PAR-3/aPKC with respect to junctional recruitment, we unexpectedly found that claudin-1 is recruited to the junctions considerably later than JAM and occludin and as late as aPKCλ and PAR-3. As demonstrated previously, JAM almost completely colocalized with the spot-like AJs positive for ZO-1 formed at the very early stage of wound healing (Fig. 2A top panels). The occludin signal was also detected in most of the nascent spot-like AJs, although its signal on the ZO-1-positive dot-like AJs tended to be weaker than that of JAM (arrows in Fig. 2A, middle panels). On the other hand, many ZO-1-positive dot-like or fragmental structures at nascent cell-cell contacts showed a weak or negative signal for claudin-1 (arrows in Fig. 2A, bottom panels). Even relatively developed

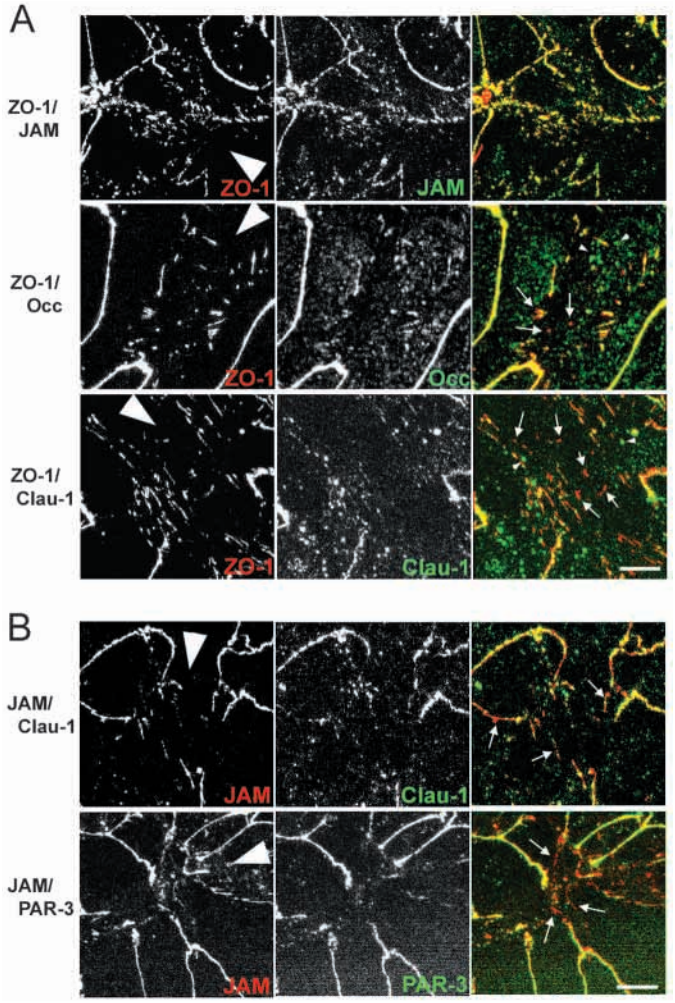

Fig. 2. Claudin-1 is a unique TJ component that is recruited into junctional regions as late as PAR-3 and aPKCλ are. The recruitment of TJ components into the cell-cell junctional regions of wound healing cells was examined by double immunostaining at intermediate stages of wound closure. In A, three TJ membrane proteins, JAM, occludin and claudin-1, were compared with ZO-1, whereas, in B, claudin-1 and PAR-3 were compared with JAM, as indicated. The combination of used antibodies (rat JAM mAb, rabbit occ pAb, rabbit claudin-1 pAb, mouse ZO-1 mAb and anti-PAR-3 pAb) is indicated in the panels, and merged views are also presented on the right. The direction of the wounds is indicated by large arrowheads, whereas ZO-1- or JAM-positive spot-like AJs, which showed negative or weak staining by corresponding other TJ proteins, are indicated by small arrows. Small arrowheads indicate occludin- or claudin-1-positive, but ZO-1 negative, granular structures sometimes observed in cytoplasm. Note that many ZO-1- or JAM-positive dot-like AJs are negative for claudin-1. Bars, 10 μm.

junctions more than 3 μm long were frequently negative for claudin-1 staining. These results suggest that claudin-1 is recruited into the junctional area after the establishment of the dot-like AJs. This was further confirmed by the direct comparison between doubly stained JAM and claudin-1 (Fig. 2B, top panels); again, many JAM-positive junctional structures of 3 μm length were often negative for claudin-1 staining. Considering the similarity of the immunostaining pattern of claudin-1 to those of PAR-3 and aPKCλ in nascent junctional areas (Fig. 1, Fig. 2B bottom panels), these results indicate that TJ components can be subdivided into at least two groups in terms of junctional recruitment, and the polarity proteins, aPKC and PAR-3, as well as claudin-1 belong to the same group whose recruitments is rather late.

Overexpression of a dominant-negative mutant of aPKCλ inhibits the development of spot-like AJs into epithelia-specific belt-like AJs

Previously, by using the adenovirus gene-transfer technique, we demonstrated that overexpression of a kinase-negative mutant of aPKCλ or ζ (aPKCkn) disturbs the junctional formation of MDCK II cells observed in the re-polarization process after a calcium switch treatment through their dominant-negative activity against endogenous aPKC (λ or ζ) kinase activity (Suzuki et al., 2001). To address the question of which steps of the epithelial junctional formation pathway are blocked by aPKCkn, we applied this aPKCλ mutant to the wound-healing assay using MTD-1A. When a confluent monolayer of MTD1A cells (2.5×10^5 cells/cm^2) was infected with adenovirus vector at MOI 600, aPKCλkn was expressed in approximately 90% of the cells showing heterogeneous expression levels (Fig. 3A, lower left panel). Similar to the situation shown for MDCK II cells in previous work (Suzuki et al., 2001), junctional structures monitored by ZO-1 staining were hardly affected by the expression of aPKCλkn unless the infected cells were subjected to regeneration of cell-cell adhesion by a calcium switch treatment (data not shown) (see ZO-1 staining at cell-cell boundaries in non-wounded regions of aPKCλkn-expressing cells in Fig. 3A, lower right panel). When monolayers of MTD1-A cells expressing aPKCλkn or LacZ were wounded, they showed apparently normal wound closure within 6-10 hours under phase-contrast microscope observation (data not shown). In LacZ-expressing cells, continuous ZO-1 staining was completely restored in the healed regions (Fig. 3A, upper right panel). This was also the case when nPKCεkn was used as a negative control instead of LacZ (data not shown). However, in cells expressing aPKCλkn, the completion of TJ formation monitored by ZO-1 was significantly inhibited in cells burying the wound (Fig. 3A, lower panels). Closer inspection demonstrated that in cells that participated in burying the wound, ZO-1 staining was broadly observed in dot-like structures or in very short fragments at cell-cell borders, and this staining pattern did not change even after the wound was completely healed (30 hours after wounding, Fig. 3B). These cells also exhibited aberrant E-cadherin staining that revealed dot-like discontinuous structures instead of belt-like AJs as observed in LacZ-expressing cells. Comparison of ZO-1 and E-cadherin staining in a single confocal plane confirmed that many ZO-1-positive dot-like AJs, if not all, are also positive for E-cadherin (Fig. 4, top panels). It was further demonstrated that α-catenin and nectin also showed colocalization with ZO-1 in these structures with higher frequency (Fig. 4, middle and bottom panels). Together with the fact that many F-actin bundles are running into these dot-like structures (see below), these results indicate that the ZO-1-positive dot-like structures induced by aPKCλkn expression are structurally identical to the spot-like AJs observed during the normal wound healing process, which appear only in newly formed cell-cell contacts between

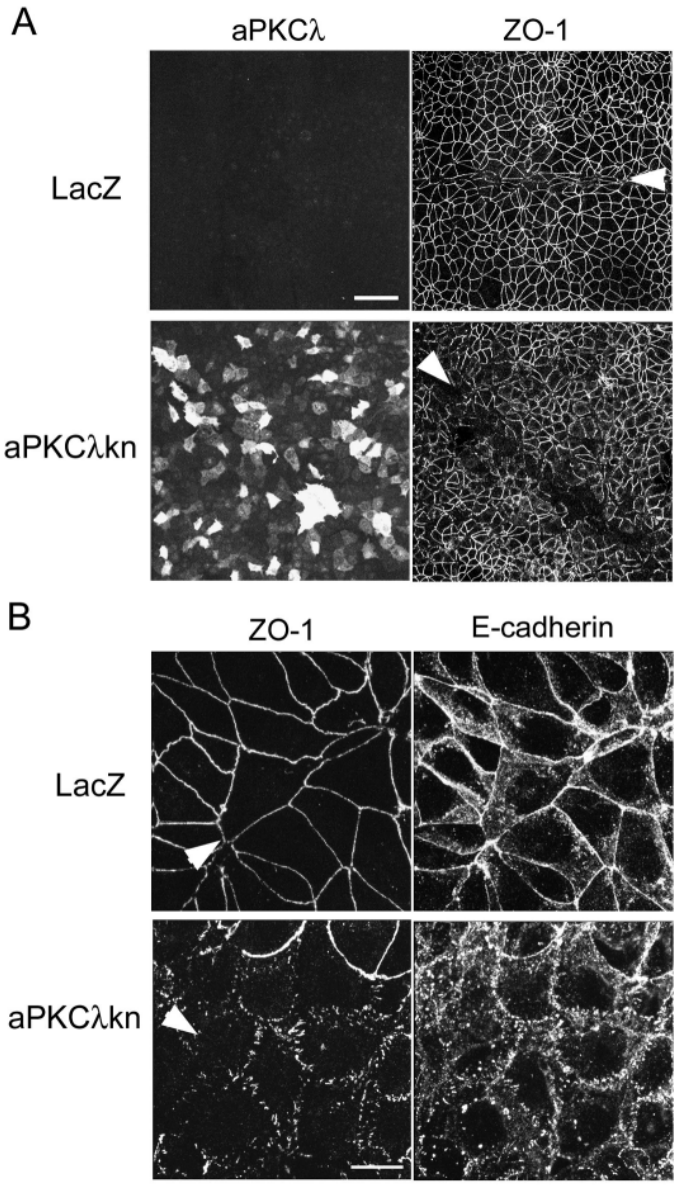

Fig. 3. Ectopic expression of the dominant-negative mutant of aPKCλ (aPKCλkn) inhibited the regeneration of TJ in the wound-healing area. Confluent monolayers of MTD1-A cells were infected with adenovirus vectors carrying LacZ or aPKCλkn cDNA, and the ectopic protein expression was induced in the normal growth media for 18 hours. The wound-healing assay was performed as described in the legend to Fig. 1 (large arrowheads indicate the direction of the wounds) and, 10 hours (A) or 30 hours (B) after wounding, cells were fixed and doubly stained with anti-aPKCλ pAb and anti-ZO-1 mAb (A) or anti-ZO-1 pAb and anti-E-cadherin mAb (B). As shown in A, although the expression levels are virtually heterogenous, aPKCλkn was expressed in almost 90% of the cells at levels higher than the endogenous one (under the photographic conditions used here, fluorescence signals of endogenous aPKCλ cannot be detected). Note that when aPKCλkn was expressed, ZO-1 staining disappeared in cells burying the wound, although the completion of wound healing was confirmed by phase-contrast microscopy (data not shown) (see aPKCλ staining in cells burying the wound). Enlarged views in B revealed that ZO-1 is broadly localized in dot-like structures in the wound-healing region of aPKCλkn-expressing cells, where E-cadherin staining also displayed rough, discontinuous appearance. Bars, 100 μm (A) or 20 μm (B).

encountering cells in the first row of the wound margin. Taken together, the results in Figs 3 and 4 suggest that the inhibition of aPKCλ kinase activity does not suppress the formation of the primordial spot-like AJ complexes; rather, it blocks their development into the epithelia-specific belt-like AJs.

The cortical F-actin bundle formation process is trapped in an intermediate state in aPKCλkn-expressing cells

Epithelia-specific belt-like AJs are characterized by their ability to align with a thick actin cable called the cortical (peripheral) bundle (Hirano et al., 1987). Consistent with the results in Fig. 4, rhodamine-phalloidin staining revealed that the formation of the cortical F-actin bundle was also inhibited in wound-healing cells expressing aPKCλkn (Fig. 5A, middle and bottom panels). Instead, many stress fiber-like F-actin bundles were running into the persistent spot-like AJs positive for ZO-1 in these cells. Significantly, in some cells, a loosely bundled F-actin cable was observed to run circularly underneath the membrane, from which many fine F-actin bundles emanated into the spot-like AJs. It should be noted that this distribution pattern of F-actin is strikingly similar to the intermediate state of F-actin reorganization observed at intermediate stages of the normal polarization process of MTD1-A cells, which finally develops into cortical bundles (Yonemura et al., 1995). These results strongly support the notion that aPKC kinase activity plays a critical role in this normal process of belt-like AJ development. In addition, they also suggested that the fusion of the premature spot-like AJ into belt-like AJs is coupled to F-actin reorganization so tightly, as though they were either side of the same coin, that they cannot proceed separately.

Interestingly, confocal z-sectional analysis revealed that aPKCλkn-expressing cells burying the wound also develop a columnar shape with a height comparable to that of surrounding cells, and the spot-like AJs reside at the apical tip of the lateral membranes (small arrowhead in Fig. 5B). In fact, besides being distributed on the dot-like AJs, E-cadherin and α-catenin also showed broad distribution on the lateral membrane (small arrowheads in Fig. 4, top and middle panels), suggesting that asymmetric domain formation in the lateral membrane occurred even in aPKCkn-expressing cells lacking mature belt-like AJs. In these cells, the premature cortical bundle structures linking the spot-like AJs were formed underneath the apical membrane independently of the basal stress fibers (Fig. 4B,C). Therefore, these results indicate that in aPKCλkn-expressing cells, F-actin reorganization to restore cortical bundles and the epithelia-specific columnar shape proceeds to some extent during the re-epithelialization process, but the final step involving connection of the F-actin cortical bundles closely to fused belt-like AJs is inhibited completely in these cells.

TJ membrane proteins and PAR-3 are trapped in the persistent spot-like AJs in aPKCkn-expressing cells

As shown in Fig. 6A, almost all of the ZO-1-positive spot-like AJs in aPKCλkn-expressing wound-healing cells were positive for JAM and occludin. Interestingly, claudin-1 also showed almost complete colocalization with ZO-1 in the dot-like structures induced by aPKCλkn, although during the normal

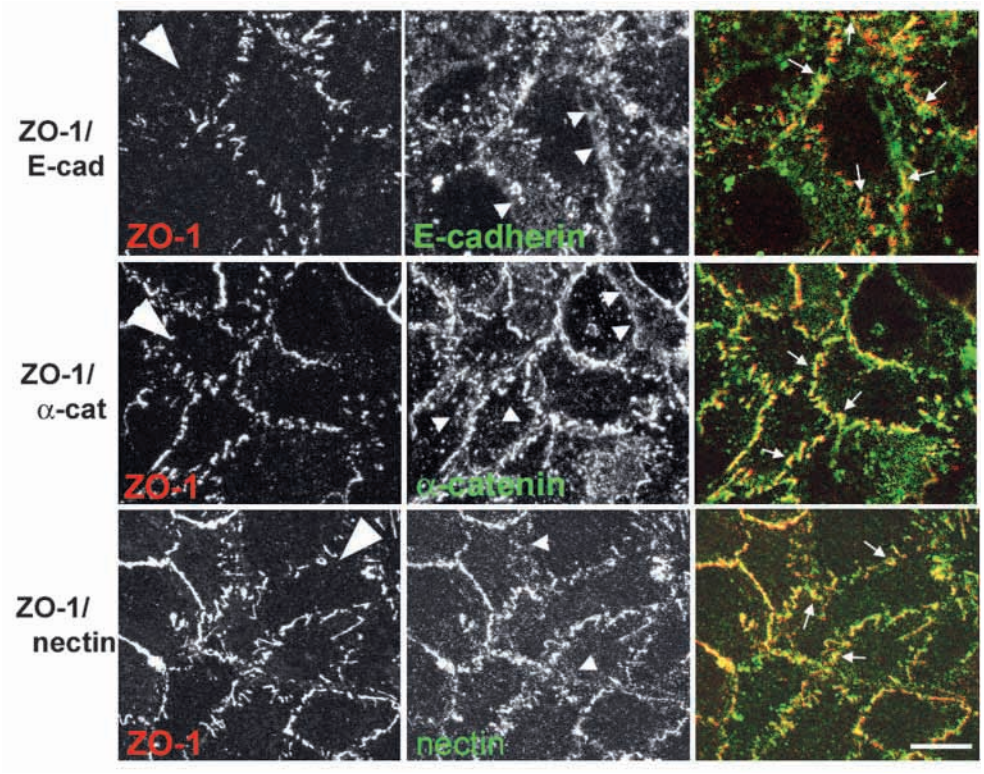

Fig. 4. Overexpression of aPKCλkn inhibited the development of spot-like AJs into belt-like AJs. aPKCλkn-expressing cells were subjected to wounding, fixed 30 hours after the wounding and doubly immunostained by the antibodies indicated. Large arrowheads indicate the direction of the wounds. The projected views of confocally obtained optical sections from the apical to the basal regions of the cells, except for merged views which were made from appropriate single confocal sections to strictly examine the co-localization of the two proteins (green: AJ proteins, red: ZO-1), are shown. Three AJ proteins, E-cadherin, α-catenin and nectin, were colocalized to some of these persistent ZO-1-positive spot-like structures (small arrows in the merged view), suggesting that these correspond to the spot-like AJs that could not develop into belt-like AJs. The structures were located at the apex of the lateral membrane (see Fig. 5B), and E-cadherin and α-catenin also distributed diffusely in the lateral membranes (small arrowheads in middle panels). Bars, 20 μm.

wound healing process, it is recruited into the junctional area rather late and is thus hardly detected in the dot-like AJs (Fig. 2). These results strongly reinforce the idea that the dot-like AJs are intermediate structures for epithelial junctional development to which TJ components including claudin-1 can be transiently recruited. Furthermore, they also indicate that aPKCλ kinase activity is not required for this translocation. Rather, aPKCλ plays a critical role in the subsequent segregation step of the junctional proteins in these primordial AJ structures into mature belt-like AJs and TJs.

We next examined the localization of PAR-3 in aPKCλkn-expressing wound-healing cells and found that a substantial part of the persistent dot-like AJs was positive for PAR-3 (Fig. 6B). Therefore, PAR-3 translocation to the spot-like AJs can also occur without aPKCλkn activity. This is consistent with the indirect observation that PAR-3 translocates to the spot-like AJs before the recruitment of aPKC into the junctional regions (Fig. 1). However, it should also be noted that in contrast with other TJ components, many ZO-1-positive spot-like AJs show weak or negative staining for PAR-3 even 30 hours after wounding (see small arrows in Fig. 6B, right panel). This may indicate that the translocation or stability of PAR-3 at the spot-like AJs partially depends on aPKC activity.

Discussion

Spot-like structures (also called puncta) positive for E-cadherin are transiently formed as an intermediate during the development of the epithelia-specific continuous cell-cell junctions (Adams et al., 1998; Vasioukhin et al., 2000; Yonemura et al., 1995). Colocalization of α-actinin, vinculin, as well as ZO-1 and termination of prominent F-actin bundles at these structures have established that these nascent AJ structures correspond to the spot-like AJs observed in fibroblastic cells (Vasioukhin et al., 2000; Yonemura et al., 1995). Using the wound healing assay of MTD1-A cells, Ando-Akatsuka et al. have extended these observations by showing that ZO-1 colocalizes with E-cadherin in the spot-like very early AJ structures prior to the recruitment of occludin, but as junctional formation proceeds, ZO-1 dissociates from E-cadherin to form TJ with occludin (Ando-Akatsuka et al., 1999). These results have created the idea that the epithelial junctional formation can be divided into two phases: first is the formation of the fibroblastic spot-like AJs at the nascent cell-cell contacts, into which several AJ and TJ proteins are transiently and sequentially recruited, and second is the differentiation of the primordial adhesion complex into epithelia-specific well segregated junctional structures such as belt-like AJs and TJs.

In this work, we reinforced this idea by finding that the overexpression of aPKCλkn results in the blockage of development of the epithelial junctional structure at the formation of spot-like AJs. The present results indicate that aPKC activity is not required for the first step of junctional development, that is, the recruitment of junctional proteins into contact sites, but is indispensable for the second step to reconstruct the nascent junctional complex into epithelia-specific mature junctional structures. Significantly, the persistent spot-like AJs in aPKCλkn-expressing cells contain multiple junctional membrane proteins, such as E-cadherin, nectin, JAM, occludin and claudin-1, confirming that the spot-like AJs are structures in which many junctional proteins gather together prior to their subsequent segregation. These membrane proteins are considered to assemble in the structures by interacting directly or indirectly (through peripheral protein such as catenins for E-cadherin) with various peripheral

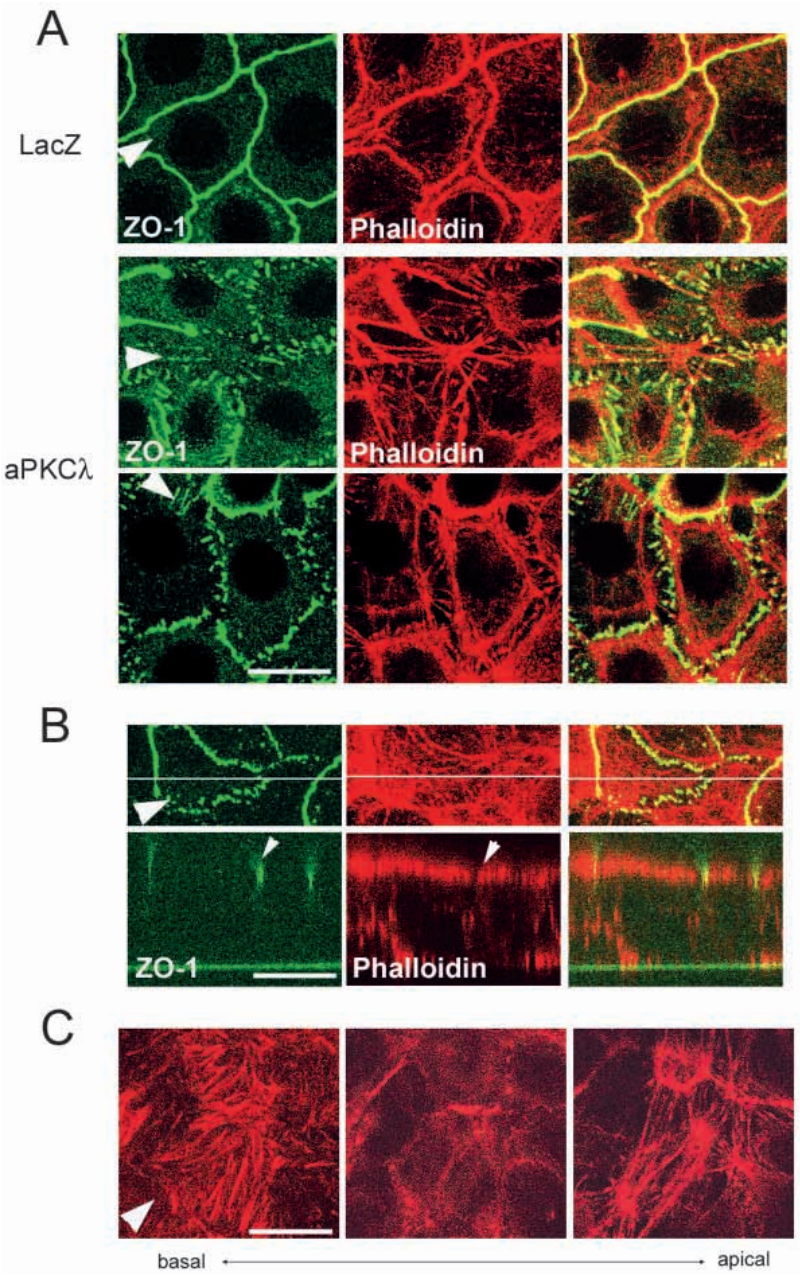

Fig. 5. Development of the cortical bundle of F-actin was blocked in an intermediate step in aPKCλ-expressing cells burying the wound. aPKCλkn-expressing cells burying the wound were doubly stained with rhodamine-phalloidin (red) and anti-ZO-1 antibody (green) 10 hours (A) or 30 hours (B and C) after wounding. Large arrowheads indicate the direction of the wounds. In A, the results for LacZ-expressing cells are also shown for comparison (top panels). Shown are projected views of confocally obtained optical sections covering apical regions (A, and top panels in B), single confocal section at basal, intermediate, or apical region (C) or z-sectional views crossing a spot-like incomplete juctional structure (small arrowhead; bottom panels of B). aPKCλkn-expressing cells do not complete cortical bundle formation of F-actin, showing stress fiber-like F-actin bundles linking ZO-1-positive spot-like AJs. In some cells, the prototype of cortical loose bundles of F-actin was observed, from which short F-actin fibers emanate into the spot-like AJs (right panels in A). Note that this kind of F-actin organization is formed in the apical surface of cells independently of the basal stress fibers (B,C), and cells with spot-like AJs also develop in height to a level comparable to that of surrounding cells with complete TJs. Bars, 10 μm.

scaffold proteins containing PDZ domains, such as ZO-1, afadin and MUPP1, which can associate with the cytoplasmic regions of the multiple membrane proteins (Ebnet et al., 2000; Hamazaki et al., 2001; Itoh et al., 1999; Itoh et al., 2001). Therefore, these results raise an intriguing possibility that aPKC is responsible for modification of the interactions between junctional membrane proteins and scaffold proteins or between membrane proteins by themselves, thereby promoting the transition of junctional structures from the first step to the second one. This is consistent with another observation in this study that aPKC is one of the later TJ components with respect to junctional recruitment.

Of course, in addition to modifying junctional proteins, aPKC may affect junctional formation by interfering with other events required for the development of belt-like AJs and TJs. In this sense, it should be noted that Vasioukhin et al. have suggested that an antiparallel pair of filopodia, at the tip of which the spot-like AJs (or puncta) are formed, physically draws the two cell surfaces together, thereby extending the zone of cell-cell contact (Vasioukhin et al., 2000). They further demonstrated that actin polymerization at the tip of the protrusions plays an active role in extending the cell-cell contact area. Therefore, the regulation of F-actin polymerization and reorganization may be another target of aPKC phosphorylation to promote belt-like AJ formation. In fact, we observed that cortical bundle formation is blocked in aPKCλkn-expressing cells at an intermediate stage (Fig. 5) (Yonemura et al., 1995). However, we also observed that even the cells expressing aPKCλkn lacking continuous junctional structures increased their heights to a level (>10 μm) comparable to those of cells in non-wounded areas. The premature spot-like AJs and the premature cortical bundles are localized at the apical tip of the lateral membranes, at which neighboring cells are in close contact. Therefore, these results indicate that the cells expressing aPKCλkn can reorganize the actin filament architecture to some extent and physically draw neighboring cell surfaces together to assume a thick epithelia-specific columnar shape. Therefore, the incomplete cortical bundle formation of F-actin may be a secondary consequence of the inhibition of the fusion of the spot-like AJs into belt-like ones.

aPKC plays an indispensable role in the establishment of cell polarity not only in the *C. elegans* and *Drosophila* early embryo but also in mammalian epithelial cells by forming an evolutionarily conserved protein complex with PAR-3 and PAR-6. We have previously demonstrated that PAR-6 may mediate signals from Rac1/Cdc42 to aPKC by interacting with both proteins and activating aPKC in a GTP-dependent manner (Yamanaka et al., 2001). Since we observed that, as in the case of MDCK,

overexpression of a PAR-6 mutant lacking aPKC-binding domain also showed effects similar to those induced by aPKCkn during wound healing of MTD1A cells (data not shown) (Yamanaka et al., 2001), aPKC is thought to function as a component of the aPKC-PAR complex in promoting belt-like AJ formation. Here, we also found that an aPKC-binding scaffold protein with three PDZ domains, PAR-3, is substantially but not completely recruited into the premature spot-like AJ complex induced by aPKCλkn, suggesting that it can translocate to the structure without aPKC activity. These results indicate that PAR-3 works as a scaffold protein to recruit aPKC and PAR-6, which can act together to stabilize the complex at the junctional area. In fact, although indirectly, we observed that PAR-3 is recruited faster than aPKC into cell-cell contact regions during the normal wound healing process (Fig. 1). This is consistent with the observation that in the *C. elegans* one-cell embryo, PAR-3 is transiently present at the cell periphery even in the absence of either PKC-3 or PAR-6, although both PKC-3 and PAR-6 absolutely require PAR-3 (Hung and Kemphues, 1999; Tabuse et al., 1998; Watts et al., 1996). Considering the fact that the cytoplasmic region of JAM binds to the first PDZ domain of PAR-3, JAM, which is recruited into the junctional region as early as ZO-1, may be a membrane target of PAR-3 (Ebnet et al., 2001; Itoh et al., 2001,). Then, aPKC may target PAR-3 anchoring to JAM. We previously suggested the possibility that aPKC translocates to the junctional region as a ternary complex with PAR-3 and PAR-6 in MDCKII cells after a calcium switch, since a considerable amount of the ternary complex was detected even in depolarized MDCK II cells cultured in low calcium medium (Yamanaka et al., 2001). We do not know the precise reason for the apparent discrepancy between the present results and the previous data. However, it is possible that the asynchronous and rapid polarization of MDCK II cells after a calcium switch made it difficult to detect subtle differences between the recruitment of aPKC and that of PAR-3. It may also reflect differences between the depolarization states of cells induced by calcium depletion or mechanical wounding.

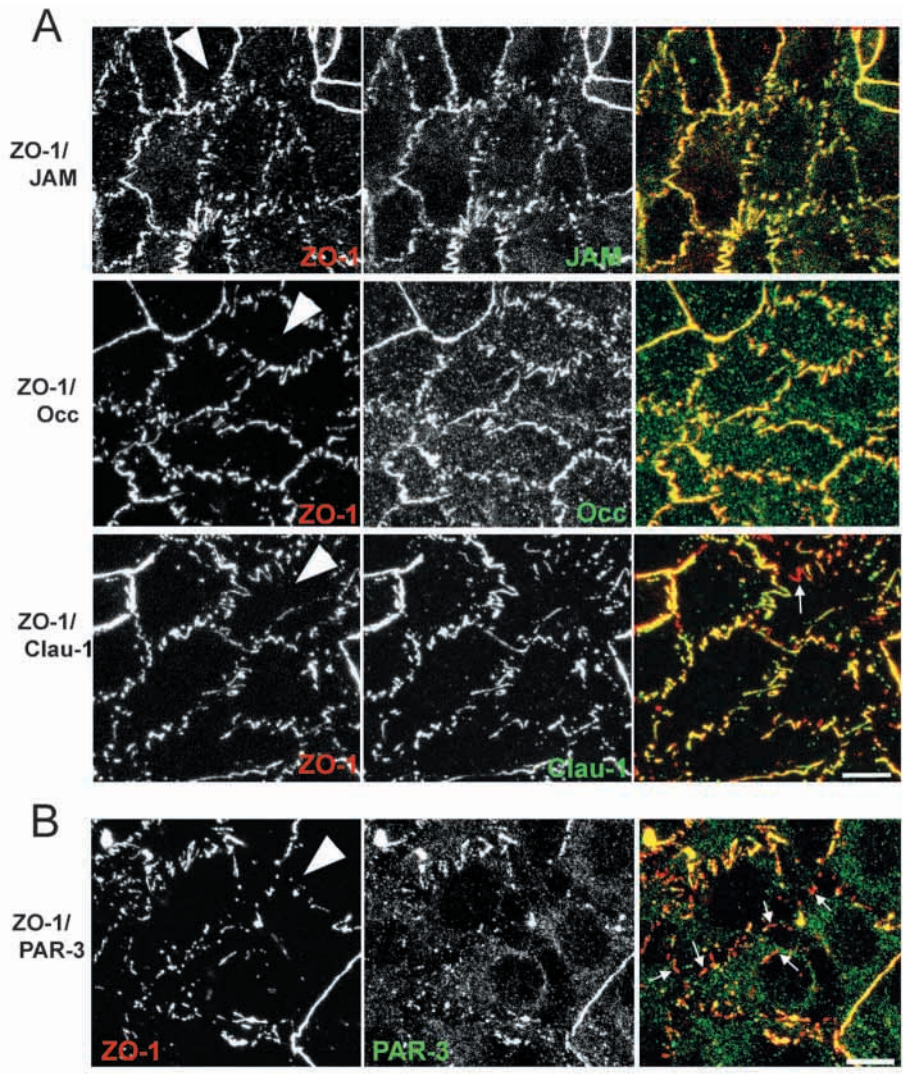

Fig. 6. Three TJ membrane proteins were also trapped in the persistent spot-like AJ of aPKCλkn-expressing cells. aPKCλkn-expressing cells were subjected to wounding, fixed 30 hours after the wounding and doubly immunostained with pAbs against TJ proteins or PAR-3 and ZO-1 mAb, as indicated. Shown are enlarged views of cells burying the wound or their merged views. JAM, occludin and claudin-1 are all colocalized with ZO-1 in the persistent spot-like AJs (A). As shown in B, a substantial number of the spot-like AJs induced by aPKCλkn are also positive for PAR-3, although the extent of the colocalization is relatively low (small arrows indicate difference in staining between PAR-3 and ZO-1). Bars, 10 μm.

In summary, the present work reveals that aPKC is required at the step where the immature cell-cell junctional complex differentiates into epithelia-specific asymmetric junctional structures. This finding supports our hypothesis that the aPKC-PAR system plays an indispensable role in the establishment of cell polarity by primarily regulating the formation of an asymmetric submembranous structure to which it anchors. Interestingly, recent progress in the genetic and molecular analysis of *Drosophila* or *C. elegans* embryos also revealed that epithelial junctions are assembled in a two-step process, that is, the formation of spot-like AJs along the lateral membrane rather randomly and their accumulation into the apical tip of the membrane and development into belt-like AJs (Michaux et al., 2001; Tepass et al., 2001). Many mutants of polarity proteins including BAZOOKA, a *Drosophila* homologue of PAR-3, have been reported to block the transition between these two steps, indicating that this transition, which is indispensable for asymmetric membrane domain differentiation is the critical step for epithelial cell polarization. Many polarity proteins are expected to interact in a complex fashion during this step. There is no doubt that the

identification of the molecular target of aPKC is one of the important steps to resolve the molecular basis for this epithelial cell polarization process.

We thank S. Tsukita and Y. Takai for providing us with the MTD1-A cell line and anti-nectin antibody, respectively. This work was supported by grants from the Japan Society for the Promotion of Science (to S.O.) and the Ministry of Education, Culture, Sports, Science and Ttechnology of Japan (to S.O. and A.S.).

References

Adams, C. L., Chen, Y. T., Smith, S. J. and Nelson, W. J. (1998). Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J. Cell Biol.* **142**, 1105-1119.

Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K. and Ohno, S. (1994). A new member of the third class in the protein kinase C family, PKC lambda, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. *J. Biol. Chem.* **269**, 12677-12683.

Ando-Akatsuka, Y., Yonemura, S., Itoh, M., Furuse, M. and Tsukita, S. (1999). Differential behavior of E-cadherin and occludin in their colocalization with ZO-1 during the establishment of epithelial cell polarity. *J. Cell Physiol.* **179**, 115-125.

Bilder, D. (2001). Cell polarity: squaring the circle. *Curr. Biol*. **11**, R132-R135.

Denker, B. M. and Nigam, S. K. (1998). Molecular structure and assembly of the tight junction. *Am. J. Physiol.* **274**, F1-F9.

Dodane, V. and Kachar, B. (1996). Identification of isoforms of G proteins and PKC that colocalize with tight junctions. *J. Membr. Biol.* **149**, 199-209.

Doe, C. Q. and Bowerman, B. (2001). Asymmetric cell division: fly neuroblast meets worm zygote. *Curr. Opin. Cell Biol*. **13**, 68-75.

Ebnet, K., Schulz, C. U., Meyer Zu Brickwedde, M. K., Pendl, G. G. and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J. Biol. Chem.* **275**, 27979-27988.

Ebnet, K., Suzuki, A., Horikoshi, Y., Hirose, T., Meyer Zu Brickwedde, M. K., Ohno, S. and Vestweber, D. (2001). The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). *EMBO J.* **20**, 3738-3748.

Enami, J., Enami, S. and Koga, M. (1984). Isolation of an insulin-responsive preadipose cell line and a mammary tumor virus-producing, dome-forming epithelial cell line from a mouse mammary tumor. *Dev. Growth Differ.* **26**, 223-234.

Gumbiner, B., Stevenson, B. and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J. Cell Biol.* **107**, 1575-1587.

Guo, S. and Kemphues, K. J. (1996). Molecular genetics of asymmetric cleavage in the early *Caenorhabditis elegans* embryo. *Curr. Opin. Genet. Dev.* **6**, 408-415.

Hamazaki, Y., Itoh, M., Sasaki, H., Furuse, M. and Tsukita, S. (2002). Multi-PDZ-containing protein 1 (MUPP1) is concentrated at tight junctions through its possible interaction with claudin-1 and junctional adhesion molecule. *J. Biol. Chem.* **277**, 455-461.

Hirano, S., Nose, A., Hatta, K., Kawakami, A. and Takeichi, M. (1987). Calcium-dependent cell-cell adhesion molecules (cadherins): subclass specificities and possible involvement of actin bundles. *J. Cell Biol.* **105**, 2501-2510.

Hung, T. J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos. *Development* **126**, 127-135.

Itoh, M., Furuse, M., Morita, K., Kubota, K., Saitou, M. and Tsukita, S. (1999). Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J. Cell Biol.* **147**, 1351-1363.

Itoh, M., Sasaki, H., Furuse, M., Ozaki, H., Kita, T. and Tsukita, S. (2001). Junctional adhesion molecule (JAM) binds to PAR-3: a possible mechanism for the recruitment of PAR-3 to tight junctions. *J. Cell Biol.* **154**, 491-497.

Izumi, Y., Hirose, T., Tamai, Y., Hirai, S., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kemphues, K. J. and Ohno, S. (1998). An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. *J. Cell Biol.* **143**, 95-106.

Michaux, G., Legouis, R. and Labouesse, M. (2001). Epithelial biology: lessons from *Caenorhabditis elegans*. *Gene* **277**, 83-100.

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**, 641-648.

Rose, L. S. and Kemphues, K. J. (1998). Early patterning of the *C. elegans* embryo. *Annu. Rev. Genet.* **32**, 521-545.

Stuart, R. O., Sun, A., Panichas, M., Hebert, S. C., Brenner, B. M. and Nigam, S. K. (1994). Critical role for intracellular calcium in tight junction biogenesis. *J. Cell Physiol.* **159**, 423-433.

Suzuki, A., Yamanaka, T., Hirose, T., Manabe, N., Mizuno, K., Shimizu, M., Akimoto, K., Izumi, Y., Ohnishi, T. and Ohno, S. (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**, 1183-1196.

Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K. J., Miwa, J. and Ohno, S. (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in *Caenorhabditis elegans*. *Development* **125**, 3607-3614.

Takahashi, K., Nakanishi, H., Miyahara, M., Mandai, K., Satoh, K., Satoh, A., Nishioka, H., Aoki, J., Nomoto, A., Mizoguchi, A. et al. (1999). Nectin/PRR: an immunoglobulin-like cell adhesion molecule recruited to cadherin-based adherens junctions through interaction with afadin, a PDZ domain-containing protein. *J. Cell Biol.* **145**, 539-549.

Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. (2001). Epithelial cell polarity and cell junctions in *Drosophila*. *Annu. Rev. Genet.* **35**, 747-784.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209-219.

Watts, J. L., Etemad-Moghadam, B., Guo, S., Boyd, L., Draper, B. W., Mello, C. C., Priess, J. R. and Kemphues, K. J. (1996). par-6, a gene involved in the establishment of asymmetry in early *C. elegans* embryos, mediates the asymmetric localization of PAR-3. *Development* **122**, 3133-3140.

Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J. Cell Biol.* **150**, 1361-1374.

Wodarz, A., Ramrath, A., Kuchinke, U. and Knust, E. (1999). Bazooka provides an apical cue for Inscuteable localization in *Drosophila* neuroblasts. *Nature* **402**, 544-547.

Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H. et al. (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. *Genes Cells* **6**, 721-731.

Yeaman, C., Grindstaff, K. K. and Nelson, W. J. (1999). New perspectives on mechanisms involved in generating epithelial cell polarity. *Physiol. Rev.* **79**, 73-98.

Yonemura, S., Itoh, M., Nagafuchi, A. and Tsukita, S. (1995). Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epithelial cells. *J. Cell Sci.* **108**, 127-142.