Polarity proteins PAR6 and aPKC regulate cell death through GSK-3β in 3D epithelial morphogenesis

Minji Kim¹, Anirban Datta¹, Paul Brakeman², Wei Yu¹ and Keith E. Mostov¹,*
¹Departments of Anatomy, and Biochemistry and Biophysics, University of California School of Medicine, San Francisco, CA 94158, USA
²Department of Pediatrics, University of California School of Medicine, San Francisco, CA, USA
*Author for correspondence (e-mail: keith.mostov@ucsf.edu)

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Summary
Epithelial cells are polarized, with an apical surface facing a lumen or outer surface and a basolateral surface facing other cells and extracellular matrix (ECM). Hallmarks of epithelial carcinogenesis include loss of polarity, as well as uncontrolled proliferation and resistance to apoptosis. Are these features controlled by a common molecular mechanism? The partitioning-defective 3 (PAR3)-PAR6-atypical PKC (aPKC) complex is a master regulator that controls polarization in many animal cells. Here we show that PAR6 is involved in apoptosis by regulating aPKC and glycogen synthase kinase 3β (GSK-3β) activity. During epithelial morphogenesis in 3D culture of Madin-Darby canine kidney (MDCK) cells, expression of an N-terminally deleted PAR6 (PAR6ΔN) leads to a significant increase in caspase-dependent cell death by downregulating aPKC activity. Accordingly, inhibition of aPKC in wild-type (WT) MDCK cells with either a cell-permeable PKCζ pseudosubstrate or RNAi promotes apoptosis, which suggests that PAR6 regulates apoptosis via an aPKC-mediated pathway. GSK-3β, a substrate of aPKC, is hyper-activated by expressing PAR6ΔN. GSK-3β inhibitors block PAR6ΔN-induced apoptosis while expression of constitutively active GSK-3β (S9A) promotes apoptosis, which is rescued by ectopic expression of aPKC. We conclude that a PAR6–aPKC–GSK-3β mechanism links cell polarity and apoptosis.

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Introduction
The PAR (partitioning defective) complex was first identified in C. elegans by its requirement for asymmetric cell division (Guo and Kemphues, 1996) and is involved in various cell polarity-related processes, such as asymmetric cell division (Betschinger et al., 2003; Cai et al., 2003), directed cell migration (Etienne-Manneville and Hall, 2003; Plant et al., 2003), axon specification (Shi et al., 2003) and tight junction formation (Lin et al., 2000; Suzuki et al., 2001). PAR6 has a central scaffolding and regulatory function through its interactions with PAR3, atypical protein kinase C (aPKC) and the Cdc42 GTPase. The N-terminus of PAR6 contains an OPC/PC motif within a PB1 domain that heterodimerizes with aPKC (Hirano et al., 2005). A deletion of the PB1 domain of PAR6 prevents its interaction with aPKC, and results in its incomplete localization (Noda et al., 2003). The PAR6 PB1 domain is followed by a CRIB motif, which engages the GTP-bound form of Cdc42 (Joberty et al., 2000; Lin et al., 2000). In addition to binding to PAR3 via a PDZ domain (Joberty et al., 2000), PAR6 interacts with at least two additional proteins, Lgl (Plant et al., 2003; Yamanaka et al., 2003) and PALS (Hurd et al., 2003), components of the Scribble-Dlg and Crumbs-PATJ polarity complexes, respectively.

Atypical PKC isozymes λ (λ in human) and ζ, which are 72% identical (Akimoto et al., 1994), have been implicated in several signaling pathways, including cell polarity, cell survival and cell differentiation. P62/ZIP, another protein that interacts with the N-terminus of aPKC, has been shown to recruit aPKC into a TNF-α receptor complex and an NGF receptor complex, transducing signals for cell survival (Mamidipudi and Wooten, 2002; Sanz et al., 1999). PKCζ has also been described to protect cells against FasL-induced apoptosis in Jurkat cells (Leroy et al., 2005). Recent studies have linked inappropriate expression of aPKCζ to the development of human non-small cell lung cancer (Regala et al., 2005). PKCζ−/− mice are known to be embryonic lethal at very early stages (Bandypadhyay et al., 2004; Soloff et al., 2004). Thus, aPKC has been implied to be closely related to the signaling pathways that regulate cell survival.

Three-dimensional (3D) epithelial culture systems, which allow epithelial cells to organize themselves into structures that resemble their in vivo architecture, have emerged as unique models that allow investigation of the orientation of epithelial cell polarity and other signaling pathways. Although lumen formation can spontaneously occur within small epithelial clusters coinciding with the establishment of apico-basal polarity (Debnath and Brugge, 2005; Hall et al., 1982; O’Brien et al., 2002), apoptosis also contributes to lumen formation in several 3D models. Indeed, apoptotic cells are present in the developing lumen of salivary gland and mammary spheroids, as well as Madin-Darby canine kidney (MDCK) cysts (Debnath et al., 2003; Hoffman et al., 1996; Huang et al., 1999; Lin et al., 1999).

Using this model system, we now demonstrate that the
PAR6-aPKC complex regulates cell death and/or proliferation during MDCK cyst formation via glycogen synthase kinase 3β (GSK-3β). PAR6ΔN expression inhibits TJ (tight junction) assembly during formation of polarity in a calcium switch assay in 2D (monolayer) culture (Gao et al., 2002). Here we used MDCK cells grown as cysts in 3D gels of extracellular matrix, which more closely resembles in vivo conditions. We embedded cells grown as cysts in collagen gels and continued for at least 13 days of culture on collagen and continued for at least 13 days of cyst formation, as shown by immunoblotting for PAR6ΔN cysts. Differences in MDCK behavior between 2D and 3D culture. Since PAR6ΔN cysts maintain a normal size compared with the control, we investigated whether proliferation also increased. We therefore immunostained with Ki-67, a marker for cycling cells and found that PAR6ΔN cysts had increased PAR6-aPKC complex plays a surprising and critical role in controlling cell survival.

Results
An N-terminally deleted PAR6 (PAR6ΔN) induces increased apoptosis

Previous work has shown that overexpression of PAR6B or a mutant PAR6B lacking the N-terminus (containing residues 102-373; PAR6ΔN) inhibits TJ (tight junction) assembly during formation of polarity in a calcium switch assay in 2D (monolayer) culture (Gao et al., 2002). Here we used MDCK cells grown as cysts in 3D gels of extracellular matrix, which more closely resembles in vivo conditions. We embedded cells that stably express Myc-tagged PAR6ΔN in collagen gels and allowed cysts to develop for 7 days. While control cysts had a uniform monolayer surrounding a hollow lumen, PAR6ΔN cysts had multilayers of cells in the wall and cells in the cysts. The PAR6ΔN cells forming the outer layer appeared at least somewhat polarized, with ZO-1 localized to the TJ region between apical and basolateral surfaces, and β-catenin at lateral surfaces (Fig. 1A). These data differ somewhat from results previously reported for MDCK cells expressing PAR6ΔN and grown as a 2D monolayer on filters. The reason for this difference is probably due to the well-known differences in MDCK behavior between 2D and 3D culture. Overexpression of wild-type PAR6 or PAR6 containing a mutation in the PDZ domain, which does not bind PAR3, yielded cysts that were not detectably different from WT MDCK (M.K., unpublished).

Interestingly PAR6ΔN cysts showed nuclear condensation and fragmentation in some cells both in the lumen and outside the cyst (Fig. 1A, arrowhead and arrow). Results were consistent among three independent clones and the effect was dependent on the level of PAR6ΔN expression (supplementary material Fig. S1). To determine whether these nuclear changes were due to apoptosis we stained cysts for cleaved caspase-3 positive cells in the lumen and periphery (80.9±2.7% of cysts), while only a few (15±2.6%) control cysts exhibited cleaved caspase-3 positive cells (Fig. 1B). An ELISA for cleaved caspase-3 showed a 3.5-fold increase in cell death (Fig. 1C). Based on their rounded morphology, condensed chromatin, and expression of activated caspases, these displaced cells were undergoing apoptotic death. The caspase-3 activity was significantly increased in PAR6ΔN cysts as early as day 5 of culture on collagen and continued for at least 13 days of cyst formation, as shown by immunoblotting for cleaved caspase-3 (Fig. 1D).

To confirm whether caspase is needed for PAR6ΔN-induced apoptosis, we added the permeable caspase inhibitor, zVAD-fmk to PAR6ΔN cysts. DMSO carrier had no effect, whereas zVAD-fmk abolished increased cell death in PAR6ΔN (Fig. 1E). This inhibitory effect of zVAD-fmk on PAR6ΔN-induced apoptosis was also confirmed by in situ cell death detection assay, based on labeling of DNA strand breaks (supplementary material Fig. S2). Taken together, these data show that PAR6ΔN causes caspase-dependent apoptosis during cyst formation. Since PAR6ΔN cysts maintain a normal size compared with the control, we investigated whether proliferation also increased. We therefore immunostained with Ki-67, a marker for cycling cells and found that PAR6ΔN cysts had increased cell death ELISA. Results are the means ± s.d. of three experiments. (D) Wild-type MDCK and PAR6ΔN lysates isolated from cysts were prepared at different time points (day 5, 7 and 13) and then analyzed for activation of caspase-3 using cleaved caspase-3 and GAPDH antibodies (loading control). (E) PAR6ΔN cysts were treated with either a carrier (DMSO) or caspase inhibitor, zVAD-fmk (50 μM) three times at day 0, 4 and 6 and immunostained for cleaved caspase-3 (red) at day 7, as described in A. Bars, 10 μm.

![Image](https://example.com/image1.png)

Fig. 1. PAR6ΔN promotes apoptosis in MDCK cystogenesis. (A,B) Wild-type MDCK and Myc-tagged PAR6ΔN-expressing stable cell lines grown on collagen for 7 days were fixed, permeabilized and immunostained for Myc (green), β-catenin (red) and ZO-1 (white) (A) and for Myc (green), actin (red) and cleaved caspase-3 (white) (B). White arrow and arrowhead indicate dead cells in A. Nuclei are shown as blue in all figures. (C) The levels of apoptosis were measured using a cell death ELISA. Results are the means ± s.d. of three experiments. (D) Wild-type MDCK and PAR6ΔN cysts had increased apoptosis (supplementary material Fig. S1). To determine whether these nuclear changes were due to apoptosis we stained cysts for cleaved caspase-3 as a marker of apoptosis. PAR6ΔN cysts exhibited cleaved caspase-3-positive cells in the lumen and periphery (80.9±2.7% of cysts), while only a few (15±2.6%) control cysts exhibited cleaved caspase-3 positive cells (Fig. 1B). An ELISA for cleaved caspase-3 showed a 3.5-fold increase in cell death (Fig. 1C). Based on their rounded morphology, condensed chromatin, and expression of activated caspases, these displaced cells were undergoing apoptotic death. The caspase-3 activity was significantly increased in PAR6ΔN cysts as early as day 5 of culture on collagen and continued for at least 13 days of cyst formation, as shown by immunoblotting for cleaved caspase-3 (Fig. 1D).
cells with a higher expression level, more of the GFP-PAR6B is cytoplasmic (supplementary material Fig. S4, arrowhead). The localization of GFP-PAR6ΔN showed a similar pattern of accumulation at the apical region of cells in cysts (supplementary material Fig. S4, lower arrow).

Lethal giant larvae (Lgl), a protein that interacts with both PAR6 and aPKC and which functions as a substrate of aPKC, is normally found at the basolateral plasma membrane in an aPKC activity-dependent manner (Chalmers et al., 2005; Hutterer et al., 2004; Yamanaka et al., 2003). We observed that overexpression of PAR6ΔN delocalized the mammalian Lgl (mLgl) from the basolateral plasma membrane partially to the cytosol and partially to apical plasma membrane (Fig. 2B, lower panel). This is consistent with the observation that the localization of aPKC is altered by overexpression of PAR6ΔN and indicates that although ZO-1 and β-catenin are correctly polarized, PAR6ΔN induces some degree of alteration in polarity in mature 3D cysts.

These data also raised the possibility that PAR6ΔN expression caused an increase in apoptosis by altering the activity of aPKC. aPKC has been implicated not only in cell polarity but also in cell survival. Earlier work has shown that the N-terminal region of PAR6 is essential for cell transformation by oncogenic Ras or Rac1 (Qiu et al., 2000). To investigate the mechanism by which PAR6ΔN mediates apoptosis, we determined that aPKC is phosphorylated at residues Thr410/403, which provides an indicator of kinase activity (autophosphorylation) of aPKC. We found that aPKC Thr410/403 phosphorylation was decreased by ~40% in PAR6ΔN cysts (Fig. 3A).

To confirm that increased apoptosis resulted from a reduction in aPKC activity, we used a cell-permeable, myristoylated PKCζ pseudosubstrate peptide (ZI), which directly inhibits aPKC autophosphorylation and transactivation. ZI is commonly used to block both aPKCζ and aPKCζ isoforms because of their high homology. MDCK cells predominantly express aPKCζ. We incubated WT MDCK with 40, 50 or 60 μM ZI (or the non-myristoylated form as a control), starting at day 4 of cyst formation, when epithelial polarity was already established. Apoptotic cells were dose-dependently increased by ZI treatment (Fig. 3B,C). Earlier addition of ZI at the time of plating showed much higher apoptosis, reaching 95% at 60 μM (M.K., unpublished).

As an alternative approach to reduce aPKC function, we transiently transfected MDCK cells with an aPKC RNAi construct (Suzuki et al., 2004). The RNAi effect peaks at day 3, reducing its level to less than 10% according to western-blot analysis (Fig. 4A). We therefore grew the cysts in Matrigel, which allows cysts to form much faster than in collagen. In control cysts after 3 days in Matrigel, ZO-1 was localized at the TJ and aPKCζ was enriched at the apical region, which is consistent with our previous results with cysts grown in collagen (Fig. 4B, upper). Cysts transfected with aPKCζ RNAi (but otherwise WT, i.e. not transfected with PAR6ΔN) lost apical staining for aPKCζ and displayed activated caspase-3 in the center of lumen (Fig. 4B, lower). The apoptotic cells were increased in a dose-dependent manner, showing 50% of cysts were affected by 5 μg of aPKCζ RNAi (Fig. 4C). Taken together, these data suggest that PAR6ΔN causes increased apoptosis by inactivation of aPKCζ and that suppression of aPKCζ induces apoptosis.
During otherwise normal cyst development, these results are consistent with a report that aPKC depletion caused increased cell death at the gastrula stage in *Xenopus* development (Chalmers et al., 2005; Moscat and Diaz-Meco, 2004). The differences may be due to our use of 3D culture in Matrigel, differing extents of depletion or other factors.

**GSK-3β functions downstream of PAR6-aPKC as the mediator of cell death signals**

A downstream target of the PAR6-PKCζ-Cdc42 complex in the regulation of migrating astrocyte polarity is GSK-3β (Etienne-Manneville and Hall, 2003). Recent studies have shown that upregulation of GSK-3β activity can lead to cell death and aberrant neuronal migration in primary neuron cultures (Maggiwar et al., 1999; Tong et al., 2001). To test whether GSK-3β is involved in PAR6-aPKC-mediated apoptosis, we examined activation of GSK-3β. Phosphorylation of GSK-3β at serine-9 inhibits its activity. In PAR6ΔN cysts, phosphorylation of GSK-3β is decreased, indicating that GSK-3β is more active (Fig. 5A). LiCl and SB216763, inhibitors of GSK-3, partially, but significantly, reversed the increased apoptosis induced by PAR6ΔN (Fig. 5B). With PAR6ΔN alone, only 25.3% of PAR6ΔN cysts had no activated caspase-3-positive cells, whereas with SB216763 or LiCl, 51.2 or 67.3% of cysts, respectively, had no activated caspase-3 positive cysts (P<0.05). This is consistent with the notion that GSK-3β has a proapoptotic function in mammalian cells (Hetman et al., 2000; Pap and Cooper, 1998) and GSK-3β function is required for apoptosis of nurse cells during gametogenesis (Rentzsch et al., 2005).

As an additional way of examining the role of GSK-3β in apoptosis, we infected MDCK cells with recombinant adenovirus expressing WT or mutant GSK-3β and confirmed expression of the constructs by western blotting of the HA epitope tag on the constructs (Fig. 6C). Ad–HA–GSK-3β(K85M) encodes a kinase-dead form of the enzyme, while Ad–HA–GSK-3β(S9A) encodes a mutant that cannot undergo inhibitory phosphorylation at Ser9. In contrast to Ad-LacZ or Ad–HA–GSK-3β(K85M), expression of Ad–HA–GSK-3β(S9A) led to increased apoptosis in MDCK cells that had not been transfected with PAR6ΔN (Fig. 6A,B). These results strongly suggest the involvement of GSK-3β-mediated cell death in cyst formation. In addition, this phenotype is partially rescued by co-expression of wild-type aPKC (Fig. 7A,B). It has been reported that treatment with a aPKC inhibitor abrogated GSK-3β phosphorylation (Wu et al., 2006). To further confirm this, we inhibited aPKC activity using ZI and measured GSK-3β activity by staining with phospho-GSK-3β. The amount of phospho-GSK-3β was decreased, as indicated by some cells that had no visible staining (arrow in Fig. 7C). Conversely, overexpression of wild-type aPKC increases GSK-3β phosphorylation (Fig. 7D, arrow indicates cells with overexpression of GFP- aPKC and increased phospho-GSK-3β). Taken together, our results strongly suggest that PAR6-aPKC regulates apoptosis through modulation of GSK-3β activity.

To analyze a potential mechanism whereby active GSK-3β exerts its apoptotic effects, we measured phosphorylation of Jun N-terminal kinase (JNK). JNK plays an important role in signal transduction pathways: JNK expression is increased in response to stress, cytokines and many anti-cancer drugs (Chen
et al., 1996; Oshima et al., 1996; Stone and Chambers, 2000), and JNK is also required for some forms of stress-induced apoptosis (Tournier et al., 2000). By contrast, the constitutive biological function of JNK is to promote cell survival or growth (Ip and Davis, 1998). In agreement with a previous study showing that JNK is activated after MDCK detachment (Hideshima et al., 2003), we found that JNK was activated in PAR6\(\Delta N\) (Fig. 7E). We attempted to inhibit JNK pharmacologically, for example with SP-600125, which is the most widely used JNK-specific inhibitor. However, we found that even a concentration of SP-600125 of 20 \(\mu M\) inhibited viability, which prevented us from determining whether there was a specific effect on GSK-3\(\beta\)-regulated apoptosis (M.K., unpublished). It has been suggested that GSK-3\(\beta\) acts as a positive mediator of stress- and proinflammatory cytokine-induced JNK activation (Kim et al., 2003). It is also interesting to note a recent report that JNK signaling is activated in polarity-deficient cells and, in the presence of oncogenic Ras, JNK switches its proapoptotic role to a progrowth effect (Igaki et al., 2006). Taken together, our results are consistent with the idea that PAR6–aPKC–GSK-3\(\beta\) altered JNK activity, which may have compromised cell survival during MDCK cyst formation.

**Discussion**

Despite recent studies revealing a link between epithelial polarization and proliferation (Aranda et al., 2006; Bilder, 2004), the role of PAR6 as an effector of signal transduction
in this area is only just beginning to be understood (Aranda et al., 2006). In the present study, we found that overexpression of an N-terminal deletion mutant of PAR6 induced significant cell death during MDCK cyst formation; this resulted from the inactivation of aPKC and hyperactivation of GSK-3β.

Lumen formation by apoptosis, which occurs in 3D cultures of kidney (O’Brien et al., 2002) and mammary epithelium (Debnath et al., 2002; Muthuswamy et al., 2001), is believed to reflect physiological death occurring during development and homeostasis of these tissues. Alterations in cell death during development result in compromised functioning of the developing organism and are also involved in a number of pathological processes, such as cystic diseases of the kidney (e.g. polycystic kidney disease) and other organs, as well as cancer. Combined inhibition of apoptosis and hyper-stimulation of proliferation leads to a lumen-filling phenotype in a model of early mammary carcinogenesis (Debnath et al., 2002). Cells in the middle of the lumen may die due to anoikis, a type of cell death caused by detachment from the basement membrane. However, PAR6ΔN produced activated caspase-positive dead cells both in the lumen and outside the cysts, a phenomenon that, to our knowledge, was not reported in work that we observe in response to PAR6ΔN expression. aPKC phosphorylates Lgl and restrict its activity to the basal side so that determinants are therefore probably less suitable for studying the connection between polarity and apoptosis.

PAR6ΔN produced only a partial disruption of polarity in cysts. ZO-1 and β-catenin were normal, although mLgl and aPKCa were mislocalized. aPKC phosphorylates Lgl and restrict its activity to the basal side so that determinants are only recruited to that portion of the cell cortex in Drosophila (Betschinger et al., 2003). Similar observations have been described in MDCK (Musch et al., 2002). This model of regulation of mLgl localization by aPKC is reminiscent of the finding that Par1 localization is also regulated in a phosphorylation-dependent manner by aPKC (Hurov et al., 2004; Suzuki et al., 2004). Despite the localization defects of aPKC and mLgl, however, PAR6ΔN does not induce defects in cyst formation (Fig. 2). This is consistent with a previous study that a non-phosphorylated form of mLgl does not markedly influence the development of a polarized phenotype in MDCK cells, although the localization of the mutant protein is affected (Musch et al., 2002). We cannot completely exclude the possibility that the mislocalization of mLgl induced by PAR6ΔN is somehow responsible for the increase in apoptosis that we observe in response to PAR6ΔN.

The mechanism by which the kinase activity of aPKC is regulated has not been clearly determined. Our finding that PAR6ΔN expression blocks autophosphorylation of aPKC and reduces the activity of aPKC (Fig. 3A) raises the possibility that PAR6ΔN expression induces a conformational change of the PAR6-aPKC complex to make the PB1 domain of aPKC free, which then functions as a dominant negative (Regala et al., 2005). Alternatively, others have proposed a model in which PAR6 exists in an equilibrium between an inactive and an active state, and activation can be artificially induced by removing the N-terminal domain of PAR6 (Gao et al., 2002).

We suggest that PAR6ΔN activates GSK-3β through the
Role of PAR6-aPKC in MDCK cyst formation

inhibition of aPKC. PKCζ has been shown to phosphorylate GSK-3β in vitro (Etienne-Manneville and Hall, 2003). Work performed in Xenopus suggested that LKB1 promotes the inhibition of GSK-3β via PKCζ (Ossipova et al., 2003). A recent report showed that PKCζ inhibitors completely abrogated GSK-3β phosphorylation (Wu et al., 2006). These results support our observation that GSK-3β has a pro-apoptotic function via aPKC.

What downstream molecules might be involved in this pathway? One candidate could be JNK. Indeed we found that JNK was activated in PAR6N cysts (Fig. 7E). Although we could obtain this correlative evidence, we were unable to inhibit JNK pharmacologically or by RNAi (M.K., unpublished) without compromising cell survival. Further work is needed to demonstrate directly a causal relationship. Another candidate could be NF-κB, which has been shown to connect tissue polarity and apoptosis resistance in mammary epithelial cells (MECs) (Weaver et al., 2002). In addition, aPKC mediates the activation of NF-κB (Sanz et al., 1999) and GSK-3β induces apoptosis mediated by inhibition of the NF-κB pathway in astrocytes (Sanchez et al., 2003). However, the incubation of WT MDCK cysts with a cell-permeable soluble peptide SN50, which specifically inhibits nuclear translocation of NF-κB, failed to induce caspase-dependent apoptosis (M.K., unpublished). This suggests that NF-κB is not involved in PAR6–aPKC–GSK-3β-complex-mediated apoptosis.

Here we provide evidence that a PAR6–aPKC–GSK-3β pathway controls not only polarization, but also caspase-dependent cell death. Overexpression of PAR6ΔN leads to reduction of aPKC activity, which in turn causes hyperactivation of GSK-3β; this sequence results in apoptosis in our 3D epithelial culture system. Our results provide a molecular mechanism connecting polarity and apoptosis during epithelial morphogenesis.

Materials and Methods

Materials

The mouse anti-Myc, anti-HA and anti-β-galatosidase (LacZ) antibodies were obtained from Santa Cruz Biotechnology, Convance and Roche respectively. Mouse anti-ZO-1, anti-Ki67 (Zymed), rabbit anti-β-catenin (Santa Cruz Biotechnology), mouse anti-phospho-JNK, rabbit anti-cleaved-caspase-3, anti-JNK, anti-phospho-PKCζ and anti-phospho-GSK-3β (Ser9) (all Cell Signaling Technology)
antibodies were used. Mouse anti-PKCα and anti-GSK-3β antibodies were purchased from BD Biosciences. Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Biodesign International. Rabbit anti-PAR6β and anti-MLC glutathione S-transferase (GST) antibodies were kindly provided by I. Macara (University of Virginia, Charlottesville, VA) and T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario), respectively. Secondary antibodies conjugated to Alexa Fluor-488, -555 and -647 were used for immunostaining experiments (Molecular Probes). Nuclei were labeled with Hoechst 33342 (Molecular Probes). z-VAD-fmk, SB216763 (Calbiochem), LiCl (Fisher) and PKCζ pseudosubstrate (Biosource) were used for immunostaining experiments (University of Virginia, Charlottesville, VA) and T. Pawson (Samuel Lunenfeld Research Institute, Toronto, Ontario). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Biodesign International. p-MLC antibodies (Ser19) were a kind gift of M. Birnbaum, University of Rochester Medical Center, Rochester, NY, and I. Macara and maintained in growth medium with 100 μg of HygromycinB, as in the previous studies (Gao et al., 2002). Mammalian PAR6β was excised from pKmyc (PAR6β) with HindIII and EcoRI and cloned into pEGFP-C3 with Xhol and EcoRI sites. GFP-PAR6β, GFP-PAR6ΔN or GFP-aPKCα was transfected was transfected by Lipofectamine2000 (Invitrogen). Cyst cultures were prepared as described previously (O'Brien et al., 2001). To treat cysts with inhibitors, z-VAD-fmk was added to the culture medium three times at day 0, 4 and 6. The treatment of aPKCζ pseudosubstrate, SB216763 or LiCl respectively was performed at day 4 and refreshed at day 6 after plating cysts in collagen gel.

RNAi
Generation of pSUPER constructs expressing shRNA targeting canine aPKC has been described previously (Suzuki et al., 2004). For RNAi in MDCK cells, pSUPER constructs expressing gene-specific shRNA were introduced into MDCK cells by nucleofection (Amaxa). 2×10^6 cells were resuspended in 100 μl of nucleofector solution with 2 μg of DNA and electroporated with program T23. After letting cells rest in MEM overnight, either control or aPKC RNAi cells were plated on a solution with 2 μg/ml of DNA. Cells were embedded and cultured in collagen gel on ultra low cluster plates (Costar) at a density of 1×10^5 cells/ml for 7 days. Cells were then analyzed using an Odyssey Infrared Imager (LI-COR).

Cell culture
Madin-Darby canine kidney (MDCK) cells were maintained in minimal essential medium (MEM) containing Earle’s balanced salt solution (Cellgro) supplemented with 5% FBS and antibiotics in 5% CO2 and 95% air. PAR6ΔN MDCKII stable cell lines were kindly provided by L. Gao (University of Virginia, Charlottesville, VA) and I. Macara and maintained in growth medium with 100 μg of HygromycinB, as in the previous studies (Gao et al., 2002). Mammalian PAR6β was excised from pKmyc (PAR6β) with HindIII and EcoRI and cloned into pEGFP-C3 with Xhol and EcoRI sites. GFP-PAR6β, GFP-PAR6ΔN or GFP-aPKC was transfected by Lipofectamine2000 (Invitrogen). Cyst cultures were prepared as described previously (O’Brien et al., 2001). To treat cysts with inhibitors, z-VAD-fmk was added to the culture medium three times at day 0, 4 and 6. The treatment of aPKCζ pseudosubstrate, SB216763 or LiCl respectively was performed at day 4 and refreshed at day 6 after plating cysts in collagen gel.

Cell death and proliferation analysis
Cells were embedded and cultured in collagen gel on ultra low cluster plates (Costar) at a density of 1×10^5 cells/ml for 7 days. Cells were then lysed for 30 minutes. The level of DNA fragmentation was quantified using the Cell Death ELISA kit (quantifying histone-associated DNA fragments) (Roche). For the cell proliferation assay, after culturing cells as described above, CyQUANT GR dye/cell lysis buffer (CyQUANT cell proliferation kit) (Molecular Probes) were added. The fluorescence was measured using a filter for 480 nm excitation and 520 nm emission.

Adenovirus infection
MDCK cysts were infected with recombinant adenovirus vectors at an MOI of 2000 cells/cyst for 7 days. Virus was removed and the cells were let for 24 hours with 5% FBS and antibiotics in 5% CO2 and 95% air. Lipofectamine2000 (Invitrogen). Cyst cultures were prepared as described previously (O’Brien et al., 2001). To treat cysts with inhibitors, z-VAD-fmk was added to the culture medium three times at day 0, 4 and 6. The treatment of aPKCζ pseudosubstrate, SB216763 or LiCl respectively was performed at day 4 and refreshed at day 6 after plating cysts in collagen gel.

Western blotting
Cysts were isolated from collagen by collagenase treatment for 20 minutes at 4°C. Lysates of isolated cysts were normalized for protein concentration using a BCA assay (Pierce Chemical). The concentration of SDS in the samples was adjusted to 0.5%, and samples were normalized based on the protein concentration of isolated cysts. Proteins were visualized using ECL reagent (Perkin Elmer Life Sciences). GAPDH was used as control for lysates of the isolated cysts. For detection of adenoviral expression, lysates were immunoblotted for primary antibody followed by a goat anti-mouse IRDye 800 and a goat anti-rabbit Alexa Fluor 680 secondary; they were then analyzed using an Odyssey Infrared Imager (LI-COR).
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