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Protein Kinase C λ/ι (PKCλ/ι): A PKC Isotype Essential for the Development of Multicellular Organisms

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PKCλ/ι belongs to the third group of the PKC family, atypical PKC (aPKC), together with PKCζ based on its sequence divergence from conventional and novel PKCs observed not only in the N-terminal regulatory domain but also in the kinase domain. Although one of the most distinct features of aPKC is its single, unrepeated cysteine-rich domain, recent studies have revealed that the N-terminal regulatory domain has additional aPKC-specific structural motifs involved in various protein–protein interactions, which are important for the regulation and the subcellular targeting of aPKC. The identification of aPKC-specific binding proteins has significantly facilitated our understanding of the activation mechanism as well as the physiological function of aPKC at the molecular level. In particular, the finding that the mammalian homologs of the *Caenorhabditis elegans* proteins, PAR-3 and PAR-6, bind aPKC unexpectedly opens a new avenue for exploring a thus far completely unrecognized critical function of aPKC, that is, as a component of an evolutionarily conserved cell polarity machinery. Together with the great progress in the genome project as well as in the genetic analysis of model organisms, these advances are leading us into the new era of aPKC study in which functional divergence between PKCλ/ι and ζ can be discussed in elaborately.

Key words: cell polarity, PI3K, PAR, PKCλ, PKCι.

Abbreviations: DG, diacylglycerol; PKC, protein kinase C; aPKC, atypical protein kinase C; PIP3, phosphatidylinositol-3,4,5-$(PO_4)_3$; PI3K, phosphoinositide 3-kinase; PDK-1, phosphoinositide-dependent kinase 1; TNFα, tumor necrosis factor α; IL-1, interleukin-1; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; ASIP, aPKC-specific interacting protein; TJ, tight junction; AJ, adherens junction

Atypical molecular structure and features of PKCλ/ι in the PKC family

The PKC λ/ι isotype was cloned as the 10th member of the PKC family in the beginning of the 1990's based on the amino acid sequence similarity of their kinase domain with those of other PKC members [human PKCι (*1*) and mouse PKCλ (*2*) are orthologs with 98% overall amino acid sequence identity and thus are referred to as PKC λ/ι, hereafter]. Similar to PKCζ that had been cloned in 1989 (*3*), it shows a unique N-terminal structure with a single cysteine-rich sequence, not repeated in tandem as observed in traditional PKC isotypes (Fig. 1) (*4*). Comparison of the amino acid sequence identities in the conserved regions also supports the notion that PKCλ/ι is a close relative of PKCζ: as for the kinase domain, PKCλ/ι shows 86% identity with PKCζ and 45–55% with other PKCs (*2*). The cysteine-rich sequence of PKCλ/ι also shows higher amino acid sequence identity with PKCζ (69%) than with other PKCs (30–40%) (*2*). Based on these results, PKCλ/ι and PKCζ were categorized into the third group of the PKC family, atypical PKC (aPKC) (*5*).

Due to its atypical N-terminal structure as well as its relatively low sequence similarity with traditional PKCs, PKCλ/ι shows unique enzymological features in the PKC family. As had been shown for PKCζ, the insensitivity to diacylglycerol (DG) and phorbol ester of PKCλ/ι, was demonstrated in a previous study on cloning (*2*). Here, the activation mechanism of PKCλ/ι is assumed to be essentially the same as that of PKCζ (see accompanying review on PKCζ), although, strictly speaking, most of the studies on this issue have been carried out using PKCζ (*6–8*). However, it should be noted that PKCλ/ι is the first aPKC that was shown to be activated downstream of phosphoinositide 3-kinase (PI3K) in intact cells (*9*). Since then, abundant evidence has been accumulated demonstrating that PKCλ/ι, as well as PKCζ, plays a critical role in transmitting signals from PI3K to particular downstream targets (see below) (*10–15*). Based on the results that phosphatidylinositol-3,4,5-$(PO_4)_3$ (PIP3), one of the lipid metabolites produced by PI3K, directly activates PKCζ *in vitro* (*8*), aPKCs were considered to be activated by the direct binding of PIP3 to the cysteine-rich sequence, which is followed by conformational change to release pseudosubstrate autoinhibition similarly to the action of DG on traditional PKCs. However, the mechanism underlying aPKC activation by PIP3 is still controversial because phosphoinositide-dependent kinase 1 (PDK1) was found to activate PKC ζ *in vitro* in a

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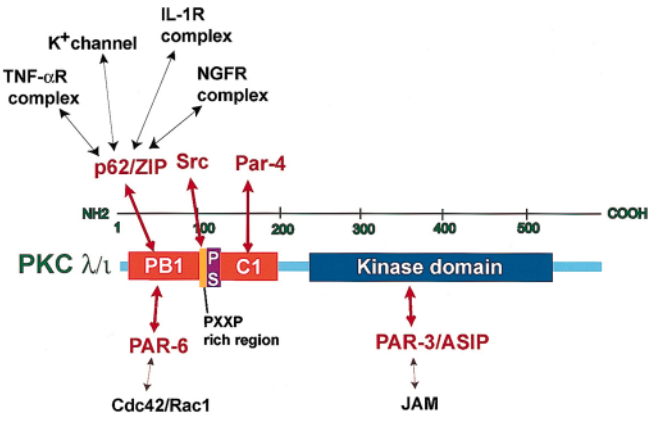

Fig. 1. **Schematic diagram illustrating the structural domains of PKCλ/ι and their interactions with aPKC-specific binding proteins (red).** Recent studies have shown that PKCλ/ι has various protein-protein interfaces including a cysteine-rich sequence (C1) in its N-terminal regulatory domain. The most N-terminal PB1 domain of aPKC interacts with OPR/PC/AID motif–containing proteins, such as PAR-6 and p62/ZIP. The PB1 domain of aPKCs is also suggested to embed the OPR/PC/AID motif within its core structure (*4*, *30*) . PS: pseudosubstrate.

PIP3-dependent manner by phosphorylating Thr (T410) in the activation loop of PKC ζ (*16–18*). The Thr residue is also conserved in PKCλ/ι (T402/403), the substitution of which to Ala has been shown to greatly reduce the kinase activity (*19*). This raises a possibility that the action of PIP3 on aPKCs is indirectly mediated by PDK-1, although recent results that the T410E mutant of PKCζ, but not its deletion form lacking the regulatory region, can be further activated by PIP3 *in vitro* suggest that the direct interaction of PIP3 with the cysteine-rich sequence also contributes to acute aPKC activation (*20*). In any case, it should be noted that the effect of PDK-1–dependent loop phosphorylation on aPKCs seems to be different from that on the traditional PKCs; in the latter case, the phosphorylation is postulated to occur chronically to induce the maturation of these PKCs for acute activation by DG (*21*).

In addition to the significant difference in the N-terminal regulatory region, aPKCs also show unique features in their catalytic domain. For example, several staurosporine-related inhibitors acting on the ATP-binding site are less efficient on aPKCs than on traditional PKCs (*22*, *23*). In this sense, it is very interesting that aPKCs contain an Ala replacement of the 6th Gly (A256/257 for PKCλ/ι) in the glycine-rich loop motif (GXGXXG) commonly found in the nucleotide-binding fold of virtually all nucleotide-binding proteins (*2*). In addition, in sharp contrast to almost all other reports on protein kinases including traditional PKCs, an Arg substitution of the invariant Lys in the ATP-binding site of PKCλ/ι (K273/274) does not affect the kinase activity (*24*). On the other hand, Mizuno *et al.* suggested that the subtle difference in the substrate-binding site also contributes to the low sensitivity of PKC ζ to several inhibitors acting on the ATP-binding site (*22*). Taken together, these data indicate that the characteristic sequences in the kinase domain of aPKCs may differentiate the enzymological properties of aPKCs from those of other PKCs. It is also noteworthy that the kinase domains of aPKCs already contain Glu (E573/574 for PKCλ/ι) instead of the second autophosphorylatable Ser found in most C-terminal hydrophobic motifs of traditional PKCs (*21*). It might also result in the uniqueness of the kinase activity and/or activation mechanism of aPKCs.

Regulation of aPKC activity through protein–protein interaction

In the past several years, various aPKC-specific binding proteins have been reported. Interestingly, most of them except PAR-3/ASIP [that binds to the kinase domain (*25*)] interact with the regulatory domain of aPKCs (Fig. 1), and some of them have been implicated in the regulation of aPKC activity. These binding proteins may compensate the low specificity of regulation by lipid metabolites, which are thought to activate several signaling molecules simultaneously.

PB1 domain–binding proteins—p62/ZIP and PAR-6. p62/ZIP and PAR-6 (one of the evolutionarily conserved cell polarity proteins named after a *C. elegans* partition defective mutant: see below) have been found to associate with the most N-terminal region of aPKCs upstream of the cysteine-rich sequence (V1 region), which is conserved in aPKCs but shows no similarity to other PKC members (*26–29*). Interestingly, the narrowed sequences of p62/ZIP and PAR-6 involved in their binding to the aPKC V1 region share a small acidic short stretch termed OPR/PC/AID (*30*, *31*), the finding of which led to the further identification of the α isoform of MEK5 as another aPKC-binding protein (*32*). This OPR/PC/AID motif is also identified in a yeast cell polarity protein, Cdc24p, and a mammalian phagocyte-specific factor, $p40^{phox}$, as an indispensable region for binding Bem1p and $p67^{phox}$, respectively (*33*). Recently, Terasawa *et al.* have predicted that the V1 region of aPKC contains a structural module named the PB1 domain (19–108 a.a. for PKCλ/ι) which was first identified in Bem1p and $p67^{phox}$ in their binding sites with Cdc24p and $p40^{phox}$, respectively (*34*, *35*). Therefore, these results suggest that the structural basis of the interaction between aPKC and p62/ZIP or PAR-6 represents an evolutionarily conserved protein–protein interaction shared by various signaling molecules, and aPKCs have acquired this versatile module during their divergence from other PKCs. Because both p62/ZIP and PAR-6 mediate the linkage of aPKCs to specific signaling complexes upon stimulation (*28*, *31*), the PB1 domain may serve to locate aPKC into different signaling pathways conferring selectivity to the kinase actions (*32*).

PAR-6 links aPKCs to small GTPases of the Rho family, Rac1 and Cdc42, in a GTP-dependent manner by binding Rac1/Cdc42 through its CRIB/PDZ domain (*28*, *29*, *36–38*). Yamanaka *et al.* demonstrated that the kinase activity of aPKC λ/ι in the PAR-6 immunocomplex is enhanced by the addition of recombinant Cdc42 preloaded with GTP-γS but not with GDP (*39*). Interestingly, when a PAR-6 mutant lacking the CRIB/PDZ domain, which can bind aPKC λ/ι but not Rac1/Cdc42, was used instead of intact PAR-6, the aPKC λ/ι kinase activity in the immunocomplex was potentiated independent of Cdc42. Therefore, it is likely that the binding of PAR-6 to the PB1 motif of aPKC λ/ι causes the sup-

pression of its kinase activity, and the binding of activated Rac1/Cdc42 to PAR-6 releases the suppression. Although the molecular basis of this regulation remains to be further clarified, it is tempting to speculate that the binding of PAR-6 may lock aPKC in a novel inactive conformation ready for activation by Rac1/Cdc42, in which the pseudosubstrate masking of the substrate-binding cavity in the kinase domain is released in a lipid-metabolite independent manner.

Cysteine-rich domain binding proteins—PAR-4 and LIP. Par-4 (prostate androgen response-4) is initially identified as the product of a gene whose expression is induced during apoptosis (note that it is different from a polarity protein, PAR-4, identified in *C. elegans*). Diaz-Meco *et al.* have found that it interacts with the single cysteine-rich sequence of aPKC λ/ι and ζ, and inhibits their kinase activity independent of lipids (*40*). The authors suggested that this interaction is important in suppressing aPKC activity that has been shown to support cell survival (see below), thereby potentiate apoptotic pathway (*41*). On the other hand, the same group has reported another aPKC-binding protein, LIP (lambda-interacting protein), which associates with the cysteine-rich domain of PKC λ/ι but not with that of ζ, and works as an activator (*42*). Although it is very interesting in terms of the first aPKC-binding protein clearly showing isotype-specificity, several data should be carefully reexamined from the present standpoint that the reported sequence does not represent the full-length cDNA but corresponds to the C-terminal portion of the recently identified protein, SMG-1, which is involved in nonsense-mediated mRNA decay (*43*).

PXXP motif-binding protein—src. Very recently, Wooten *et al.* have shown that amino acid residues of human PKC λ/ι (96–111 a.a.) rich in PXXP motif bind to the SH-3 domain of src (*44*). Since the PXXP motifs are also present in PKCζ but not in other PKCs, this binding is thought to be aPKC-specific in this sense. The association was shown to be enhanced by NGF in PC12 cells along with the formation of a signaling complex containing a NGF receptor (TrkA), src and PKC λ/ι. *In vitro*, purified PKC λ/ι was phosphorylated at several Tyr residues in the kinase domain by src and then activated. Consistently, in intact cells, PKC λ/ι becomes tyrosine-phosphorylated in the membrane in a src-dependent manner coincident with the activation by NGF. This finding may provide a novel mechanism of aPKC activation involving tyrosine phosphorylation.

Physiological function of a PKCλ/ι

To determine the physiological function as well as the activation mechanism of these unique PKC isotypes, pseudosubstrate peptides, antisense oligonucleotides and dominant negative mutants of aPKCs have been used as experimental tools to specifically inhibit aPKC activity in intact cells. Recent identification of the aPKC-specific binding proteins mentioned above has further facilitated our understanding of the molecular basis of aPKC functions. Nevertheless, at present most of the enzymatic and cell biological features revealed thus far are essentially common to PKCλ/ι and ζ , and we cannot clearly discuss functional differences between the two aPKC isotypes. It should be partially because the sequence similarity between PKC λ/ι and ζ might hamper the isotype-specific effect of dominant negative mutants or pseudosubstrate peptides. In addition, the fact that some anit-PKCζ antibodies cross-react with aPKC λ/ι also makes the situation complex. Finally, all aPKC-binding proteins reported thus far, except LIP, bind both aPKCs. Therefore, we restrict the following descriptions on the physiological functions of PKCλ/ι to a brief summary to avoid redundancy with the accompaning review on PKC ζ, and concentrate on introducing one specific topic reported mainly for PKCλ/ι in the next section. As for the functional divergence between PKCλ/ι and ζ, we will discuss it again in the last part of this review.

Similar to PKCζ, PKC λ/ι has been suggested to mediate growth-factor-provoked signals from PI3K and/or Ras to the activation of the MAPK cascade (*45*, *46*), AP-1-dependent or cyclin D1 promoter activity (*2*, *47*), DNA synthesis (*48*) and $p70^{S6\ kinase}$ (*49*), and thereby support cell proliferation, differentiation, and survival (Fig. 2); however, some of the data were based on the experiments using the *Xenopus* PKCλ/ι ortholog formerly designated *Xenopus* PKCζ. In insulin signaling, aPKC λ/ι, but not traditional PKCs, has also been demonstrated to be critical for triggering glucose transport (*13*, *50*, *51*). On the other hand, aPKCλ/ι is also shown to be involved in TNF-α/IL-1 signaling leading to NFκB activation and cell survival (*52*, *53*). Recent studies have demonstrated that p62/ZIP works as a scaffold protein to recruit aPKC λ/ι into the TNF-α receptor complex (containing TRADD, TRAF2, and RIP) (*54*) or IL-1 receptor complex (containing MyD88, IRAK, TRAF6) (*55*), and trigger aPKC λ/ι activation to phosphorylate IKKβ. p62/Zip was also shown to work in the recruitment of aPKC to the NGF receptor complex; thus it mediates the NGF signaling pathway for cell survival (*56*). In this signaling, p62/ZIP is suggested to bind both TrkA and p75, the two NGF receptors with different affinities, and thus to integrate the two signaling pathways in order to regulate cell survival or cell death (*57*). The activation of PKCλ/ι by src stated above is also suggested in this signaling (*44*). Finally, PKCλ/ι is suggested to play critical roles in cytoprotection against drug- or UV-induced apoptosis (*58*, *59*). In these cases (and even in TNF-α/IL-1 signaling), ceramide, which is induced under various apoptotic stimuli, may work as a potential second messenger to activate PKCλ/ι (*60*).

It is noteworthy that several subcellular localizations including the nucleus, cytoplasmic vesicles (*27*, *61*), and cell-cell junctional structure (*25*) have been reported for aPKC λ/ι. For example, aPKCλ/ι was demonstrated to show rapid nucleocytoplasmic shuttling in response to stimuli, such as PDGF and NGF (*9*, *62*). In this regard, it is interesting that PKC λ/ι and ζ have functional nuclear import and export signals in their sequences (*19*). These results suggest that aPKCs are directly involved in nuclear events, such as phosphorylation of transcriptional factors or nuclear matrix components (*63*, *64*).

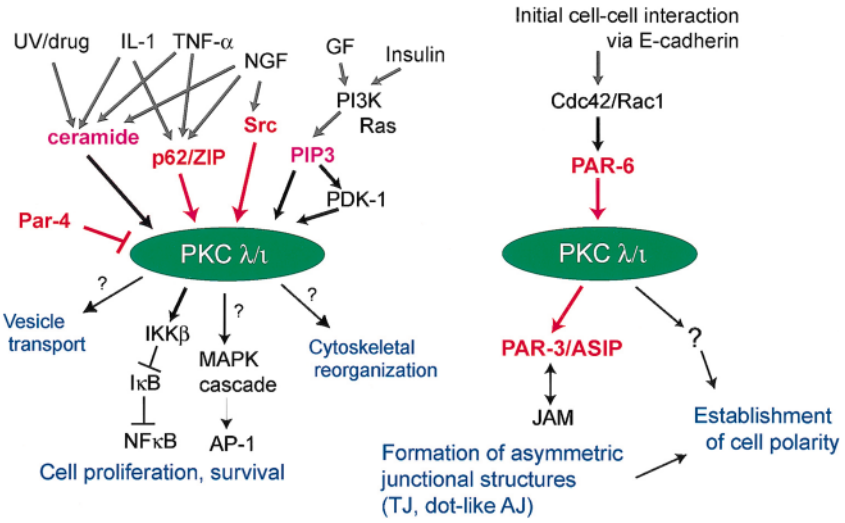

Fig. 2. **Summary of the signal transduction pathways revealed thus far to involve PKCλ/ι.** Inputs of external signals are shown by gray arrows and the details of the initial signaling pathways are omitted (GF: growth factors). aPKC-binding proteins and the interaction are presented in red. Since PDK-1 was demonstrated to bind not only aPKCs but also traditional PKCs, it is shown in black similarly to other upstream proteins such as PI3K and Ras. Lipid metabolites suggested to activate PKCλ/ι are shown in purple. aPKCλ/ι has been demonstrated to play critical roles for cell survival signaling, and recent identification of aPKC-specific binding proteins, such as p62/ZIP, Par-4, and Src, has deepened our understanding of the molecular basis of the signaling (left). On the other hand, the identification of PAR-3/ASIP and PAR-6 led to the unexpected finding that aPKCs play an indispensable role in establishing cell polarity, which is crucial for the development of multicellular organisms (right). The correlation between these two signaling cascades is not known yet.

PKCλ/ι is indispensable to establish epithelial cell polarity

In 1998, a mammalian homolog of a *C. elegans* cell polarity protein, PAR-3, was identified as an aPKC-specific interacting protein, ASIP (*25*). This led to the unexpected finding that aPKC is a critical component of an evolutionarily conserved cell polarity machinery (*65*). PAR-3/ASIP is a scaffold protein with three PDZ domains in its N-terminal region, the first of which has been demonstrated to bind the C-terminal PDZ binding consensus of a transmembrane component of the tight junction (TJ), JAM, in mammalian epithelial cells (*66*). By anchoring to the conserved aPKC-binding region of PAR-3/ASIP (*67*), the stable aPKC–PAR-6 complex stated above is recruited to TJ in epithelial cells (Fig. 3) and dot-like adherens junctions (AJs) in fibroblasts (*28*). Direct evidence that aPKC is indispensable for cell polarization processes is firstly demonstrated genetically in the *C. elegans* embryo (*68*): anterior-posterior cell polarization of the zygote which is required for the subsequent asymmetric cell divisions was severely impaired similarly to *par-3* or *par-6* mutants when *C. elegans* aPKC (PKC-3) was knocked down by RNA-mediated interference. The role of mammalian aPKC in cell polarization events was then shown in cultured epithelial cells, which are one of the most polarized cells in adult (*28*): overexpression of a dominant negative mutant of PKCλ/ι in cultured MDCK cells disrupted the apico-basal polarity monitored by the asymmetric distribution of Na^+/K^+-ATPase or apically incorporated fluorescence-labeled phospholipids. In these cells, the formation of TJ, one of the epithelia-specific cell–cell junctional structures indispensable for the formation and maintenance of epithelial cell polarity was also impaired. Importantly, the effect of the dominant negative mutant of PKCλ/ι was observed only when cell-cell adhesion, and thus cell polarity, was reset by manipulating the calcium ion concentration in culture medium, suggesting that aPKC plays a critical role in the signal transduction pathway from cell-cell adhesion to cell polarization processes. Since Rac1 and/or Cdc42 have been shown to be activated upon cell-cell adhesion through E-cadherin (*69*, *70*), they might activate aPKC through their interaction with PAR-6 as described above (Fig. 2). On the other hand, the downstream target of aPKC for the development of epithelial cell polarity remains to be clarified. However, it was shown that aPKC phosphorylates a conserved Ser (S827) in the aPKC-binding region of PAR-3/ASIP in vivo and this phosphorylation is important for TJ formation (*67*). Detailed analysis of the wound-healing process of another epithelial cell line, MTD1A, also suggested that the dominant negative mutant of PKCλ/ι inhibits the differentiation of the fibroblastic, primordial dot-like adherens junction (AJ) into the epithelia-specific belt-like AJ and TJ (*71*). Together with the fact that knocking down of PKC-3 in the *C. elegans* embryo results in disruption of the asymmetric anterior cortex localization of PAR-3 and PAR-6 (*68*, *72*), these results suggest that aPKC kinase activity generally plays important roles in establishing asymmetric submembranous structures to which the aPKC-PAR-3-PAR-6 complex itself localizes (Fig. 3).

Functional divergence between PKCλ/ι and PKCζ

The establishment and regulation of cell polarity are essential for the development of multicellular organisms from a zygote. In fact, as genetically demonstrated, the embryos of *C. elegans* or *Drosophila* having a single aPKC are arrested in their early stage of embryogenesis when they have defects in the aPKC function (*72*, *73*). Considering that yeast does not have aPKC (*4*, *74*), it is tempting to speculate that the divergence of aPKC from acient PKCs may be one of the critical events for the appearance of multicellular organisms. On the other hand, the presence of the two distinct members of aPKCλ/ι and ζ in mammals showing ~70% overall amino acid sequence identities raises an issue that they might function redundantly in this aPKC function to establish cell polarity. In fact, a dominant negative mutant of PKCζ also disrupted TJ formation in cultured epithelial cells similarly to that of PKCλ/ι (*28*). However, the

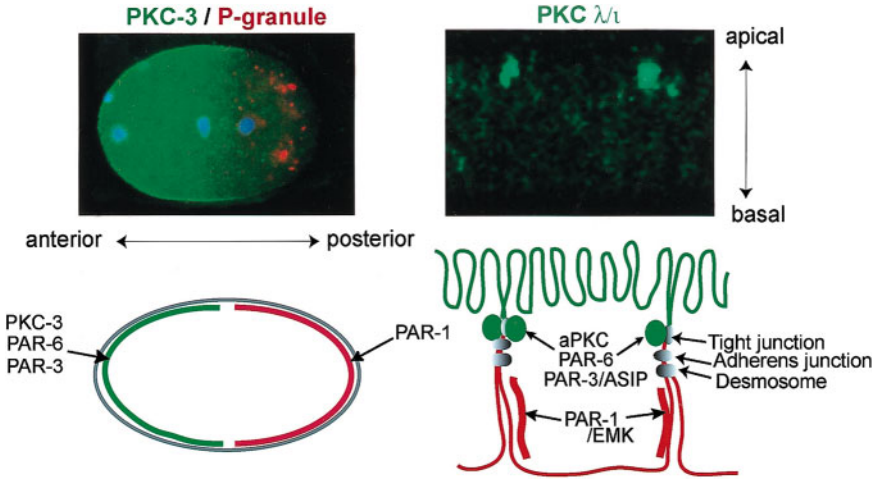

Fig. 3. **The asymmetric distribution of aPKC in *C. elegans* one-cell embryo (left) and mammalian epithelial cells (right).** As a ternary complex with PAR-3 and PAR-6, *C. elegans* aPKC (PKC-3: green) is asymmetrically localized to the anterior cortex in the one-cell embryo, whereas mammalian PKCλ/ι is localized to the most apical tip of the cell-cell contact region by anchoring to TJ in epithelial cells. P-granule (red) is a posterior marker. In both cells, the asymmetric localizations of aPKC, PAR-3 and PAR-6 are mutually dependent: defect in one of the proteins causes the mislocalization of the other proteins and results in the disruption of cell polarity. aPKC is required to establish the asymmetric submembranous structures important for the subsequent organization of cell polarity, to which the aPKC/PAR3/PAR6 complex is localized. PAR-1 is another PAR protein, which is shown to work downstream of aPKC, PAR-3 and PAR-6 in *C. elegans*. Interestingly, in both cells, PAR-1 is shown to localize asymmetrically at a site opposite to the aPKC/PAR3/PAR-6 complex (*83*).

answer is no in vivo. Recent data from PKCλ/ι- and ζ-knock-out mice demonstrated that PKCζ-knock-out mice are viable showing impaired NFκB signaling and immune response (*75*, *76*), whereas PKCλ/ι-knock-out mice die at an early embryonic stage (Akimoto *et al*. unpublished results). Ectodermal epithelia of the PKCλ/ι null embryo show deficiency in their cell polarity as observed for aPKC mutants of *C. elegans* and *Drosophila*. There data clearly indicate that duplications of an ancient aPKC gene probably occurred during early vertebrate evolution resulting in the functional divergence between the two aPKCs, and aPKCλ/ι remained to function in cell polarity dominantly. Consistently, the PKCλ/ι ortholog has been solely identified in *Xenopus* (*77*) and *zebra fish* (*78*), which show higher amino acid sequence identity with mouse PKC λ/ι (85 and 88%, respectively) than with mouse PKCζ (71 and 70%, respectively), and the mutations in the *zebra fish* PKC λ/ι gene (*heart and soul*) were demonstrated to result in embryonic lethality showing abnormality in the polarized epithelia of the retina, neural tube and digestive tract (*78*, *79*). *Xenopus* PKCλ/ι is demonstrated to redistribute asymmetrically in the animal hemisphere during oocyte maturation together with PAR-3/ASIP, suggesting its possible involvement in the establishment of oocyte polarity as demonstrated in *Drosophila* (*80*). The differential contribution of these two aPKC isotypes in the whole body might be due to their different expressions. Another possibility is that there might be critical functional differences that have been overlooked in previous studies especially due to the low specificity of the experimental tools used as stated above. Several reports have already suggested the possible functional differences between PKCλ/ι and ζ (*19*, *23*, *42*, *47*, *81*, *82*), although some of them compared mammalian PKCζ and *Xenopus* PKCλ/ι and thus need further confirmation. In any case, we should accumulate more evidence based on carefully designed experiments, especially those using (conditional) knock-out-mice–derived cells or cells subjected to individual aPKC depletion by RNA mediated interference in order to draw a valid conclusion.

The GenBank accession numbers of the amino acid sequences used in this paper are: BAA32499 for *mouse* PKC λ/ι; AAA60171 for *human* PKCλ/ι; AAK91291 for *zebra fish* PKC λ/ι; AAA75362 for *Xenopus* PKCλ/ι; AAA39983 for mouse PKCζ.

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