An Atypical PKC Directly Associates and Colocalizes at the Epithelial Tight Junction with ASIP, a Mammalian Homologue of *Caenorhabditis elegans* Polarity Protein PAR-3

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**Abstract.** Cell polarity is fundamental to differentiation and function of most cells. Studies in mammalian epithelial cells have revealed that the establishment and maintenance of cell polarity depends upon cell adhesion, signaling networks, the cytoskeleton, and protein transport. Atypical protein kinase C (PKC) isoforms PKCζ and PKCλ have been implicated in signaling through lipid metabolites including phosphatidylinositol 3-phosphates, but their physiological role remains elusive. In the present study we report the identification of a protein, ASIP (atypical PKC isoform–specific interacting protein), that binds to aPKCs, and show that it colocalizes with PKCλ to the junctional complex in cultured epithelial MDCKII cells and rat intestinal epithelia. In addition, immunoelectron microscopy revealed that ASIP localizes to tight junctions in intestinal epithelial cells. Furthermore, ASIP shows significant sequence similarity to *Caenorhabditis elegans* PAR-3. PAR-3 protein is localized to the anterior periphery of the one-cell embryo, and is required for the establishment of cell polarity in early embryos. ASIP and PAR-3 share three PDZ domains, and can both bind to aPKCs. Taken together, our results suggest a role for a protein complex containing ASIP and aPKC in the establishment and/or maintenance of epithelial cell polarity. The evolutionary conservation of the protein complex and its asymmetric distribution in polarized cells from worm embryo to mammalian-differentiated cells may mean that the complex functions generally in the organization of cellular asymmetry.

Key words: ASIP • atypical PKC • par • cell polarity • tight junction

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The stimulus-coupled turnover of membrane lipids is an important event during cellular signal transduction (Nishizuka, 1995). Members of the protein kinase C (PKC) family are among the prominent candidates that transduce a variety of extracellular stimuli to the cellular signaling networks through lipid-derived second messenger molecules. PKC comprises at least 10 closely related isoforms that can be subdivided into three classes: ePKC (PKCα, PKCβI, PKCβII, and PKCγ), nPKC (PKCδ, PKCε, PKCη, and PKCθ), and aPKC (PKCζ and PKCλ) (Nishizuka, 1995). The cPKC and nPKC isoforms can be positively regulated by a variety of lipid-derived messenger molecules including diacylglycerols, and are receptors for phorbol esters such as TPA. Unlike other PKC isoforms, aPKCs do not seem to be regulated directly by Ca²⁺, phorbol esters, or diacylglycerols (Nishizuka, 1995). Some studies have shown that ceramide regulates the activity of PKCζ (Lozano et al., 1994; Müller et al., 1995). Purified PKCζ is also regulated by phosphatidylinositol 3,4,5-trisphosphate (PI-3,4,5-P₃), a product of phosphatidylinositol 3-kinase (PI3-kinase) (Nakanishi et al., 1993), and PKCλ is activated upon PDGF receptor stimulation through a pathway involving PI3-kinase (Akiyama et al., 1996; Toker and Cantley, 1997). Stimulation of insulin receptors also results in PKCζ activation through the PI3-kinase pathway (Standaert et al., 1997; Bandypadhyay et al., 1997). A series of studies using a pseu-
dosubstrate peptide or kinase-deficient dominant negative forms of PKCζ and PKCα suggest that atypical PKC iso-
types (aPKC) plays roles distinct from those of the cPKC
and nPKC isoforms in a variety of cellular functions. These
include *Xenopus* oocyte maturation (Domínguez et al.,
1992; Berra et al., 1993), proliferation and survival of fi-
broblasts (Berra et al., 1993; Diaz-Meco et al., 1996), dif-
erentiation of PC12 (Wooten et al., 1994) and leukemic
cells (Ways et al., 1994), activation of mitogen-activated
protein kinase (MAPK) (Berra et al., 1995) and gene ex-
pression (Lozano et al., 1994; Akimoto et al., 1996; Xu et
al., 1996), and insulin-induced glut4 translocation (Stan-
daert et al., 1997). Furthermore, several proteins have
been shown to interact directly with aPKC isoforms (Diaz-
Meco et al., 1994; Diaz-Meco et al., 1996a; Diaz-Meco et
al., 1996b; Puls et al., 1997; Sanchez et al., 1998). However,
the downstream effectors and the exact cellular function
of aPKCs remain elusive. In this paper we present evi-
dence consistent with a hypothesis that atypical PKC iso-
type–specific interacting protein (ASIP) and aPKC com-
plex is involved in establishment or maintenance of cell
polarity.

Cell polarity is the reflection of complex mechanisms
that establish and maintain functionally specialized do-
mains in the plasma membrane and cytoplasm, and is fun-
damental to differentiation and function of most cells
(Drubin and Nelson, 1996; Caplan, 1997). Cell polarity is
being studied in model genetic systems like *Caenorhabdi-
tis elegans* and in tissue culture in epithelial cells. Studies
of asymmetric cell division in embryogenesis have pro-
vided evidence that transient asymmetric distribution
of proteins at the cell periphery is essential for cell polar-
ity (Knoblich, 1997). In early *C. elegans* embryos, PAR pro-
teins such as PAR-3 are required for embryonic polarity,
and become localized asymmetrically at the periphery of
the one-cell embryo (Etemad-Moghadam et al., 1995; Guo
and Kemphues, 1996). The cue that triggers cell polariza-
tion and determines the axis of polarity is provided by the
sperm (Goldstein and Hird, 1996). Mutations in the *par-3*
gene affect the asymmetric distribution of other proteins
involved in cell fate determination and the orientation of
mitotic spindles in successive cell cycle (Guo and Kem-
phues, 1996; Bowerman et al., 1997). How the sperm cue
triggers asymmetric distribution of PAR proteins is not
clear; neither is it clear how the asymmetric distribution
of PAR proteins leads to other cellular asymmetries.

Mammalian epithelial cells provide an experimental sys-
tem that has revealed essential features of cell polarity
(Eaton and Simons, 1995; Drubin and Nelson, 1996; Gumbi-
er, 1996). Epithelial cells respond to asymmetric cell
adhesion to organize cytoskeletal and membrane proteins
into distinct apical and basal-lateral membrane domains;
this apical/basal polarity provides a basis for directed
transport across the epithelium. Tight junctions are spe-
cialized structures that play an essential role in epithelial
cell polarity by creating a barrier to diffusion between cells
in the epithelial sheet and forming an intramembrane dif-
fusion fence that restricts intermixing of apical and basa-
lateral membrane components (Balda and Matter, 1998).
As in the *C. elegans* one-cell embryos, establishing of cell
polarity in epithelial cells starts with a cortical spatial cue.
The spatial cue in epithelial cells is cell adhesion. E-cad-
herin–mediated cell–cell contact and the contact between
integrins and the extracellular matrix trigger the special-
ized assembly of actin-based cytoskeleton and signaling
networks around the adhesion receptors and tight junc-
tions, and position other cytoskeletal complexes and pro-
tein-sorting compartments (Eaton and Simons, 1995; Dru-
bin and Nelson, 1996; Gumbiner, 1996). How adhesion
receptors trigger the establishment of cellular asymmetry
is not clear; neither is it clear how tight junctions reinforce
and maintain the cellular asymmetry.

During experiments to clarify the role of aPKC isoforms,
we searched for aPKC-interacting proteins using an inter-
action cloning approach using purified recombinant PKCζ
as a probe. In the present study, we show that a novel pro-
tein, ASIP, interacts with aPKC isoforms, and that the in-
teraction involves the kinase domain of aPKC and occurs
within a region of 225 amino acids of ASIP. ASIP shows
significant sequence similarity to a *C. elegans* polarity pro-
tein, PAR-3. Furthermore, the direct interaction with
aPKC is conserved from worm PAR-3 to mammalian
ASIP. Endogenous ASIP and PKCα form a complex in
NIH3T3 cells and epithelial MDCKII cells. In addition,
ASIP colocalizes with PKCα to the tight junction and ad-
herens junction–containing junctional complex in MDCKII
cells. ASIP also colocalizes with ZO-1, a tight junction
protein, in MDCKII cells, and localizes to the tight junc-
tion in rat intestinal epithelium.

**Materials and Methods**

**Materials and Chemicals**

PKC expression vectors encoding PKCα (YK504), PKCβ (M241), PKCζ
(M246), PKCα (MLNP45), and PKCARD (MLRD) have been described previ-
ously (Akimoto et al., 1994; Ohno et al., 1994; Akimoto et al., 1996;
Izumi et al., 1997). PKCα-KDI encoding amino acid (a.a.) residues 191–586
of PKCα was constructed by K. Akimoto. Tag-ASIP (SRHIS-ASIP) en-
codes the entire ASIP sequence fused downstream of the six histidine res-
idenues and a 12–amino acid sequence from the T7 gene 10 leader sequence.
Anti-PKCζ (ΦRb2) was raised against the COOH-terminal peptide of
mouse PKCζ (from residues 578–592). Anti-glutathione-S-transferase
(GST) was raised against the purified protein for glutathione-S-trans-
ferase. Monoclonal antibodies (mAb) of anti-PKCs and anti-PKCα were
purchased from Transduction Laboratories (Lexington, KY), and anti-T7
tag was from Novagen. The mAb of PKCα was purchased from Transduc-
tion Laboratories. This antibody was generated against the kinase domain
of PKCα, a human homologue of mouse PKCα, and specifically recognizes
PKCs, but not PKCζ. The anti-PKCα (κ2) antibody is a polyclonal anti-
body against the D2/D3 region of mouse PKCα, and anti-PKCα (κ2) recognize
cellular PKCα specifically, and not PKCζ (data not shown). The anti-ASIP antibody (C1 and C2) was raised against
the GST fusion protein of aPKC-binding domain (a.a. residues 712–936,
and P1 was raised against the GST fusion protein of the third PDZ
domain (a.a. residues 584–708). Anti-ZO-1 and MDCKII cells were kindly
provided by Dr. S. Tsuchida (Kyoto University, Kyoto, Japan). FITC-con-
gjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibodies
were purchased from E.Y. Laboratories, Inc. (San Mateo, CA) and Amer-
sham Life Science, Inc. (Arlington Heights, IL), respectively.

**Purification and 32P-labeling of PKCζ**

Mouse PKCζ (2.1 μg) purified from recombinant baculovirus-infected
Sf21 cells (Fujise et al., 1994) was labeled with 32P by autophosphorylation
in 155 μl of kinase buffer containing 20 mM Tris-HCl, pH 7.5, 5 mM
MgCl2, 10% glycerol, 0.6 μCi/ml [γ-32P]ATP, and 40 μg/ml phosphati-
dyserine at 30°C for 2 h. After the reaction, the [32P]-labeled PKCζ
was separated from the unreacted ATP by gel filtration on Sephadex G-50
equilibrated with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM DTT,
and 0.5% Triton X-100, and was used as a probe for screening a λEXlox cDNA expression library.

**Isolation of cDNA Clones Encoding ASIP**

Poly(A)+ RNA was isolated from NIH3T3 cells, and randomly primed cDNA was synthesized using a HindIII primer adapter (Novagen Inc., Madison, WI). cDNA was ligated to λEXlox vector arm (Novagen Inc.), and was packaged in phage particles using a Phage Marker System (Novagen Inc.). This library contains ~4.95 × 10^6 independent clones. Approximately 1.8 × 10^7 clones were plated at 10,000 phages per plate using *Escherichia coli* strain BL21 (DE3) pLysE as host cells. After incubation for 6 h at 37°C, the plaques were covered with Hybond-C Extra (Amersham Life Science, Inc.), impregnated with 10 mM IPTG, and allowed to grow for an additional 3.5 h at 37°C. The filters were then washed with TBS buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl), blocked with 5% skim milk at 4°C overnight, and incubated with 1 mM ATP solution (TBS buffer containing 1 mM ATP) at room temperature for 2 h to saturate any autophosphorylation sites or ATP-binding sites on the proteins. The filters were then incubated with [γ-32P]-labeled PKC (1000 cpm/ml) in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 50 μg/ml phosphatidylserine, and 1% BSA for 5 h at room temperature. After washing in TBS buffer, the filters were exposed for autoradiography. Positive phage clones were converted to plasmids (λEXlox + cDNA insert) using *E. coli* strain BM25 according to the manufacturer’s instructions. The initial isolate (clone I-1), which encodes amino acid residues 710–924, was used as a probe to screen a rat 3Y1-ASAP II cDNA library to obtain a cDNA clone encoding the full-length ASIP coding sequence. The nucleotide sequence of the ASIP cDNA has been submitted to the Genbank/EMBL databank with accession no. AB005549.

**Northern Blot Analysis**

Total RNA preparations from various mouse tissues were obtained as described (Osaeda et al., 1992). Poly(A)+ RNA preparations from various cell lines were isolated using a QuickPrep mRNA Purification Kit (Pharmacia Biotech, Inc., Piscataway, NJ). Northern blot analysis was performed according to the standard protocol.

**FISH Mapping of ASIP on Chromosomes**

Lymphocytes isolated from human blood were cultured in α-MEM containing 7% calf serum, penicillin, 10% FBS and phytohemagglutinin at 37°C for 24 h. Lymphocyte cultures were treated with BrdU (0.18 mg/ml: Sigma Chemical Co.) to synchronize the cell population. The synchronized cells were washed three times with serum-free medium to release the block, and were recultured at 37°C for 6 h in α-MEM containing thymidine (2.5 μg/ml: Sigma Chemical Co.). The cells were harvested, and slides were made by standard procedures including hypotonic treatment, fixation, and air-drying. For in situ hybridization and FISH detection, the rat ASIP cDNA probe was biotinylated with dATP using a BioNick labeling kit (Pharmacia Biotech, Inc., Piscataway, NJ). Northern blot analysis was performed according to the standard protocol.

**Immunofluorescence Microscopy**

MDCKII and NIH3T3 cells plated on glass coverslips were fixed with methanol-acetone (1:1) for 10 min at −20°C, and were then air-dried. The cells were incubated with TBS (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% calf serum for 30 min at room temperature, incubated with primary antibodies for 45 min at 37°C, and washed three times for 5 min each with PBS. After the first incubation, the cells were incubated for 45 min at 37°C with secondary antibodies (FITC-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibodies), and were washed three times for 5 min each with PBS. Samples were viewed using an Olympus BX50. Images were captured with a Princeton Instruments digital camera. Images were manipulated using Adobe Photoshop software.

**Immunocytochemistry of Rat Intestinal Epithelium and Hepatic Bile Capillary**

Rat (10-wk-old) tissue samples were frozen in liquid nitrogen, and the frozen sections (~7 μM) were cut in a cryostat. The samples were mounted on glass slides, fixed with methanol/acetic acid (1:1) for 10 min at −20°C, and air-dried. After the samples were incubated with TBS containing 10% calf serum for 10 min at room temperature, they were incubated with primary antibodies for 45 min at 37°C, and were washed three times for 5 min each with PBS. After the first incubation, the cells were incubated for 45 min at 37°C with secondary antibodies (FITC-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse antibodies) and washed three times for 5 min each with PBS. Samples were viewed using an Olympus BX50. Images were captured with a Princeton Instruments digital camera (Princeton Instruments, Trenton, NJ). Images were manipulated using Adobe Photoshop software.
Immunoelectron Microscopy

Small pieces of rat small intestine were fixed in 2% formaldehyde (freshly depolymerized from paraformaldehyde; Merck & Co., Whitehouse Station, NJ) in 0.1 M sodium phosphate buffer, pH 7.4, and 5% sucrose for 30 min at 4°C. After rinsing, the tissue specimens were infused with a mixture of sucrose and polyvinylpyrrolidone (Sigma Chemical Co.; Tokuyasu, 1989) and frozen rapidly in liquid nitrogen. Ultrathin (50–70 nm) cryosections were prepared as described (Liou et al., 1996) and subjected to indirect immunolabeling with 10 nm colloidal gold-conjugated goat anti-rabbit IgG antibody (Amersham Life Science, Inc., Arlington Heights, IL). The specimens were embedded in a mixture of 2% methylcellulose (Nacalai Tesque, Kyoto, Japan) and 0.4% uranyl acetate (Griffiths et al., 1986).

Results

Molecular Cloning, Primary Structure, and mRNA Expression of ASIP

To identify proteins that interact directly with aPKC, we screened an NIH3T3 cDNA expression library using purified and autophosphorylated PKCζ as a probe. One cDNA found, clone I-1, encoded a truncated form of a novel protein, and we next screened a rat fibroblast 3Y1 cDNA library to obtain a full-length cDNA clone encoding the entire protein-coding sequence. This cDNA was 5,500 nucleotides in length, and contained a 1,337–amino acid open reading frame. The open reading frame is preceded by 259 nucleotides containing an in-frame termination codon at nucleotide position -96 (compared with A at the putative initiation codon), and is followed by a 1,227-nucleotide 3’ noncoding sequence. Since the encoded protein shows binding for aPKC isotypes of the PKC family (see below), we named it ASIP (aPKC-specific interacting protein). The sequence of ASIP does not contain any apparent structural motif suggesting catalytic activity, but contains three PDZ domains that are potentially involved in protein–protein interactions (Saras and Heldin, 1996; Sheng, 1996) in addition to the aPKC-binding region (see below).

A database search for sequence similarity revealed Caenorhabditis elegans PAR-3 as the only protein with a significant overall sequence similarity (Fig. 1, a and b). PAR-3 localizes asymmetrically to the periphery of the C. elegans one-cell embryo, and is required for embryonic cell polarity (Etemad-Moghadam et al., 1995). The similarity between ASIP and PAR-3 involves an NH2-terminal conserved region (CR1), the three PDZ domains (CR2), and a conserved sequence (CR3) in the aPKC-binding region. A Drosophila EST cDNA clone, AA439413, also contains a sequence that shows significant similarity to the sequence of CR1 (Fig. 1 c).

Northern analysis of the ASIP mRNA from mouse tissues and mammalian cell lines shows that there are at least two different mRNA sizes, and that both are expressed ubiquitously in adult tissues and cell lines in slightly different manners (Fig. 2 A). The two kinds of ASIP mRNAs might be products of the same gene produced by alternative splicing, promoter usage, poly(A) addition, or they may be products of a related gene. Human chromosome mapping of the gene for ASIP using rat ASIP cDNA as a probe (Fig. 2 B, a and b) shows a single chromosomal localization on chromosome 10, band p11.2. This result suggests that the human ASIP gene is a single gene, although the possibility of the presence of tandem aligned multiple genes can not be excluded.

Figure 1. Sequence similarity between ASIP and PAR-3. (a) Amino acid sequence alignment of rat ASIP and C. elegans PAR-3. Alignment was done using the GeneStream align program (http://genome.eerie.fr/bin/align-guess.cgi). Residues with black and shaded backgrounds indicate amino acid identity and similarity, respectively. PDZ domains (wavy underlining) and the aPKC-binding region (double underlining; see Fig. 5) are indicated. Arrows indicate potential PKC phosphorylation sites in the CR3. Dashes represent sequence gaps. (b) Schematic view of the ASIP and PAR-3 structures. Typical conserved regions—CR1, CR2, and CR3—are shown. The aPKC-binding region shown is sufficient for a direct interaction with aPKC (see Fig. 5). (c) Amino acid sequence alignment of the NH2-terminal region of rat ASIP with C. elegans PAR-3 and Drosophila AA439413. Residues with a black background indicate amino acid identity. The amino acid sequence of Drosophila AA439413 is encoded by residues 137–401 of the nucleic acid sequence. (d) Amino acid sequence alignment of the PDZ domain of ASIP with the third PDZ domain of rat PSD95 and human DLG. Residues with a black background indicate amino acid identity. The six β sheets (βA–βF) and the two α helices (αA and αB) present in the structure of the third PDZ domain of rat PSD95 (Doyle et al., 1996) and human DLG (Cabral et al., 1996) are indicated in the upper alignment. r, rat; c, C. elegans; d, Drosophila; h, human.
Association Between ASIP and aPKC in COS Cells

To confirm the association between aPKC and ASIP in intact cells and to examine the binding specificity of ASIP to various PKC isoforms, we coexpressed ASIP and PKC isoforms in COS cells, and performed immunoprecipitation experiments. When epitope-tagged-ASIP (tag-ASIP) is immunoprecipitated from cells transfected with tag-ASIP and PKCζ, the overexpressed exogenous PKCζ (lane tag-ASIP/PKCζ) coprecipitates with ASIP along with the endogenous PKCζ or PKCα (lane tag-ASIP; Fig. 3 a). The reverse experiment, where PKCζ is first immunoprecipitated and the immunoprecipitates are then probed with anti-tag antibody, yields a tag-ASIP band (Fig. 3 b). Similar experiments using other PKC isoforms indicate that PKCα can also interact with ASIP in COS cells (Fig. 3 c), while PKCζ, a conventional PKC, and PKCδ, a novel PKC, do not (Fig. 3, d and e). Furthermore, the kinase domain of PKCα coprecipitates with ASIP (Fig. 3 f). These results indicate that ASIP interacts with aPKC isoforms, and that the kinase domain of aPKC is sufficient for the interaction.

Direct Association of ASIP and C. elegans PAR-3 with aPKC

The original mouse ASIP cDNA (clone I-1), identified by screening for protein interactions, contains only a part of ASIP from amino acid residues 710–924; this region is sufficient for direct association with aPKC. An overlay assay where rat ASIP was expressed as a GST fusion protein, fixed on a filter, mixed with purified recombinant PKCζ, and probed with an anti-PKCζ antibody (Fig. 4), clearly indicates that the interaction between ASIP and aPKC is a direct one, and that the region from amino acids 712–936 of ASIP is sufficient for the interaction. Importantly, as suggested by the sequence similarity between ASIP and C. elegans PAR-3, the overlay assay also revealed that mouse PKCζ binds to GST-PAR-3 (a.a residues 678–932; Fig. 4). These regions in the two proteins are in the same relative position with respect to the PDZ domains, and contain a small segment (CR3) with 73% amino acid identity that fits the consensus for phosphorylation by PKC (Fig. 1, a and b).

In Vivo Association of ASIP with PKCα in MDCKII Epithelial Cells and NIH3T3 Fibroblasts

The experiments described above establish that ASIP and aPKC interact with each other directly in vitro and in COS cells. To evaluate the physiological significance of this interaction, we next examined the interaction in vivo. First, we raised anti-ASIP antibodies against two portions of the protein in three rabbits. One anti-serum, P1, was raised against the third PDZ domain (amino acid residues 584–708), and the others, C1 and C2, were raised against the aPKC-binding region (712–936). All three antisera recognized bands of 180 and 150 kD on Western blots of proteins from MDCKII cells, a single band of 180 kD in NIH3T3 cells, and a major band of 150 kD in COS cells. The 180-kD band comigrates with the major band in COS cell extracts transfected with full-length ASIP cDNA (Fig. 5 a), indicating that the 180-kD band corresponds to ASIP encoded by the full-length cDNA sequence. Importantly, both the 180-kD and 150-kD bands disappear in the presence of excess amounts of antigen (Fig. 5 a). Although the present results strongly suggest that the 150-kD band observed in MDCKII and COS cells is a splicing variant of ASIP or the product of a related gene, its exact nature remains to be clarified in future experiments.

For immunohistochemical determination of the distribution of different aPKC isoforms, we searched among available antibodies that can distinguish PKCα from PKCζ. We selected anti-PKCα mouse monoclonal antibody (mAb), which specifically detects PKCα (data not shown). Unfortunately, in our hands all the available antibodies directed against PKCζ also recognize PKCα (data not shown), so we have restricted our analysis to PKCα, which complicates understanding of the distribution of PKCζ reported in previous publication. Western analysis using the anti-PKCα (mAb) revealed a single band migrat-
as well as PKCα. (b) The reverse experiment to a. Cell extracts were immunoprecipitated with anti-PKCζ (Rb2) antibody. The immunoprecipitates were probed with anti-T7 tag antibody. Tag-ASIP is coprecipitated with PKCζ (c–e) Cell extracts were immunoprecipitated with anti-T7 tag antibody followed by anti-PKCa (mAb; c), PKCd (mAb; d), or PKCc (mAb; e). PKCa, but not PKCd or PKCc, can interact with ASIP in COS cells. (f) The PKCa-kinase domain (KD) coprecipitated with ASIP in COS cells. COS cells were transiently transfected with the expression vectors shown at the top, and were immunoprecipitated by anti-T7 tag antibody. The immunoprecipitates were probed with anti-PKCζ antibody (λ2) which recognizes both PKCARD and PKCαKD.

To test the association of ASIP with PKCa in vivo, we used the ASIP-specific antibody, C2, and PKCa (mAb). Immunoprecipitation of NIH3T3 cell extracts with anti-ASIP antibodies followed by Western blotting using anti-PKCa antibody detected a band corresponding to PKCa (lane 1). In MKDKII cells, immunoprecipitates obtained using anti-ASIP antibodies (C2) contain PKCa as well as the two PKCa bands (180 kD and 150 kD; Fig. 5 c). These results provide strong evidence that ASIP associates with PKCa in vivo. Whether endogenous PKCζ also interacts with ASIP remains to be clarified because of the lack of PKCζ-specific antibodies.

**Colocalization of ASIP with PKCa at the Junctional Complex MDCKII and NIH3T3 Cells**

*C. elegans* PAR-3 becomes localized asymmetrically in the anterior periphery, and affects the asymmetric distribution of other cytoplasmic components. Conservation of the overall amino acid sequence and the aPKC-binding activity between ASIP and *C. elegans* PAR-3 led to our recent findings that a *C. elegans* homologue of aPKC, PKC-3, is required for establishing embryonic polarity, that PKC-3 localizes in the anterior periphery of the *C. elegans* one-cell embryo in a manner very similar to that of PAR-3, and that PAR-3 and PKC-3 are mutually required for their asymmetric distributions (Tabuse et al., 1998). These findings not only provide strong evidence that aPKC and PAR-3 cooperate together to determine cell polarity in the *C. elegans* embryo, but also suggest the physiological importance of the interaction between ASIP and aPKC. The conservation of the biochemical relationship between ASIP/PAR-3 and aPKC/PKC-3 led us to investigate whether ASIP and PKCa are involved in regulating cell polarity as observed in *C. elegans* embryos.

In mammals, cell polarity has been studied intensively using cultured epithelial cells such as MDCKII cells. To evaluate the above possibility that the protein complex of
ASIP and aPKC is involved in regulating epithelial cell polarity, and to test further whether ASIP and PKCz physically interact in vivo, we examined the cellular distribution of ASIP and PKCz in MDCKII cells by double-label immunofluorescence microscopy. The results were as expected and exciting in that ASIP and PKCz showed overlapping localizations in vivo. Furthermore, they localize at junctional complexes including tight junctions and adherens junctions.

As shown in Fig. 6, ASIP staining (green) appears as a noncontinuous belt-like structure along the cell–cell contact region (Fig. 6 a). PKCz staining (red) appears in a very similar pattern in addition to punctate staining throughout the cytoplasm (Fig. 6 b). Importantly, there is considerable overlap in the staining patterns for ASIP and PKCz at the cell–cell contact region (orange/yellow) (Fig. 6 c). Furthermore, ASIP staining (green; Fig. 6 d) almost completely overlaps in double immunostaining with ZO-1 (red; Fig. 6, e and f), a protein known to localize at the tight junctions of epithelial cells. Confocal dissection revealed that signals for both antibodies are concentrated in the cell–cell contact region facing the apical membrane domain (data not shown). These results not only strongly support the in vivo association of ASIP and PKCz, but also suggest that both proteins are components of the epithelial junctional complex. Light microscopy doesn’t allow us to distinguish whether this staining reflects localization to the tight junction, the adherens junction, or to both. As described above, the anti-ASIP antibodies used in the present study all detect two ASIP bands, at 180 kD and 150 kD in MDCKII cell extracts. Overexpression of the 180-kD band by introducing full-length ASIP cDNA results in an increase in the staining at the cell–cell contact region (data not shown), clearly indicating that the 180-kD ASIP localizes to the junctional complex in MDCKII cells.

Essentially similar results are obtained for confluent cultures of NIH3T3 cells (Fig. 7), where adherens junctions are formed at the cell–cell contact region (Reynolds et al., 1996). In NIH3T3 cells, however, localization of ASIP and PKCz to the cell–cell contact region is not so complete as observed for MDCKII cells. More precisely, PKCz staining (red) mostly appears as a punctate pattern throughout the cytoplasm (Fig. 7 b), whereas localization of ASIP is mostly concentrated in the cell–cell contact region (Fig. 7 a). Importantly, there is again considerable overlap in the staining patterns for ASIP and PKCz in the cell–cell contact region (orange/yellow; Fig. 7 c). In addition, the ASIP staining (green; Fig. 7 d) also overlaps with that of ZO-1 (Fig. 7, e and f) in the cell–cell contact region in NIH3T3 cells. ZO-1 has been reported to localize at adherens junctions in confluent fibroblast cultures (Howarth et al., 1992; Itoh et al., 1993; Yonemura et al., 1995). Since NIH3T3...
cells express only the 180-kD ASIP, the ASIP staining in NIH3T3 cells is derived from this isoform. ASIP staining in nonconfluent cultures of NIH3T3 cells appears as punctate structures distributed in the cytoplasm in a manner similar to that of PKCα (data not shown).

Ca2+-dependent Distribution of PKCα and ASIP at the Junctional Complex of MDCKII Cells

To examine the requirement of cell–cell contact for the localization of PKCα and ASIP at the junctional complex in MDCKII cells, we monitored the localization of PKCα and ASIP during the formation and disappearance of cell–cell contacts. Previous experiments have shown that Ca2+-depletion from the culture medium of MDCKII cells results in the disappearance of cell–cell contacts and loss of tight junctions and adherens junctions (Gonzalez-Mariscal et al., 1985; Rajasekaran et al., 1996; Yamamoto et al., 1997). Furthermore, adding Ca2+ to the culture medium causes formation of cell–cell contacts with functional junctional complexes. As shown in Fig. 8, a and b, Ca2+-depletion from the culture medium results in cell rounding and disappearance of the specific staining for ASIP (green) and PKCα (red) at the cell surface. Most of the staining shows a cytoplasmic distribution that is slightly different for PKCα and ASIP. These results suggest that colocalization of ASIP and PKCα require the presence of cell–cell contacts. Fig. 8, c and d, show that adding Ca2+ results in reconcentration of PKCα and ASIP in the cell–cell contact region within 2 h. These properties of ASIP and PKCα are very similar to those of ZO-1 and E-cadherin (data not shown; Rajasekaran et al., 1996), and support the notion that ASIP and PKCα are peripheral components of cell–cell junctions.

Localization of ASIP and PKCα at Tight Junctions in Intestinal Epithelial Cells

To analyze the subcellular localization of the ASIP-PKCα complex in epithelial cells, we examined the distribution of ASIP and PKCα in several rat tissues with epithelial junctional complexes. Small intestine is a typical tissue with differentiated epithelial cells containing well-developed cell–cell junctions. As shown in Fig. 9, a and b, ASIP staining (green) is concentrated at the lumen side of the epithelial cells where tight junctions and adherens junctions are concentrated. Fig. 9 c shows another example of well-differentiated cell–cell junctions at the bile capillary formed from hepatocytes. Again, ASIP staining is concentrated at the cell–cell junctions. Fig. 9, d, e, and f show enlarged views of the intestinal epithelium, clearly indicating localization of both ASIP (green) and PKCα (red) staining at the cell–cell junctions; double staining indicates colocalization of the two proteins (Fig. 9 f). Fig. 9, g, h, and i show the results of similar experiments using antibodies for ASIP (green) and ZO-1 (red), indicating colocalization of ASIP and ZO-1. These results not only confirm the association between ASIP and PKCα in vivo, but also demonstrate the strong correlation between the ASIP-PKCα complex and the junctional complex.

We next used immuno-gold EM to examine whether ASIP localizes to tight junctions or adherens junctions in rat epithelia. Fig. 10 shows examples indicating that ASIP localizes to tight junctions, but not to adherens junctions in

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**Figure 7.** Colocalization of ASIP with aPKC and ZO-1 at cell junctions in NIH3T3 cells. Confluent NIH3T3 cells were doubly stained with anti-ASIP (C2) antibody (a and d, green) and anti-PKCα antibody (mAb) (b, red) or anti-ZO-1 (e, red) followed by FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG antibodies. The yellow and orange staining in c and f indicates the colocalization of ASIP and PKCα or ZO-1. Bars (c and f), 10 μm.

**Figure 8.** Immunofluorescence localization of ASIP and aPKC in Ca2+ switch experiments with MDCKII cells. Subconfluent MDCKII cells were transferred to low Ca2+ medium (growth medium containing 4 mM EGTA) for 6 h (a and b), and were then transferred back to normal Ca2+ medium for 2 h (c and d). The cells were doubly stained with anti-ASIP (C2) antibody (a and c) and anti-PKCα (mAb) antibody (b and d), followed by FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG antibodies. Bar (d), 10 μm.

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intestinal epithelia. This feature is very similar to that of ZO-1. These results provide strong evidence that ASIP and aPKC are peripheral components of tight junctions in epithelial cells.

Discussion

ASIP is a Physiological Partner of PKCλ in Differentiated Mammalian Cells

Protein kinase C plays a major role in intracellular signaling through lipid-derived second messengers (Nishizuka, 1995). However, the existence of multiple protein kinase C isoforms, their different sensitivities to lipids and calcium, and their diverse tissue and subcellular distribution suggest that different isoforms may have quite different physiological roles (Nishizuka, 1995; Mochly-Rosen and Gordon, 1998). Although overexpression studies and the use of pseudosubstrate peptides or dominant negative mutants of PKC isoforms have provided evidence consistent with the involvement of specific PKC isoforms in distinct cellular functions, their roles remain mostly elusive. As an alternative way to sort out these roles, efforts are being made to identify and study proteins that interact directly with different PKC isoforms. For example, attempts to understand the role of the atypical class of PKCs have led to the identification of several interacting proteins. These include p62/ZIP (PKCζ interacting protein), LIP (PKCα interacting protein), PAR-4 (which is induced upon cell apoptosis and is completely different from C. elegans polarity gene par-4), and Ras (Puls et al., 1997; Diaz-Meco et al., 1996a; Diaz-Meco et al., 1996b; Diaz-Meco et al., 1994). Demonstrating that these interactions are physiologically relevant is the next step.

In this study, we identified a novel protein, ASIP, that interacts with aPKC isoforms, PKCζ and PKCλ, but not with other PKC isoforms including cPKC (PKCα) and nPKC (PKCδ). This interaction is direct and involves the kinase domain of aPKC binding to ASIP within a 225–amino acid region. Importantly, this interaction is shown for endogenous proteins in NIH3T3 cells and in MDCKII cells. Furthermore, we provide evidence that a protein complex containing ASIP and PKCλ is a peripheral component of tight junctions in epithelial cells. Quite interestingly, ASIP shows strong overall sequence similarity to the C. elegans polarity protein PAR-3, and the interaction between ASIP and aPKC is conserved for PAR-3 and aPKC. As described above, this led to our recent findings that a C. elegans homologue of aPKC, PKC-3, is required for establishing embryonic polarity, binds to PAR-3 in COS cells, and colocalizes with PAR-3 at the anterior periphery of the one-cell embryo (Tabuse et al., 1998).

ASIP as well as PAR-3 does not contain any sequence motifs, suggesting catalytic activity, but does contain three PDZ domains in CR2. PDZ domains (also called DHR or GLGF) are found in many proteins such as the Drosophila Dlg, PSD-95, and ZO-1, leading to the current understanding that PDZ domains serve as protein–protein interaction domains to localize protein complexes to the cell periphery (Saras and Heldin, 1996; Sheng, 1996). Furthermore, analysis of the InaD protein, which contains five PDZ domains each interacting with a different protein in

Figure 9. Colocalization of ASIP with aPKC and ZO-1 at cell junctions in rat intestinal epithelium and hepatic bile capillaries. (a) Phase contrast images of frozen cryosections of rat small intestine. lum, lumen; epi, epithelium. (b and c) The localization of ASIP immunofluorescence in frozen sections of rat small intestine (b) and liver (c). b shows the same fields as a. The arrowhead in c shows a bile capillary. (d–i) Enlarged view of the intestinal epithelia. The samples were doubly stained with anti-ASIP (C2) antibody (d and green) and anti-PKCλ (mAb) antibody (e, red) or anti-ZO-1 (h, red) followed by FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG antibodies. The yellow and orange staining in f and i indicates colocalization of ASIP and PKCλ or ZO-1. Bars (b and c), 20 μm; bars (f and i), 5 μm.
The Drosophila phototransduction pathway, suggested that PDZ domain–containing proteins may serve as scaffolds upon which components of signaling complexes are assembled (Tsunoda et al., 1997). In addition to CR2, the CR1 region is conserved from worm to mammals, and may promote self-association of ASIP since the CR1 motif can interact with itself in the yeast two-hybrid system (T. Hirose, Y. Izumi, and S. Ohno, unpublished results). Thus, we propose that ASIP and PAR-3 serve as scaffolds for aPKC and other proteins to organize a signaling complex. Candidates for additional components in the ASIP/PAR-3 signaling complex include the previously reported aPKC-binding proteins, proteins localizing at tight junctions (see below), and the putative mammalian homologues of C. elegans of par-5 and par-6, genes required for the proper localization of PAR-3 (Watts et al., 1996; Guo and Kemphues, 1996).

ASIP and aPKC are Peripheral Components of Cell–Cell Junctions

Immunofluorescence microscopy reveals that ASIP and PKCα colocalize to the most apical end of the cell–cell contact region in MDCKII cells, and to the apical junction in intact epithelial cells in rat intestine. Evidence that this distribution reflects association with the tight junction comes from colocalization of ASIP with the tight junction component ZO-1 in both cases examined, the requirement for Ca^{2+} to maintain this localization in MDCKII cells, and immunoelectron microscopy in intestinal epithelia. The localization of PKCα to the cell–cell contact region is consistent with the previous observation that the signals obtained using anti-PKCζ antibodies overlap with those of ZO-1 in MDCKII cells (Saxon et al., 1994; Stuart and Nigam, 1995; Dodane and Kachar, 1996).

Tight junctions are the most apical intercellular junctions of epithelial and endothelial cells, and create a regulatable semipermeable diffusion barrier between individual cells (Balda and Matter, 1998). On a cellular level, they form an intramembrane diffusion fence that restricts the intermixing of apical and basolateral membrane components, leading to the asymmetric distribution of proteins and other cytoplasmic structures. These functions of tight junctions are dynamically regulated, but the molecular mechanism remains elusive. Identification of proteins localizing at tight junctions is starting to help us to understand the function of and regulation of tight junctions. These include proteins localizing at tight junctions such as ZO-1 (Stevenson et al., 1986; Willott et al., 1993; Itoh et al., 1993), ZO-2 (Gumbiner et al., 1991; Jesaitis and Goode, 1994), and p130/ZO-3 (Balda et al., 1993; Haskins et al., 1998), which share sequence similarities to Drosophila tumor suppressor DlgA, a tumor suppressor and peripheral component of septate junctions, the Drosophila version of mammalian tight junction (Woods et al., 1996).

In addition, cingulin (Citi et al., 1998), 7H6 (Zhang et al., 1993), Rab3B (Weber et al., 1994), symplekin (Keon et al., 1996), and AF-6 (Yamamoto et al., 1997) are known to be present at tight junctions. However, limited information is available concerning the roles these proteins play in the function and regulation of the tight junction.

Although some tight junction proteins such as ZO-1, ZO-2, p130/ZO-3, and cingulin are phosphorylated, there are no correlations between phosphorylation level and tight junction assembly (Balda et al., 1993; Citi and Deniseno, 1995). The presence of the consensus PKC phosphorylation sequence in the CR3 of ASIP and PAR-3 suggests that PKCα regulates this complex through phosphorylation. Consistent with this notion, ASIP domains, expressed as GST-fusion proteins in E. coli, are efficiently phosphorylated by recombinant mouse PKCζ (data not shown). The identification of ASIP and PKCα as proteins locating at tight junctions has the potential to accelerate understanding of tight junction regulation or function.

We have shown that ASIP colocalizes with ZO-1 to the cell–cell contact region in confluent cultures of NIH3T3 cells, which do not form tight junctions. Since ZO-1 localizes to the adherens junction in nonepithelial cells (Howarth et al., 1992; Itoh et al., 1993; Yonemura et al., 1995), it appears that ASIP and PKCα are components of adherens junctions in cells that do not form tight junctions. Not all of the PKCα colocalizes with ASIP at putative junctional complexes. Punctate staining is detectable in the cytoplasm of MDCKII cells and confluent NIH3T3 cells. One possibility is that this cytoplasmic staining represents PKCα localization to the lysosome-targeted endosomal compartment as was recently described for PKCα and its binding partner ZIP/p62 (Sanchez et al., 1998).

Analogy Between Epithelial Cell Polarity and That in Worm Embryogenesis

The conservation of a physical interaction between aPKC and ASIP/PAR-3 in worms and mammals raises the intriguing possibility that they are components of a widely used signaling complex whose role might include the determination of cell polarity. Another component of the C. elegans polarity system, PAR-1, is a member of a conserved family of kinases with several mammalian members. Rat family members MARK1 and MARK2 have been shown to phosphorylate microtubule-associated proteins, and the overexpression of MARK1 or MARK2 disrupts microtubule arrays in CHO cells (Drewes et al., 1997). Two other family members, a human protein, Kp78, and a mouse protein, EMK, localize asymmetrically in polarized epithelial cells (Parsa, 1988; Böhm et al., 1997). In-
Interestingly, the mouse homologue of MARK2, EMK, is distributed on the lateral surface of MDCK cells, and this distribution ends at the tight junction where ZO-1 localizes (Böhm et al., 1997). Taken together with the findings on C. elegans PKC-3 (Tabuse et al., 1998), the present study supports earlier speculation about a possible parallel between C. elegans embryos and mammalian epithelial cells (Fig. 11; Böhm et al., 1997).

As clearly demonstrated in the present study, ASIP and aPKC are components of tight junctions in epithelial cells, and may be involved in the function of tight junctions. They also localize to adherens junctions in cells without tight junctions, and are expressed in cells without cell–cell junctions. These results support the hypothesis that the protein complex of ASIP and PKCα is involved in the organization of cell polarity in epithelial cells. Alternatively, the protein complex might be a type of conserved biochemical machine used for diverse cellular functions. Clarification of the nature of the ASIP–aPKC protein complex will greatly enhance our understanding of the function of cell–cell junctions in the organization of cells, and hence the mechanism regulating cell growth and differentiation in multicellular organisms.

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