Meiotic maturation induces animal-vegetal asymmetric distribution of aPKC and ASIP/PAR-3 in *Xenopus* oocytes

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SUMMARY

The asymmetric distribution of cellular components is an important clue for understanding cell fate decision during embryonic patterning and cell functioning after differentiation. In *C. elegans* embryos, PAR-3 and aPKC form a complex that colocalizes to the anterior periphery of the one-cell embryo, and are indispensable for anterior-posterior polarity that is formed prior to asymmetric cell division. In mammals, ASIP (PAR-3 homologue) and aPKC, form a complex and colocalize to the epithelial tight junctions, which play critical roles in epithelial cell polarity. Although the mechanism by which PAR-3/ASIP and aPKC regulate cell polarization remains to be clarified, evolutionary conservation of the PAR-3/ASIP-aPKC complex suggests their general role in cell polarity organization. Here, we show the presence of the protein complex in *Xenopus laevis*. In epithelial cells, XASIP and XaPKC colocalize to the cell-cell contact region. To our surprise, they also colocalize to the animal hemisphere of mature oocytes, whereas they localize uniformly in immature oocytes. Moreover, hormonal stimulation of immature oocytes results in a change in the distribution of XaPKC 2-3 hours after the completion of germinal vesicle breakdown, which requires the kinase activity of aPKC. These results suggest that meiotic maturation induces the animal-vegetal asymmetry of aPKC.

Key words: *Xenopus laevis*, PKC, ASIP, PAR, Asymmetry, Polarity, Oocyte, Meiotic maturation

INTRODUCTION

The asymmetric localization of cytoplasmic components is the basis for the cell fate decision in embryonic patterning and cell function in differentiated cells. Genetic studies on asymmetric cell division in *C. elegans* and *Drosophila* provide insights into the mechanisms of cell polarity and emphasize the importance of cortically localized polarity organizing molecules, adapter molecules and the actin cytoskeleton in controlling the unequal segregation of cell-fate determinants and spindle orientation. In *C. elegans*, par genes play critical roles in the establishment of the cell polarity required for asymmetric divisions in early embryogenesis. Embryogenesis in *Caenorhabditis elegans* involves successive asymmetric cell divisions. Anterior-posterior (A-P) polarity is established during the first cell cycle after fertilization and requires a dynamic rearrangement of the cytoplasm along the A-P axis, which is defined by an extrinsic cue provided by sperm (Kirby et al., 1990; Goldstein and Hird, 1996). This process requires par genes (Kemphues et al., 1998). *Par* mutants show defects in the timing of early cleavage and the distribution of the cytoplasmic ribonucleoprotein P-granules, and the asymmetric distribution of cell fate-determining proteins such as GLP-1, a Notch homologue, and SKN-1, a DNA binding protein. *Par* mutants other than *par-1* also show defects in spindle orientation. Among six *par* genes (Morton et al., 1992; Cheng et al., 1995; Watts et al., 1996), all have been cloned (Levitan et al., 1994; Guo et al., 1995; Etemad-Moghadam et al., 1995; Boyd et al., 1996; Hung and Kemphues, 1999; Watts et al., 2000). *PAR-1* is a serine-threonine protein kinase. *PAR-2* is an unknown protein. *PAR-3* and *PAR-6* are adapter-like PDZ domain-containing proteins. Interestingly, *PAR-1* and *PAR-2* localize transiently to the posterior periphery of the one-cell embryo, whereas *PAR-3* and *PAR-6* localize to the anterior periphery. *PAR-3* and *PAR-6* are mutually required for their proper localization and for the localization of *PAR-1*, suggesting a possible functional link between these *par* gene products. However, the mechanism by which the sperm cue triggers the asymmetric distribution of PAR proteins is not clear, nor is it clear how the asymmetric distribution of PAR proteins leads to other cellular asymmetries.

Mammalian epithelial cells also provided an experimental system in which essential features of cell polarity have been revealed (Eaton and Simons, 1995; Drubin and Nelson, 1996; Gumbiner, 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 the basis for directed transport across the epithelium. As in *C. elegans* one-cell embryos, the establishment of cell polarity in epithelial cells starts with a cortical spatial cue. The spatial cue in epithelial cells is cell
adhesion. E-cadherin-mediated cell-cell contact and contact between integrins and the extracellular matrix (ECM) trigger the specialized assembly of the actin-based cytoskeleton and signaling networks around the adhesion receptors and tight junctions, and position other cytoskeletal complexes and protein-sorting compartments (Eaton and Simons, 1995; Drubin and Nelson, 1996; Gumbiner et al., 1996). How adhesion receptors trigger the establishment of cellular asymmetry is not clear, nor is it clear how tight junctions reinforce and maintain cellular asymmetry.

Protein kinase C (PKC) describes a family of serine/threonine protein kinases that comprises at least 10 isotypes in mammals. Studies in cultured cells revealed that each isotype responds to and is activated by one or more of a variety of lipid metabolites, including diacylglycerols and phosphatidylinositol 3,4,5-trisphosphate, a product of phosphatidylinositol-3 kinase (PI-3 kinase). Thus, PKC isotypes seem to play distinct roles in cell regulation, although their exact roles in cell signaling remain to be clarified. Among three PKC subfamilies, cPKC, nPKC and aPKC, which are conserved from C. elegans to mammals, the aPKC isotype has a unique position. Our recent experiments on C. elegans and mammalian epithelial cells have provided evidence to suggest a general role of aPKC in the regulation of cell polarity. The initial finding was a novel PDZ-containing protein, ASIP (Atypical protein kinase C isotype Specific Interacting Protein), that associates directly with atypical PKC and colocalizes at the cell-cell junction, the tight junction (Izumi et al., 1998). The close sequence similarity between ASIP and C. elegans PAR-3 prompted us to examine the role of aPKC (PKC-3) and its interaction with PAR-3 in C. elegans embryogenesis. As expected, the depletion of PKC-3 (aPKC) by RNA-mediated interference (RNAi) demonstrated that in addition to the six par genes, pck-3 is also required for asymmetric cell division. The pck-3 (RNAi) embryo shows a phenotype very similar to that of par-3. Importantly, PKC-3 and PAR-3 associate directly and colocalize to the anterior periphery of the one-cell embryo, and both are required for the posterior localization of PAR-1 (Tabuse et al., 1998; Y. Sugiyama et al., unpublished).

The evolutionary conservation of aPKC and PAR-3/ASIP from nematodes to mammals suggests a general role for the protein complex involving aPKC and ASIP/PAR-3 in fundamental cellular functions, including regulation of the spatial control of cytoplasmic materials or cell polarity. However, huge differences exist between cell polarity in the C. elegans embryo and in mammalian epithelial cells. The present study was undertaken to close this gap by identifying and characterizing the proteins or protein complex in Xenopus oocytes, in which cell polarity also plays a critical role in early embryogenesis. Here, we have identified aPKC and its binding protein ASIP/PAR-3 in Xenopus by using specific antibodies. We describe their distribution in oocytes and early embryos, and their reorganization during meiosis. Moreover, we verify these phenomena by using ectopic molecules in vitro. Our results also suggest that animal-vegetal polarization is established during meiosis after germinal vesicle breakdown (GVBD) prior to fertilization.

**MATERIALS AND METHODS**

**Antibodies**

The anti-aPKCζ antibody C-20 (Santa Cruz) is an affinity-purified rabbit polyclonal antibody raised against the C terminus (aa 573-592) of mouse aPKCζeta (Josef et al., 1999). The anti-ASIP antibody was raised against the GST-ASIP fusion protein containing amino acid (aa) residues 712-936 of ASIP and affinity purified (Izumi et al., 1998). The anti-Tag antibody was purchased from Santa Cruz as Omni probe (M-21) raised against (His)6 peptide sequences. Secondary antibodies were obtained from BIO-RAD (anti-Rabbit HRP) and Jackson ImmunoResearch (anti-Rabbit FITC).

**Immunoprecipitation and immunoblotting**

For the identification of proteins by western blot analysis, *Xenopus* oocytes and embryos were chemically dejellied with 3.5% cysteine hydrochloride (pH 7.8), washed with Steinberg’s solution (SS; 60.00 mM NaCl, 0.67 mM KCl, 0.34 mM Ca(NO₃)₂, 0.83 mM MgSO₄·7H₂O, 10.00 mM Hepes, pH 7.4) (Asashima et al., 1990), and homogenized in four volumes of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na₃ VO₄, 10 mg/ml leupeptin, 1 mM PMSF, 2 mg/ml aprotinin, 1% Triton X-100. After a 30 minute incubation on ice, the extracts were sonicated for 30 seconds and clarified by centrifugation at 15,000 g for 30 minutes. Extracts were electrophoresed in 4%-20% or 8.5% polyacrylamide-SDS gels, transferred, and probed with the indicated antibodies using an ECL detection system (Amersham) as described (Izumi et al., 1998). For immunoprecipitation, extracts were prepared as described above using two volumes of lysis buffer. Extracts were preincubated with Protein G-Sepharose (Pharmacia Bio-tech, Inc., Piscataway, NJ, USA) for 1 hour at 4°C to remove non-specific binding to Protein G-Sepharose, and incubated with antibodies preabsorbed on Protein G-Sepharose (Pharmacia Bio-tech, Inc., Piscataway, NJ, USA) for 2 hours at 4°C. The immunocomplexes on Sepharose were washed six times with lysis buffer, after which the proteins were analyzed by western blotting.

**Immunocytochemistry and immunohistochemistry**

*Xenopus* kidney epithelial A6 cells were cultured in 50% Leibovitz L-15 medium containing 15 mM Hepes, 1 mM L-glutamine (Sigma) 0.01% Kanamycin, pH 7.4. Confluent A6 cells plated on glass coverslips were washed three times with Stainburg’s solution and fixed with cold methanol for 30-60 minutes at 4°C. After blocking in 3% normal goat serum/TBS for 2 hours at room temperature, specimens were incubated with the primary antibodies for 2 hours at room temperature and washed three times for 10 minutes each at room temperature with TBS. After the first incubation, the cells were incubated for 2 hours at room temperature with the secondary antibodies (FITC-conjugated goat anti-rabbit antibody) and washed three times for 10 minutes each with TBS. Samples were viewed under an Olympus BX50 microscope. Images were captured with a Princeton Instruments digital camera. Images were manipulated using Adobe Photoshop software.

Albino adult *Xenopus* ovaries were preserved for immunocytochemical staining by freeze-substitution (Shiurba et al., 1991). Sections were cut at a thickness of 8 μm and mounted on poly-L-lysine coated glass slides (Matunami glass IND., Ltd, Japan). Sections were deparaffinized in xylene, rehydrated in a graded ethanol series, and washed in deionized water for 5 minutes. The samples were then treated with 0.3% hydrogen peroxide in absolute methanol for 30 minutes to inhibit endogenous peroxidase activity. The slides were rinsed in water and washed in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 10 minutes. Non-specific protein binding was blocked by incubation with 3% normal goat serum (NGS) in TBS for 2 hours. Working dilutions of primary antibodies (1:200 anti-aPKC) were prepared in TBS with 1% normal goat serum (NGS). For negative controls, primary antibodies were pre-absorbed with 200 μg/ml of the corresponding peptide overnight at 4°C, then applied to sections overnight at 4°C. Sections were washed in TBS for 30 minutes. Then, a peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) secondary antibody, diluted 1:2000 in
TBS containing 1.5% NGS, was applied for 2 hours. Sections were washed again for 30 minutes, and developed for 8-30 minutes in 0.25 mg/ml 3,3′-diaminobenzidine (DAB) chromogen, 0.05% hydrogen peroxide in 50 mM Tris-HCl, pH 7.4. Finally, the sections were dehydrated in ethanol and xylene, and glass coverslips were applied with Permount (Fisher Scientific, Fair Lawn, NJ, USA) for photomicrography.

Whole-mount immunohistochemistry
Albino or wild-type *Xenopus* oocytes and dejellied embryos were fixed for longer than overnight at room temperature in Dent fixative (20% DMSO, 80% methanol) (Klymkowsky and Hanken, 1991). The fixed oocytes and embryos were bleached for 12 hours to 4 days in 10% hydrogen peroxide diluted in Dent fixative to block endogenous peroxidase. Samples were washed with TBS for 30 minutes at 4°C, then they were incubated overnight at 4°C in blocking solution (3% normal goat serum (NGS) diluted in 95% calf serum/5% DMSO). After blocking, the samples were incubated with primary antibodies overnight at 4°C. Working dilutions of primary antibodies (1:200 anti-ASIP; 1:200 anti-nPKCζ; 1 ng/ml of normal rabbit IgG) were applied for 2 hours at 4°C, and clarified by centrifugation at 15,000 C, washed five times at 4°C in secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), diluted 1:2000 as for primary antibodies. The samples were washed five times for 60 minutes each, and developed for 30 minutes in 0.25 mg/ml 3,3′-diaminobenzidine tetrahydrochloride, 0.05% (v/v) hydrogen peroxide in TBS. The reactions were stopped by dehydration with methanol. Images were captured under a KEYENCE digital microscope (VH-6300) and manipulated using Adobe Photoshop software.

Oocyte preparation and culture
Stage I-VI albino and wild-type *Xenopus* oocytes were selected manually from ovaries treated with 1 mg/ml collagenase (Sigma) in 100 mM sodium phosphate buffer (pH 7.4) for 30 minutes at 20-23°C. Oocytes at each stage were washed three times with OR-2 (82.50 mM NaCl, 2.50 mM KCl, 1.00 mM CaCl2, 1.00 mM MgCl2, 1.00 mM Na2HPO4, 5.00 mM Hepes, pH 7.2) solution and stored in OR-2 medium (Gibco) containing 15 mM Hepes, 1 mM L-glutamine (Sigma), 0.01% Kanamycin, pH 7.4.

Plasmids and in vitro transcription
The mammalian expression vectors used to prepare the His-T7 fusion protein expression constructs have been described previously (Akimoto et al., 1998); aPKCζ-WT encodes full-length mouse PKCζ cDNA; aPKCζ-KN encodes the full-length aPKCζ with a point mutation at the ATP binding site (K273E). His-T7-fused aPKCζ (Tag-aPKCζ-WT) and its mutant (Tag-aPKCζ-KN) were constructed by fusing aPKCζ-WT or aPKCζ-KN downstream of the six histidine residues and a 12 aa sequence from the T7 gene 10 reader sequence. The appropriate restriction enzyme digestion fragments (Tag-aPKCζ-WT, Tag-aPKCζ-KN) were subcloned into the BglII site of pSP64T vector, provided by Dr D. A. Melton. Template plasmids were digested with Smal and capped mRNA was synthesized in vitro by mMASSAGE mMACHINE (Ambion) as described (Krieg et al., 1989). RNA samples were dissolved in Gurdon’s buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.5) and microinjected into the marginal zone of prepared stage-6 oocytes in 3% Ficoll-OR2 solution.

Microinjection
Immature stage VI *Xenopus* oocytes were selected after the ovaries were treated with 1 mg/ml collagenase in 100 mM sodium phosphate buffer (pH 7.4) for 30 minutes at 20-23°C. Stage-6 oocytes were microinjected with 20 nl of sample (0.1-2.0 mg/ml mRNA in Gurdon’s buffer) in 3% Ficoll-modified Barth’s solution containing 88.0 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO3, 0.3 mM Ca(NO3)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 0.01% Kanamycin, pH 7.4, using an injector. After injection, the oocytes were immediately transferred to L-15 medium, cultured for 18 hours, and then exposed to 4 µM progesterone (Gotoh et al., 1995). More than 30 oocytes were scored for the appearance of a white spot in the animal pole to judge germinal vesicle breakdown (GVBD).

Whole-mount in situ hybridization
*Xenopus* albino embryos at various stages were fixed in MEMFA (100 mM MOPS, 2 mM EGTA, 1 mM MgSO4, 3.7% formalin) for 8 hours, and transferred into 100% methanol. The XaPKC gene fragment is obtained by PCR using the following oligonucleotide primers to the *Xenopus* aPKCζ: 5′-CCAGCCCATCAGATCATTC-3′ and 5′-AGATTCGCTATTGTGTCGCC-3′. Digoxigenin (DIG)-labeled RNA probes in sense and antisense orientations were prepared from the XaPKC gene fragment subcloned into pGEM T easy vector, using a DIG RNA Labeling Kit (Boehringer Mannheim). Whole-mount in situ hybridization was performed as previously described (Harland, 1991) with the following modifications: the embryos were washed with maleic acid buffer (MAB; 100 mM NaCl, pH 7.5) instead of the PBS-based solutions, and then incubated in MAB with anti-DIG antibody (Boehringer-Mannheim) and 20% bovine serum. Hybridization was detected with an alkaline-phosphatase coupled anti-DIG antibody and visualized using BM purple as a substrate (Boehringer Mannheim).

RESULTS

XaPKC and XASIP in *Xenopus* oocytes, embryos and epithelial cells
A previous study identified a cDNA clone (accession number A5A75362) encoding a *Xenopus* homologue of the mammalian aPKC isotypes PKCζ and PKCζ (Dominguez et al., 1992). Since the sequence is more similar to PKCζ, we call it XaPKCζ. To examine the expression of XaPKCζ, we used an antibody, C-20, directed against the C-terminal 20 amino acids of mouse PKCζ (anti-aPKC antibody) since this antibody efficiently recognizes PKCζ, whose corresponding sequence is identical to that of XaPKCζ (Fig. 1A). The antibody recognized an 80 kDa band in extracts prepared from *Xenopus* oocytes, unfertilized eggs and *Xenopus* epithelial cell line A6, and concentrated in immunoprecipitates of the same antibody (Fig 2A). These results indicate that the band is XaPKCζ or its close homologue (XaPKC), and is maternally expressed throughout oogenesis. The presence of the 80 kDa band in extracts of A6 epithelial cells also indicates that XaPKC shows zygotic expression (Fig. 2A).

The conservation of the amino acid sequence (Fig. 1B) and the polarized localization of ASIP/PAR-3 in mammalian epithelial cells and *C. elegans* one-cell embryos (Fig. 1C,D: Izumi et al., 1998; Etemad-Moghadam et al., 1995; Tabuse et al., 1994) raises the intriguing possibility that they are orthologues sharing a conserved cellular function regulating cell polarity. This implies the presence of their *Xenopus* counterpart. To test this notion, we performed western analysis of the total lysate of unfertilized egg extracts using an anti-ASIP antibody raised against rat ASIP. Unfortunately, we failed to detect any clear bands recognized specifically by
the antibody (data not shown). We then carried out
immunoprecipitation experiments using extracts of unfertilized
egg. Western analysis of the immunoprecipitates by the same
antibody revealed a band migrating at around 180 kDa (Fig.
2B, upper panel). The 180 kDa band disappeared in the
presence of excess antigen, GST-ASIP 712-936 (data not
shown). Importantly, the same ASIP-immunoprecipitates
contained XaPKC (Fig. 2B, lower panel). Thus, the 180 kDa
band in the ASIP-immunoprecipitates fulfills at least two
criteria for a Xenopus homologue of ASIP (XASIP). First, the
180 kDa band contains an aa sequence similar to rat ASIP.
Second, the 180 kDa band forms a complex with XaPKC.
These suggest an evolutionary conservation of the interaction
between aPKC and ASIP/PAR-3 (Izumi et al., 1998; Tabuse et
al., 1998). Reciprocal experiments in which XaPKC was first
immunoprecipitated and probed with anti-ASIP antibody failed
to detect the 180 kDa XASIP band (Fig. 2B, upper panel). A

![Fig. 1. Schematic structure of aPKC and ASIP/PAR-3/Bazooka and their intracellular localization. (A) Comparison of the C-terminal sequence of mouse aPKC isotypes and XaPKC. The anti-aPKC antibodies used in the present study were raised against the C-terminal 20 aa sequence of mouse PKCζ. The sequence shares significant sequence identity to that of mouse PKCζ and XaPKCζ. (B) Schematic structure of mammalian ASIP, C. elegans PAR-3 and Drosophila Bazooka. The N-terminal domain (CR1), three PDZ domains (CR-2) and the aPKC binding region (CR-3) are evolutionarily conserved. The polyclonal antibody against ASIP was raised against the aPKC binding region of ASIP. (C) Cellular localization of PKC-3 and PAR-3 in a C. elegans 1-cell embryo. The arrow indicates the anterior-posterior axis. (D) Cellular localization of aPKC and ASIP in polarized epithelial cells. The arrow indicates the apical-basal axis.](image1)

![Fig. 2. Identification of XaPKC and XASIP in Xenopus. (A) Western analysis of XaPKC in Xenopus oocytes, eggs and epithelial A6 cells. XaPKC was identified as an 80 kDa band by an anti-mouse PKCζ antibody. XaPKC is expressed maternally throughout oogenesis, in unfertilized eggs and in epithelial A6 cells. Samples loaded as oocyte and egg extracts were estimated to contain 25 μg protein per lane. (B) Immunoprecipitation analysis of XaPKC and XASIP. XASIP is identified as a 180 kDa band in anti-ASIP immunoprecipitates of egg extracts (upper panel). The 80 kDa XaPKC was detected in the anti-ASIP immunoprecipitates as well as in the anti-aPKC immunoprecipitates. Samples loaded into each lane correspond to extracts of 30 eggs (anti-aPKC I.P.) or 50 eggs (anti-ASIP I.P.).](image2)
similar situation occurs with mammalian counterparts and is explained by the presence of excess amounts of aPKC compared with ASIP (Izumi et al., 1998).

**Localization of XaPKC and XASIP in the epithelial cell-cell contact region**

In order to test whether the antibodies used in the above experiments could be used for immunohistochemical analysis, we stained *Xenopus* epithelial A6 cells. Previous studies have shown that A6 cells form a tight junction, where ZO-1 staining localizes to the cell-cell contact region (Merzdorf et al., 1997). As shown in Fig. 3B, the immunofluorescence staining of A6 cells with anti-aPKC antibody gave clear staining at the cell-cell contact region in addition to the cytoplasm. This is very similar to the result obtained for mammalian epithelial cells such as MDCK cells stained by the same antibody (Izumi, 1998). Taken together with the results of the western analysis showing the presence of an 80 kDa band (Fig. 2A), we concluded that the anti-aPKC antibody could be used for the immunohistochemical detection of XaPKC.

The anti-ASIP antibody stained the cell-cell contact region and an unknown perinuclear structure (Fig. 3D). Since only the staining at the cell-cell contact region disappeared in the presence of excess antigen (Fig. 3C), the perinuclear staining is likely to be non-specific. These results further support the notion that the 180 kDa band recognized by the anti-ASIP antibody is the *Xenopus* ASIP, XASIP, and that XaPKC and XASIP colocalize at the cell-cell contact region in epithelial cells, as in the case of their mammalian counterparts (Izumi et al., 1998).

**Uniform localization of XaPKC and XASIP in immature oocytes**

In *C. elegans*, PAR-3 and aPKC form a complex, colocalize to the anterior periphery of the one-cell embryo, and are required for anterior-posterior polarity of the one-cell embryo. In *Xenopus*, the first body axis is the animal vegetal axis, which is believed to be formed during the early stages of oogenesis. This suggests that XaPKC and ASIP might show asymmetric distributions along the animal-vegetal axis. To examine this possibility, we stained sections of ovary isolated from albino *Xenopus*. XaPKC staining could be detected in some oocytes, where it appeared to be distributed throughout the cytoplasm (Fig. 4B). Interestingly, some of the oocytes stained strongly and the rest stained weakly. Taken together with the results of the western blot analysis, where the 80 kDa XaPKC band is most abundant in stage-3 oocytes (Fig. 2A), this suggests that oocytes showing strong cytoplasmic staining are at around stage 3, although we...
were unable to confirm this. To analyze further the distribution of XaPKC in oocytes, we next stained oocytes isolated at various stages by whole-mount immunohistochemistry, which indicates cortical protein distribution (Fig. 4D). Oocytes at various stages showed uniform staining for XaPKC (Fig. 4D). Taken together, these results suggest that XaPKC distributes uniformly throughout the cytoplasm during the different stages of oogenesis, and this is also detected by whole-mount staining. Similar uniform staining for XASIP in isolated oocytes was also obtained (data not shown), suggesting that the distribution of XASIP in immature oocytes is also uniform.

**Asymmetric colocalization of XaPKC and XASIP in the animal hemisphere in mature oocytes and early embryos**

As described above, XaPKC staining in oocytes failed to give any clues about asymmetry. However, the staining of XaPKC in mature oocytes (unfertilized eggs) showed a striking staining pattern, with clear asymmetry (Fig. 5A-C). Since we used an albino strain to obtain clear staining patterns, the animal-vegetal axis cannot be distinguished, but it is possible to observe which hemisphere is stained in 8-cell embryos. The XaPKC staining in 8-cell embryos is restricted to the four animal blastomeres and is not detected in the four vegetal blastomeres (Fig. 5E,F). Further, in stage-10.5 embryos, the XaPKC staining distributes to the presumptive ectoderm area but not to the yolk plaque (data not shown). Thus, the stained hemisphere in unfertilized eggs must be the animal hemisphere. Very similar staining patterns were obtained for XASIP (Fig. 5D), suggesting the colocalization of the two proteins. This is consistent with the formation of a complex between these proteins. Importantly, such asymmetric staining was not observed in stage-6 oocytes. Both antibodies for XaPKC and XASIP stained uniformly in stage-6 oocytes (Fig. 5G,H).

**Asymmetric distribution of XaPKC in the animal hemisphere after oocyte maturation in vitro**

The results shown above clearly demonstrate that, as far as cortical proteins are concerned, XaPKC and XASIP stainings distribute uniformly in stage-6 oocytes, but almost exclusively to the animal hemisphere in mature oocytes (unfertilized eggs). This implies that the change in the distribution of XaPKC and XASIP occurs during the maturation of stage-6 oocytes.

Oocyte maturation can be demonstrated in vitro by progesterone stimulation. To confirm the above implication, we isolated stage-6 oocytes and examined whether XaPKC staining changes during hormone-induced in vitro maturation. For this purpose we used wild-type oocytes to monitor maturation in terms of germinal vesicle breakdown (GVBD). The animal hemisphere of wild-type oocytes had a very high
staining background, but the presence or absence of the staining could be distinguished by focussing on the vegetal hemisphere. If XaPKC distributes uniformly, the vegetal pole would be stained by anti-aPKC antibody. On the other hand, if XaPKC distributes to the animal hemisphere, the vegetal pole would not be stained. We counted oocytes showing loss of staining in the vegetal pole. This made it possible to determine whether the change in the distribution of XaPKC occurs in vitro and, if it does, to determine when it occurs. As shown in Fig. 6B, XaPKC distribution changes to the animal hemisphere 2-3 hours after GVBD.

We wished to develop a system to determine which sequences in aPKC are required for complex localization during oocyte maturation, so we next examined whether ectopic aPKC behaves similarly to endogenous XaPKC. For this purpose, we constructed a fusion protein in which His-T7 epitope is fused N-terminal to aPKCα (Tag-aPKCα). Injection of the mRNA encoding the Tag-aPKCα fusion protein resulted in the expression of the band visualized by western blotting with anti-Tag antibody (Fig. 7C). Next, we stained the RNA-injected oocytes with anti-Tag antibody. As shown in Fig. 7Ah, stage-6 oocytes show a loss of staining in the vegetal hemisphere only after hormone stimulation. This provides a strong evidence that XaPKC distribution changes to the animal hemisphere in response to hormone stimulation.

The kinase domain and its activity in XaPKC is responsible for the hormone-induced change in the asymmetric distribution of XaPKC

The above results showing that ectopic aPKC has similar pattern of hormone-induced change in its distribution suggests the possibility of identifying the sequence involved in the change in distribution. Thus, we examined the behavior of two aPKC constructs. Importantly, Tag-aPKCα-KN, a point mutant lacking the kinase activity, distributed uniformly before and after hormone stimulation, indicating that the kinase

Fig. 7. Change in the distribution of the ectopic Tag-aPKCα fusion protein during hormone-induced maturation of oocytes. (A) Wild-type oocytes were injected with RNA synthesized in vitro, incubated with (+) or without (−) 4 μM progesterone for 16–18 hours, and stained with anti-Tag antibody after fixation and bleaching. (B) The ratio of staining in both hemisphere with anti-Tag antibody, indicating the Tag-aPKCα distribution in immature or mature oocytes. These experiments were reproduced more than three times. (C) Western analysis of the oocyte extracts prepared 18 hours after mRNA injection with anti-Tag antibody.

Fig. 8. Albino unfertilized eggs and 8-cell embryos analyzed by whole-mount in situ hybridization. Eggs and embryos were fixed in MEMFA and hybridized with DIG-labeled sense (control) or anti-sense probes. (A,B,E,F) Animal views, (C,G) vegetal views and (D,H) equatorial views of eggs and embryos. Scale bar, approximately 250 μm.
activity of aPKC is required for the change in the distribution (Fig. 7). On the other hand, another tagged aPKC KD, a deletion mutant lacking the regulatory domain, showed a very similar phenotype to the wild-type aPKC, i.e. uniform distribution before stimulation and hormone-induced change in the distribution to the animal hemisphere (data not shown). This suggests that the kinase domain of aPKC is responsible for the hormone-induced change in the distribution of aPKC.

Asymmetric distribution of aPKC mRNA in unfertilized eggs

The results described above show the change in the asymmetric distribution of the XaPKC protein and, most probably, the XASIP protein. The next question is whether the protein distribution relates to mRNA localization. Therefore, we examined the localization of the XaPKC mRNA in unfertilized eggs and 8-cell embryos. As shown in Fig. 8, the XaPKC mRNA distribution shows an asymmetric gradient along the animal-vegetal axis; the animal pole signal is strongest while the vegetal hemisphere is weakest. The pattern of XaPKC mRNA distribution is clearly different from that of the XaPKC protein in that the XaPKC mRNA distribution shows a gradient whereas that of the XaPKC protein shows exclusive distribution in the animal hemisphere.

DISCUSSION

The Xenopus egg has a single axis of asymmetry prior to fertilization, the animal-vegetal axis. It is widely accepted that this asymmetry, the polarized localization of maternal factors in the egg, is the earliest step in embryonic patterning. Molecules that trigger certain developmental programs are specifically sequestered in defined regions of the egg cytoplasm, and the differential segregation of maternal factors establishes the initial differences between cell groups, which are then amplified by intercellular signaling (Kume et al., 1993). The idea that vegetally localized cytoplasmic factors are shifted towards the future dorsal side after fertilization prompted the examination of the role of vegetally localized factors in the formation of the dorsoventral axis. Molecules that show animal-vegetal asymmetry include cytoskeletal components (Gard and Klymkowski, 1998; Gard et al., 1997) and maternal mRNAs (VegT etc.) (Mowry et al., 1998; Zhang et al., 1996, 1998). However, specific proteins that localize asymmetrically in the mature egg, as well as the molecular mechanisms underlying the localization process, are only now being unraveled.

In the present study, we examined the presence and polarized distribution of the Xenopus versions of C. elegans, Drosophila, and mammalian polarity proteins, aPKC and ASIP/PAR-3/Bazooka, and demonstrated the presence of XaPKC in oocytes of various stages, eggs and cultured A6 cells using an aPKC-specific antibody. Quite interestingly, immunocytochemical visualization of XaPKC revealed that it distributes exclusively to the animal hemisphere in mature oocytes, whereas it distributes throughout immature oocytes, including stage-6 oocytes, as far as the cortical protein distribution is concerned. Moreover, this change in the distribution of XaPKC was demonstrated in vitro during progesterone-induced maturation of full-grown stage-6 oocytes. Analyses of ectopic Tag-aPKC fusion proteins revealed that the kinase activity of aPKC is critical for the change in the distribution. Antibodies raised against mammalian ASIP also recognized a 180 kDa protein, XASIP, and XASIP-immunoprecipitates of oocyte extracts contain XaPKC, confirming the presence of XASIP, although the cDNA for XASIP remains to be identified. Thus, XaPKC and XASIP presumably form a complex, as in the case of their C. elegans and mammalian counterparts. The XASIP protein visualized using anti-mammalian ASIP antibodies shows very similar distribution to that of XaPKC, suggesting that the two proteins form a complex in oocytes during hormone-induced asymmetric distribution.

In addition to the asymmetric distribution of the XaPKC and XASIP proteins, we also showed the asymmetric distribution of the XaPKC mRNA, which could explain the asymmetric distribution of the XaPKC protein. However, XaPKC mRNA distribution cannot be the sole cause of XaPKC protein distribution. As shown in Fig. 7, although Tag-aPKC-l-WT showed a change in the distribution induced by meiotic maturation, Tag-aPKC-l-KN did not show this change (Fig. 7A,B). Tag-aPKC-l-KN, which lacks kinase activity, differs from Tag-aPKC-l-WT by only one amino acid at the ATP binding site, and the mRNAs encoding them differ by only nucleotide. Thus, an asymmetric distribution of Tag-aPKC-l-WT must reflect a mechanism operating at the protein level, where the aPKC kinase activity occurs, indicating that the asymmetric distribution of the XaPKC protein is not solely dependant on a mechanism related to mRNA localization. Further, the disappearance of vegetal aPKC during maturation must be caused by the redistribution and/or degradation of aPKC, also processes in which the kinase activity of aPKC is required.

Polarization and meiotic maturation of oocytes in Xenopus

The most detailed studies of animal-vegetal asymmetry involve the cytoskeletal components, microtubules (MTs) and microfilaments (MFs) (Gard and Klymkowsky, 1999). Histological studies show that the asymmetry of the cytoskeleton progresses throughout oogenesis and meiotic maturation. A series of studies of keratin distribution using specific inhibitors of microfilaments and microtubules, cytochalasin B and nocodazole, suggests that cortical keratin network formation is regulated by MFs and MTs in stage-6 oocytes (Gard et al., 1997). On the other hand, studies of maternal mRNA localization show the presence of an additional mechanism of animal-vegetal asymmetry. Some maternal mRNA localizes to the vegetal cortex (Mowry and Cote, 1999; Zhang et al., 1996, 1998). Studies on the sorting system of maternal mRNA reveal two distinct mechanisms, an early pathway and a late pathway. The early pathway is based on a mitochondrial cloud (MC) during the early stages of oogenesis, stages 1-6. The late pathway depends on cytoskeletal elements (Mowry and Cote, 1999). These observations suggest that maternal mRNA localization is established in stage-6 oocytes. Our present demonstration that XaPKC mRNA distributes in an asymmetric manner with a gradient along the animal-vegetal axis suggests experiments to analyze further the mechanism regulating maternal mRNA localization in oocytes.
In marked contrast to the localization of mRNAs, however, little is known about proteins that localize asymmetrically (Denegre et al., 1997). XaPKC and XASIP, which distribute uniformly at all stages up to early stage-6 oocytes and reorganize their distribution to show animal-vegetal asymmetry prior to fertilization, are quite unique in their distribution and emphasize the importance of the meiotic maturation process for the completion of animal-vegetal polarization. The hormone stimulation of full-grown stage-6 oocytes arrested in prophase I of meiosis results in maturation and progression to metaphase II arrest. This process, meiotic maturation, involves a drastic change in structural and biochemical events that include plasma membrane flattening, redistribution of cortical granules, germinal vesicle disassembly, meiotic spindle formation, marked changes in cytoskeleton and mRNA distribution, changes in pH, phosphorylation and dephosphorylation, and ion flux (Bement and Capco, 1990). For example, Vg1 mRNA is redistributed from the vegetal subcortical region of stage-6 oocytes into the whole vegetal hemisphere in mature oocytes (Weeks and Melton, 1987). Further, XIP3R localizes in the cytoplasm of the animal hemisphere in a well-organized endoplasmic reticulum-like structure and intensively in the perinuclear region of stage-6 oocytes, whereas it is densely enriched in the cortical region of both hemispheres (Kume et al., 1993). Importantly, the cortical cytoskeletal network disassembles and causes dynamic changes in membrane organization with the most extensive changes in the animal hemisphere (Bement and Capco, 1990; Gard et al., 1997).

Meiotic cell cycle progression depends upon maturation-promoting factor (MPF), which consists of two subunits, Cdc2 and cyclin B (Dumphy et al., 1988; Gautier et al., 1988, 1990). Meiotic maturation is initiated by active MPF, which is triggered by Cdc25-dependent T14/Y15 dephosphorylation (Izumi and Maller, 1995). On the other hand, the other factor that initiates meiotic maturation is identified as a protooncogene c-mos product, Mos. Meiotic cell cycle progression requires the de novo synthesis of Mos, which is regulated by translational control, polyadenylation of c-mos mRNA (see Sagata et al., 1997, for a review). The activation of MPF requires de novo synthesized Mos for the initiation of maturation and entry into meiosis II. Recent experiments have shown the importance of the correct subcellular localization of cyclin B2 for the initiation of bipolar spindle formation (Yoshitome et al., 1998). These observations suggest an intracellular mechanism regulating the distribution of specific proteins and mRNAs during oocyte maturation, but the exact nature of this regulation remains to be clarified.

Studies in mammalian cells have demonstrated that aPKCα is activated downstream of the PDGF receptor, through the phosphatidylinositol 3-kinase pathway (Akimoto et al., 1996). Microinjection of the pseudosubstrate peptide of XaPKC or the overexpression of the kinase-deficient mutant of XaPKC, results in the inhibition of oocyte maturation (Domínguez et al., 1992; Berra et al., 1993; Camero et al., 1995). This suggests a role of XaPKC in oocyte maturation. Although we are not in a position to speculate further about the role of XaPKC in the maturation process, our present demonstration of a change in the asymmetric distribution of XaPKC and XASIP and the requirement for the kinase activity of aPKC for the maturation-induced change in the localization of aPKC suggest a role for XaPKC and XASIP in the completion of oocyte maturation.

### Conservation of the aPKC-ASIP complex from nematodes to vertebrates

A kinase-deficient mutant of aPKCα blocks the formation of tight junctions and the proper localization of apical and basolateral membrane proteins (A. Suzuki, T. Yamanaka, T. Hirose, K. Hashiba, Y. Izumi, T. Ohnishi and S. Ohno, unpublished). The presence of XaPKC and XASIP in the cell-cell contact region of Xenopus epithelial A6 cells is consistent with the idea that the protein complex plays a role in the polarization of epithelial cells in general. The asymmetric distribution of XaPKC and XASIP in the animal hemisphere of Xenopus mature eggs suggests a possible analogy to the asymmetric distribution of PKC-3 and PAR-3 in C. elegans one-cell embryos. Although mature C. elegans eggs exhibit some asymmetry, the sperm pronucleus/centrosome complex specifies the posterior pole of the one-cell embryo (Goldstein and Hird, 1996). After entering an egg, the sperm pronucleus generates a microfilament-dependent flux, with internal cytoplasm flowing towards it and more cortical cytoplasm flowing away. This cytoplasmic flow continues while the maternal pronucleus migrates towards its paternal partner, and cytoplasmic ribonucleoproteins, called P-granules, also move posteriorly (Hird et al., 1996). P-granules contain developmental regulators such as MEX-3, PIE-1 and PGL-1, which are segregated to the germline and required for the specification of germline fate (Bowerman and Shelton, 1999). PKC-3 (aPKC) and PAR-3, as well as other 5 par gene products, are required for establishing, maintaining or elaborating A-P asymmetries after fertilization. Despite the large differences in asymmetry between C. elegans one-cell embryos and Xenopus eggs, the conservation of the asymmetric distribution implies its general role in cellular asymmetry that may involve the signal-induced redistribution of cellular components.

Recent studies on Drosophila have revealed a further analogy, bazooka has been identified as a gene required for normal zonula adherens and polarized blastoderm epithelium. Bazooka is a homologue of PAR-3 (and ASIP, see below) and distributes to the apical cortical cytoplasm of epithelial cells and neuroblasts. The bazooka mutant phenotype fails to coordinate the axis of cell polarity, manifested as defective spindle orientation, mispositioning of the daughter cell after division, and misdistribution of fate-determining proteins such as Prospero through direct interaction with Insuteable. (Muller and Wieschaus, 1996; Kuchinke et al., 1998). Bazooka is also a maternal protein and its role in oocyte asymmetry remains an interesting subject, since Drosophila oogenesis has been studied extensively. Our present finding of the dynamic change in the distribution of XaPKC and XASIP will permit a detailed biochemical analysis of the mechanism underlying the cellular asymmetry organization that is conserved from C. elegans to mammals and from oocytes to differentiated epithelial cells.

### Note added in proof

A recent study has identified the Xenopus par-6 gene (XPAR-6) (Choi et al., 2000). Interestingly, the XPAR-6 mRNA staining shows an enhanced signal in the animal hemisphere of...
eggs. This asymmetric distribution parallels that of the XaPKC mRNA.

The Drosophila par-1 gene has recently been cloned (Shulman et al., 2000; Tomancak et al., 2000). Drosophila PAR-1 protein localizes at the posterior pole of the oocyte and colocalizes with Stauken protein and oskar mRNA. Drosophila par-1 gene is required for appropriate oskar mRNA and Stauken protein localization, and may depend on the machinery involved in the organization of the microtubule cytoskeleton. Furthermore, PAR-1 protein mislocalizes in oskar and stauken mutants. This also supports the existence of the evolutionary conserved polarity establishing mechanism involving the par and aPKC proteins.

REFERENCES


