# The aPKC–PAR-6–PAR-3 cell polarity complex localizes to the centrosome attracting body, a macroscopic cortical structure responsible for asymmetric divisions in the early ascidian embryo

**Solenn Patalano*, Gérard Prulière, François Prodon, Alexandre Paix, Philippe Dru, Christian Sardet and Janet Chenevert‡**

BioMarCell, Laboratoire de Biologie de Developpement, UMR 7009 CNRS, University Pierre and Marie Curie, Observatoire, Villefranche-sur-mer 06230, France

*Present address: Center for Genomic Regulation, Passeig Maritim, Barcelona 08003, Spain
‡Author for correspondence (e-mail: chenevert@obs-vlfr.fr)

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## Summary

**Posterior blastomeres of 8-cell stage ascidian embryos undergo a series of asymmetric divisions that generate cells of unequal sizes and segregate muscle from germ cell fates. These divisions are orchestrated by a macroscopic cortical structure, the 'centrosome attracting body' (CAB) which controls spindle positioning and distribution of mRNA determinants. The CAB is composed of a mass of cortical endoplasmic reticulum containing mRNAs (the cER-mRNA domain) and an electron dense matrix, but little is known about its precise structure and functions. We have examined the ascidian homologues of PAR proteins, known to regulate polarity in many cell types. We found that aPKC, PAR-6 and PAR-3 proteins, but not their mRNAs, localize to the CAB during the series of asymmetric divisions. Surface particles rich in aPKC concentrate in the CAB at the level of cortical actin microfilaments and form a localized patch sandwiched between the plasma membrane and the cER-mRNA domain. Localization of aPKC to the CAB is dependent on actin but not microtubules. Both the aPKC layer and cER-mRNA domain adhere to cortical fragments prepared from 8-cell stage embryos. Astral microtubules emanating from the proximal centrosome contact the aPKC-rich cortical domain. Our observations indicate that asymmetric division involves the accumulation of the aPKC–PAR-6–PAR-3 complex at the cortical position beneath the pre-existing cER-mRNA domain.**

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## Introduction

Asymmetric divisions ensure the unequal partitioning of cell fate determinants and the spatial regulation of cleavage planes (Ahringer, 2003; Betschinger and Knoblich, 2004; Horvitz and Herskowitz, 1992). A conserved set of polarity regulators, the PAR proteins, have been described as key actors controlling cell polarity and asymmetric divisions in worms, flies and mammals (Macara, 2004; Ohno, 2001). PAR proteins appear to function by creating specialized cortical domains, which in turn control spindle positioning and determinant localization (Cheeks et al., 2004; Munro et al., 2004). Three of these polarity proteins, the kinase aPKC, and PDZ domain-containing proteins PAR-6 and PAR-3, can form a complex which is activated by the small G protein CDC42 (Betschinger and Knoblich, 2004; Etienne-Manneville and Hall, 2003). The aPKC–PAR-6–PAR-3 complex localizes asymmetrically along the cell periphery and regulates cell polarity in a wide range of cell types, including *C. elegans* zygotes, *Drosophila* oocytes and neuroblasts, and *Xenopus* eggs (Macara, 2004; Ohno, 2001). In mammalian epithelial cells the aPKC–PAR-6–PAR-3 complex associates with tight junctions where it functions to establish apical-basolateral polarity, but the specific structures that anchor the PAR complex at the cortex in early embryos are not well understood.

In ascidian embryos, two posterior blastomeres undergo a series of asymmetric divisions that separate muscle cell fate from germline precursors, beginning at the 8-cell stage (Nishida, 2002; Nishida, 2005) (Fig. 1). These three successive asymmetric divisions are directed by a macroscopic cortical structure, the centrosome attracting body (CAB) situated in the posterior cortex of B4.1 blastomeres and of the smaller daughter cells produced by each unequal cleavage (red patch in Fig. 1). The role of the CAB in unequal cleavage has been demonstrated by elegant micromanipulation experiments using the large *Halocynthia* zygote (Hibino et al., 1998; Nishida, 1994; Nishikata et al., 1999): when posterior fragments of the embryo are removed no CAB is formed and the B4.1 cells cleave equally, whereas fusion of posterior fragments to an

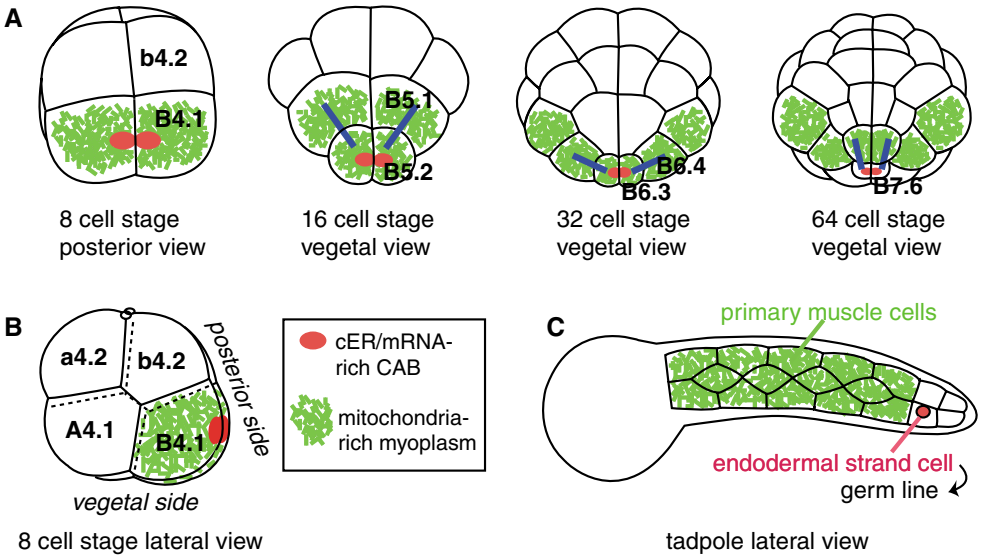


**Fig. 1.** Asymmetric divisions directed by the centrosome attracting body (CAB) in the posterior of the ascidian embryo. (A,B) In the 8-cell stage embryo, the two posterior vegetal blastomeres (B4.1 cells) contain both the CAB (cER-mRNA-rich domain in red) and the myoplasm (mitochondria-rich domain in green). (A) B4.1 divides asymmetrically to form a large (B5.1) and a small (B5.2) daughter cell which inherits the CAB. The small daughter cell divides asymmetrically again, generating B6.4 (large cell) and B6.3, the small cell, which contains the CAB and undergoes a final asymmetric division to yield a very small cell (B7.6) which ceases to divide. Blue bars indicate the daughters formed by each CAB-directed asymmetric division. (C) Final position of myoplasm and cER-mRNA domains in the tadpole larva. The myoplasm-containing cells give rise to tail muscle. The B7.6 cells containing the cER-mRNA domain form part of the endodermal strand and are thought to give rise to the germline after metamorphosis (Takamura et al., 2002; Tomioka et al., 2002).

anterior position causes extra CAB formation and ectopic unequal cleavage. The CAB also has an important function in mRNA localization. Over two dozen maternal mRNAs collectively called *postplasmic/PEM* mRNAs have been found to localize to the CAB and segregate with it into the smaller cells (Nakamura et al., 2003; Sardet et al., 2005; Sasakura et al., 2000; Yamada et al., 2005; Yoshida et al., 1996). *Postplasmic/PEM* mRNAs encode a variety of proteins and include the muscle determinant *macho1*, the germline marker *Vasa*, and an mRNA encoding a G protein exchange factor (Fujimura and Takamura, 2000; Nishida and Sawada, 2001; Satou and Satoh, 1997).

The precise structure of the CAB and how it functions to attract the centrosome and localize mRNA determinants are not well understood. It is primarily composed of a tightly packed mass of cortical endoplasmic reticulum (cER) which adheres strongly to the plasma membrane and encases an electron dense matrix (EDM) reminiscent of germ plasm (Iseto and Nishida, 1999; Prodon et al., 2005; Sardet et al., 2003). At least some of the *postplasmic/PEM* RNAs are bound to the network of cortical ER, so that this region is called the cER-mRNA domain (Nakamura et al., 2005; Prodon et al., 2005; Sardet et al., 2003). The cER-mRNA domain is inherited from the oocyte, acquires a posterior position during reorganizations of the first cell cycle, and is partitioned into B4.1 blastomeres of the 8-cell stage embryo, at which point it forms part of the CAB (Nishida, 2005; Roegiers et al., 1999; Sardet et al., 2005; Sardet et al., 2003; Yoshida et al., 1996). The mechanism by which the CAB causes unequal cleavage in B4.1, B5.2 and B6.3 cells is thought to involve capture of plus ends of microtubules emanating from one centrosome. This model is supported by the observations that a conspicuous bundle of microtubules extends from the nucleus to the region of the CAB (Hibino et al., 1998) and that the migration of the nucleus toward the posterior cortex requires microtubules but not actin (Nishikata et al., 1999). An antibody to vertebrate kinesin labels the CAB region (Nishikata et al., 1999), but no other proteins localized specifically to the CAB, which could provide clues to how it functions, have been reported.

We have examined the distribution of the PAR polarity complex in the ascidian embryo. We demonstrate that aPKC, PAR-6 and PAR-3 accumulate in the CAB, forming a thin cortical layer sandwiched between the plasma membrane and the cER-mRNA domain. Astral microtubules connect the nucleus to the PAR domain. The polarized distribution of the aPKC, PAR-6 and PAR-3 proteins correlates with the onset of unequal cleavages, suggesting that the aPKC–PAR-6–PAR-3 complex plays an important role during asymmetric divisions in the ascidian embryo.

## Results

### In vivo observation of the CAB during asymmetric division

Embryos of many solitary ascidian species display the same invariant cleavage pattern and cell lineage (Nishida, 2005; Satoh, 1994). The presence of the CAB appears to be an additional conserved feature of ascidian embryos. The term CAB was first employed to designate a refractive spot that persists in the posterior of *Halocynthia roretzi* embryos following detergent extraction (Hibino et al., 1998) and it was recognized that this peripheral domain corresponds to the site of localization of *PEM* transcripts (Sardet et al., 2005; Yoshida et al., 1996). The CAB has since been documented in embryos of *Halocynthia roretzi*, *Ciona intestinalis* and *Phallusia mammillata* by a variety of methods including electron microscopy, in situ hybridization to *postplasmic/PEM* mRNA probes, or labelling of ER on isolated cortices (Iseto and Nishida, 1999; Nishikata et al., 1999; Prodon et al., 2005; Sardet et al., 2003). This cortical structure of distinct composition had also been observed in the original description of ascidian embryonic development as a ‘notch of clear protoplasm’ beneath the myoplasm in the highly pigmented *Styela* embryo (Conklin, 1905).

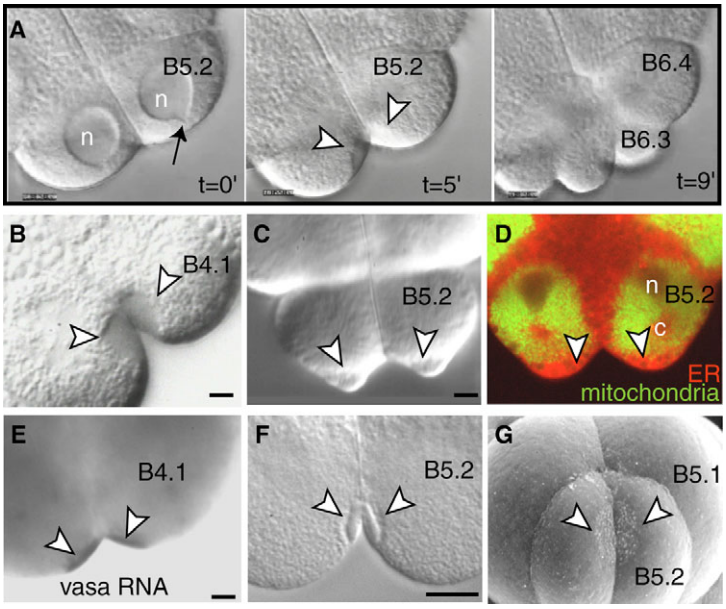


**Fig. 2.** Visualization of the CAB in posterior vegetal blastomeres of *Phallusia mammillata* embryos. (A-D) Live *Phallusia* embryos viewed by DIC optics (A-C) or by fluorescence confocal microscopy (D). (A) Images extracted from a time-lapse sequence (see Movie 1 in supplementary material). t=0: the interphase nucleus (n) appears pinched (arrow) as it migrates towards the CAB; t=5′ (5 minutes): the CAB (arrowhead in all panels) appears as a smooth zone, which compacts and thickens during prophase; t=9′ (9 minutes): the spindle positions asymmetrically, causing unequal cleavage into large (B6.4) and small (B6.3) cells. (B) The CAB is visible as a smooth cortical zone (arrowhead) late in the cell cycle. (C) A transient surface protrusion forms at the position of the CAB early in the cell cycle. (D) Embryo double labelled for ER (red) and mitochondria (green). The nucleus (n) and centrosome (c) are oriented toward the cER-rich CAB. (E) In situ hybridization showing localization of a *postplasmic/PEM* mRNA in the CAB (antisense probe for *Phallusia Vasa*). (F) Embryo fixed with formaldehyde viewed by DIC optics. (G) Scanning electron micrograph shows surface structures protruding at the position of the CAB. The names of the relevant blastomeres are indicated as drawn in Fig. 1. Bars, 10 μm.

In the exceptionally transparent embryos of *Phallusia mammillata* we find that the CAB is readily visible by DIC optics in living embryos as a smooth translucent oval-shaped zone in the cortex of posterior vegetal blastomeres (Fig. 2). This zone is especially apparent during prophase and metaphase when the CAB forms a compact dome shape, protruding up to 10 μm into the cytoplasm (Fig. 2B). Asymmetric division of posterior blastomeres in a 16-cell stage embryo was analyzed by time-lapse microscopy (Fig. 2A: sequence of three images extracted from Movie 1 in supplementary material). In this and other films, the interphase nucleus appears to stretch and, led by a centrosomal aster region, migrates towards the CAB at the approximate speed of 2 μm per minute. During the nuclear migration, the surface at the position of the CAB ripples and forms a transient protrusion (Fig. 2C), possibly reflecting tension generated by localized interactions between microtubules and the cortex. In vivo labelling showed that the cortical domain towards which the centrosome orients is rich in ER and poor in mitochondria (Fig. 2D). Antisense RNAs for *postplasmic/PEM* mRNAs such as *Phallusia Vasa* (Fig. 2E) or *Phallusia macho-1* (not shown) hybridized to this cER-rich domain. The CAB is also apparent as a discrete cortical structure in unlabelled fixed *Phallusia* embryos by DIC optics (Fig. 2F). Scanning electron microscopy revealed a concentration of distinct surface blebs at the position of the CAB (Fig. 2G), which may correspond to the surface protrusions observed by transmission electron microscopy in *Ciona intestinalis* and *Halocynthia* (Iseto and Nishida, 1999).

## Ascidian aPKC protein sequences define a distinct group

The search for polarity proteins in the *Ciona intestinalis* genome has revealed one coding sequence each for aPKC, PAR-6 and PAR-3 (Sasakura et al., 2003), but the localization and function of the corresponding proteins have not yet been addressed in ascidian embryos.

We cloned the cDNA encoding *Phallusia mammillata* aPKC and found its deduced protein sequence to be highly similar to aPKCs from ascidian species for which sequence information is available (*Ciona intestinalis*, *Ciona savigny* and *Halocynthia roretzi*) (Fig. 3). Alignment of protein sequences demonstrates that ascidian aPKCs contain characteristic PB1 and kinase domains, but interestingly aPKCs from the four ascidian species examined all lack a central region, which includes the cysteine-rich C1 domain. The function of the C1 domain in aPKC appears to be to bind phosphatidylinositol (3,4,5)-trisphosphate PtdIns(3,4,5)$P_3$, which causes an activating conformation change, and this domain is also proposed to interact with distinct activating and inhibitory partners (Diaz-Meco et al., 1996; Suzuki et al., 2003). All other known aPKC proteins contain this cysteine-rich motif, including those from nematodes, insects, echinoderms, hemichordates and vertebrates. This indicates that the C1 domain was specifically lost in urochordates during evolution, and that ascidians may have developed a distinct mechanism for regulating aPKCs independent of the PtdIns(3,4,5)$P_3$ pathway. In place of the cysteine-rich domain, ascidian aPKCs possess a short conserved sequence not found in other aPKCs (boxed in Fig. 3), which may define a site for interaction with ascidian-specific partners.

## aPKC protein but not mRNA localizes to the CAB

The temporal and spatial distribution of aPKC protein in ascidian embryos was determined using an antibody made to the conserved C-terminal 20 amino acids, which specifically recognizes aPKC in a wide variety of vertebrate and invertebrate species. On immunoblots of *Phallusia* eggs the aPKC antibody detected a single band of approximately 65 kDa, the predicted size of the ascidian aPKC homolog. This signal disappeared when the antibody was pre-incubated with the immunogenic peptide but not with an unrelated peptide (Fig. 4A). The aPKC protein was found to be present maternally and to persist throughout embryonic development (Fig. 4B).

When used for imunofluorescence analysis, the aPKC

antibody weakly and uniformly labelled the cortex of all cells of *Phallusia* embryos, from the egg to the tadpole (not shown). A polarized enrichment of aPKC protein in the posterior cortex of the embryo appeared gradually from the 2-cell stage and became systematically detectable at the 4-cell stage (Fig. 4C). At the 8-cell stage, aPKC protein accumulated strongly in the pair of posterior vegetal B4.1 blastomeres at the position of the CAB (Fig. 4D,E). The amount of aPKC protein did not appear to increase during these cleavage stages, or thereafter (Fig. 4B), indicating that its localisation is a result of redistribution of maternal protein. This polarized cortical enrichment of aPKC protein persisted in embryos of 16-, 32- and 64-cell stages in the B5.2, B6.3 and B7.6 blastomeres, respectively (Fig. 4F-H). The aPKC antibody was also found to label the CAB in embryos of the widely studied ascidian species *Ciona intestinalis* (not shown).

High magnification surface views showed that the aPKC protein is present in large particles (approximately 1 μm diameter) near the cell surface, and that these particles are densely packed at the position of the CAB (Fig. 4I, and z

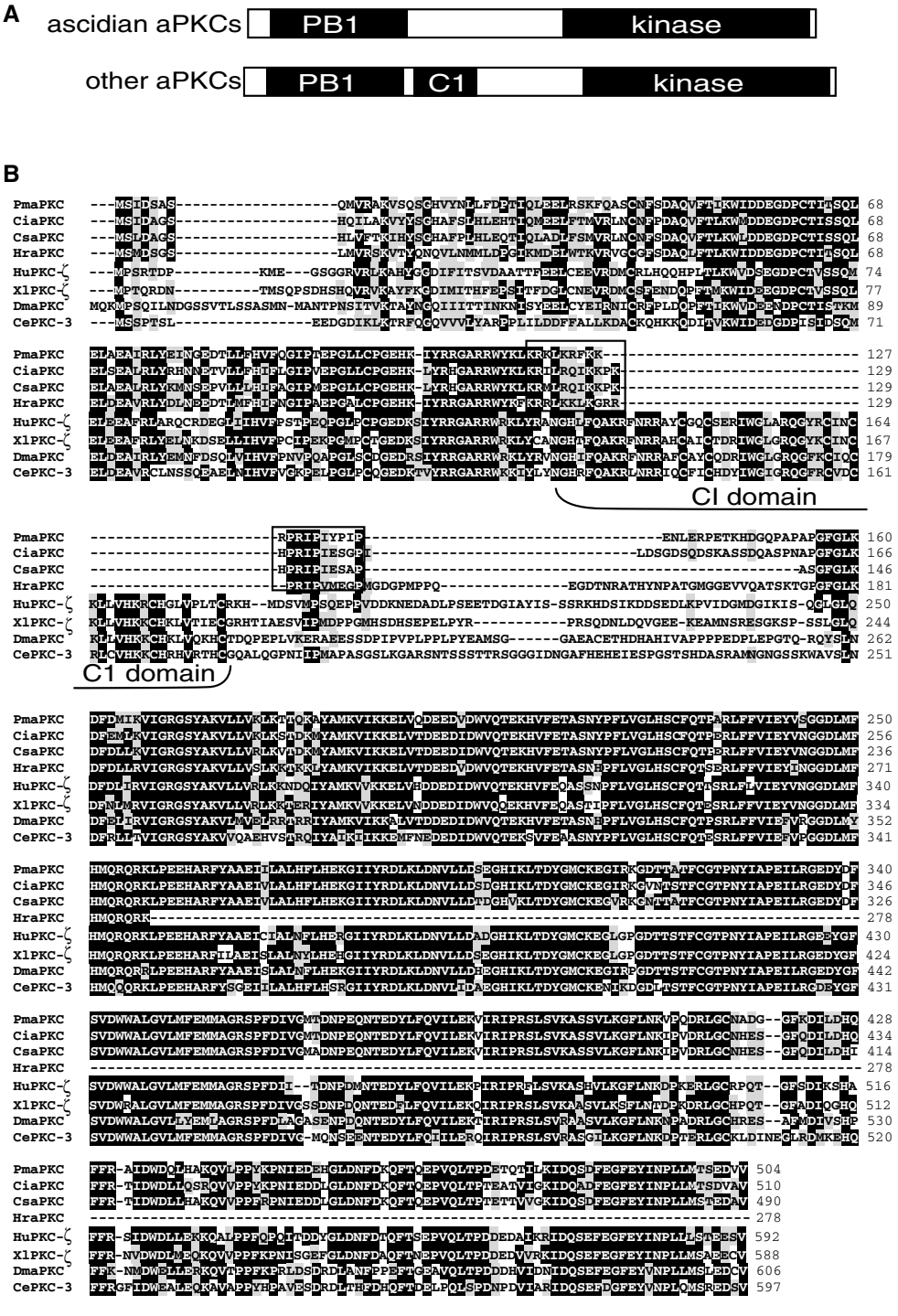


**Fig. 3.** Ascidian aPKCs lack a conserved cyteine-rich domain. (A) Domain structure of aPKCs. PB1, Phox and Bem protein interaction domain which binds PAR-6 protein; Kinase, serine threonine kinase domain; C1, cysteine-rich domain which binds InsPtd(3,4,5)$P_3$. (B) Comparison of aPKC proteins from four ascidian species (*Phallusia mammillata,* Pm; *Ciona intestinalis,* Ci; *Ciona savigny,* Cs; and *Halocynthia roretzi,* Hr) with aPKCs from human (Hu), frog (Xl), fly (Dm), and worm (Ce). The C1 domain usually present in aPKC but lacking in ascidian aPKCs is underlined. The two boxed areas indicate a normally contiguous region of homology specific to ascidian aPKCs. PmaPKC was obtained by cloning from a cDNA library (this study, GenBank AY987397). CiaPKC was obtained by assembling sequence information from Sasakura et al. (Sasakura et al., 2003) and CLSTR05328r1 from the Ghost genome browser (ghost.zool.kyoto-u.ac.jp). CsaPKC was obtained by analysis of genomic sequence information in http://www2.bioinformatics.ttl.org.sg:8082/Ciona_savignyi/ and http://www.broad.mit.edu/annotation/ciona/. HraPKC sequence was obtained from 5′ EST data from the Magest genome site accessible via Aniseed (aniseed-ibdm.univ-mrs.fr). The HraPKC protein appears truncated in the kinase domain because the sequence encoding the C-terminal half of the protein is not available.

series in Movie 2 in supplementary material). Like the CAB itself, the patch of localized aPKC protein changed size and shape during the cell cycle (Fig. 4J). In B4.1 cells of 8-cell stage *Phallusia* embryos (cell diameter 85 μm) the surface dimensions of the aPKC-rich disc measured on average 25×15 μm (length×width) during interphase and compacted to 15×11 μm at anaphase. In 16- and 32-cell stage embryos, the CAB continued to cycle between a widespread state in interphase and a compacted form in mitosis. In B5.2 cells (diameter 40 μm) the aPKC-rich disc measured on average 20×14 μm in interphase, compacting to 11×7 μm at anaphase. In addition to this cycle of relaxation/condensation, the overall size of the CAB diminished with each unequal cleavage, as the posterior-most cells become smaller.

If *aPKC* mRNA were a *postplasmic/PEM* type of mRNA localized in the CAB, then translation of the localized mRNA could account for the polarized distribution of aPKC protein. In order to address this possibility, we examined the presence and distribution of the *aPKC* mRNA. Northern blotting shows a single band for *aPKC* which is maternal and persists throughout embryonic development (Fig. 4K). The *aPKC* mRNA is equally present in all cell lineages and is distributed ubiquitously throughout the cytoplasm of all blastomeres (Fig. 4L-N). *aPKC* mRNA is not enriched in the CAB (arrowhead in Fig. 4N). Thus the polarized accumulation of aPKC protein at the CAB is not a consequence of localized mRNA; rather it is probably due to active redistribution of aPKC protein located elsewhere in the cortex or in the cytoplasm.

## PAR-6 and PAR-3 proteins colocalize with aPKC at the CAB

To determine whether in ascidian embryos aPKC protein might act in a complex with PAR-6 and PAR-3 proteins, we examined the distribution of PAR-6 and PAR-3 proteins during asymmetric divisions.

We first cloned the cDNA encoding *Phallusia* PAR-6 and found it to encode a protein of predicted molecular mass 45 kDa, highly similar to *Ciona* PAR-6 (Fig. 5B). Antibodies produced against an N-terminal PAR-6 peptide and affinity-purified against either the immunogenic peptide or against the entire PAR-6 fusion protein detected a single major band of the expected size on immunoblots of *Phallusia* protein extracts (Fig. 5C). The N-terminal PAR-6 antibody principally labelled the posterior vegetal blastomere surfaces at the position where the CAB is situated (Fig. 5D). A second PAR-6 antibody produced against a C-terminal peptide gave an identical staining pattern (Fig. 5E). Similar to the mRNA encoding aPKC, *PAR-6* mRNA was found to be maternal, ubiquitously distributed, and present in a constant amount throughout embryonic development (not shown).

We next examined the distribution of the PAR-3 protein using an antibody raised against the N-terminal 297 amino acids of *Drosophila* PAR-3 (Wodarz et al., 1999), a region

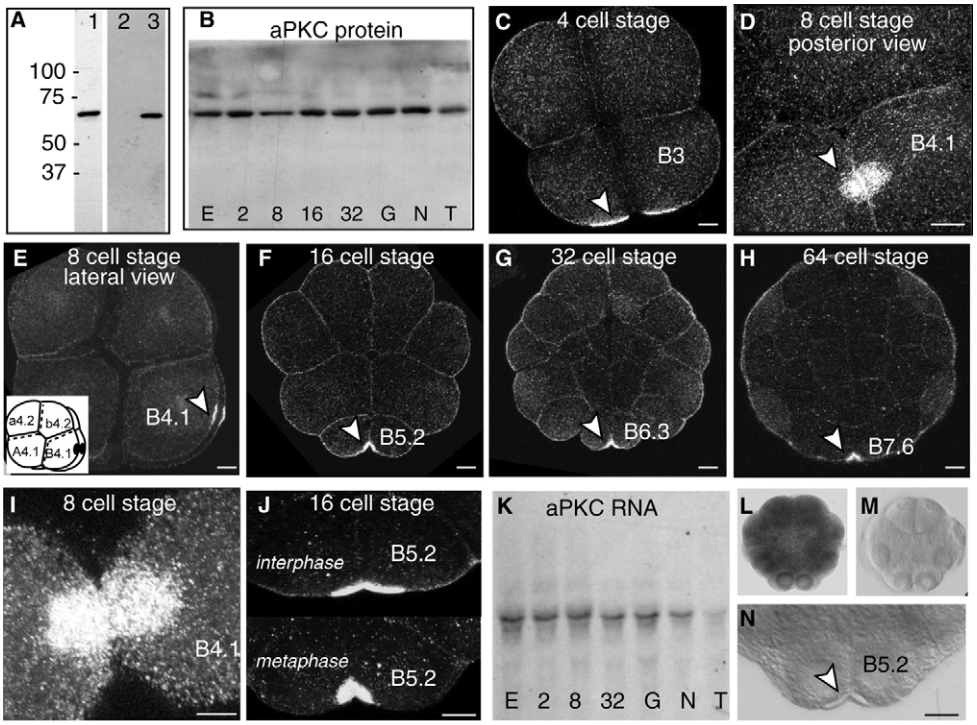


**Fig. 4.** aPKC protein but not its mRNA localizes to the CAB. (A) aPKC antibody recognizes a single 65 kDa protein in *Phallusia mammillata* egg extracts (lane 1). The signal disappears when aPKC antibody is pre-incubated with the specific antigenic peptide (lane 2) but not with an unrelated peptide (lane 3). (B) Immunoblot of protein extracts from different developmental stages (E, egg; 2, 2 cells; 8, 8 cells; 16, 16 cells; 32, 32 cells; G, gastrula; N, neurula; T, tadpole). (C-J) Confocal images of *Phallusia* embryos immunolabelled for aPKC protein. Arrowhead indicates the pair of CABs. Stages can be compared with drawings in Fig. 1 and posterior vegetal blastomere name is indicated (B4.1, B5.2, B6.3, B7.6). Each image represents a projection of two to four confocal sections containing the brightest aPKC label. (C) 4-cell stage, vegetal view. (D) 8-cell stage, posterior surface view. (E) 8-cell stage, lateral view; the two halves of the embryo are projected into one plane so that both left and right CABs are visible. Inset shows a drawing of the orientation of the embryo as depicted in Fig. 1B. (F) 16-cell stage, vegetal view. (G) 32-cell stage, vegetal view. (H) 64-cell stage, vegetal view. (I) 8-cell stage, high magnification posterior surface view. Movie 2 in supplementary material shows the complete series of confocal z sections corresponding to this image. (J) B5.2 blastomeres from two 16-cell stage embryos showing cell-cycle-dependent compaction of the CAB. (K-N) *aPKC* mRNA is maternal and ubiquitously distributed. (K) Northern blot. *aPKC* probe was hybridized to RNA prepared from *Phallusia* embryos at the indicated stages (E, unfertilized egg; 2: 2 cells; 8: 8 cells; 32: 32 cells; G: gastrula; N, neurula; T, tadpole). (L-N) In situ hybridization using *aPKC* antisense probe (L,N) or negative control sense probe (M). The CAB is visible as in most fixed embryos (arrowhead in N) but not labelled by the probe for *aPKC* mRNA. Bars, 15 μm.

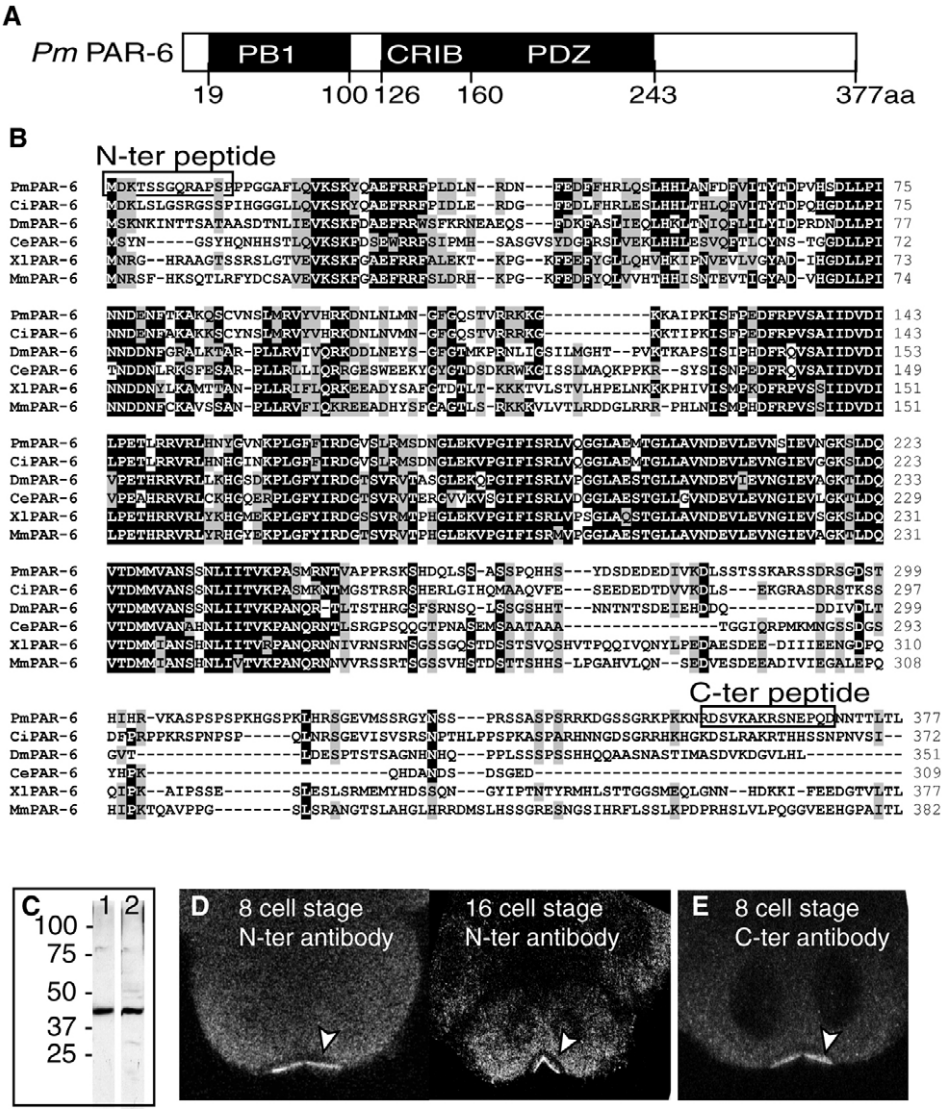


**Fig. 5.** PAR-6 protein localizes to the CAB. (A) Domain structure of PAR-6 proteins. PB1, Phox and Bem protein interaction domain, which binds aPKC protein; CRIB, CDC42 interacting domain; PDZ, protein interaction domain, which binds PAR-3 protein. (B) Comparison of PAR-6 sequences from *Phallusia mammillata* (Pm), *Ciona intestinalis* (Ci), *Caenorhabditis elegans* (Ce), *Drosophila melanogaster* (Dm), *Xenopus laevis* (Xl) and *Mus musculus* (Mm). Boxed areas indicate N-terminal (MDKTSSGQRAPSP) and C-terminal (RDVSVKAKRSNEPQD) peptides used to raise antibodies. (C) A single 45 kDa band is detected on a western blot of *Phallusia* egg extract using sera purified against the synthetic peptide (lane 1) or against the PAR-6 fusion protein (lane 2). (D,E) Both PAR-6 antibodies directed against either the N-terminal or C-terminal peptides label the surface of posterior vegetal blastomeres at the position of the CABs (arrowheads).

which precedes the three PDZ domains and contains several well conserved elements. A coding sequence highly homologous to the first 80 amino acids of this region was detected by analyzing the raw sequence data of the *Ciona* genome (Fig. 6A). This N-terminal PAR-3 homology domain lies just upstream of the reported *Ciona* PAR-3 coding sequence (Sasakura et al., 2003) and probably corresponds to the true amino terminus of ascidian PAR-3 (Fig. 6B). On immunoblots of *Phallusia* extracts, the PAR-3 antibody reacted with a protein of approximately 120 kDa, a size characteristic of PAR-3 proteins (Fig. 6C). The PAR-3 antibody labelled the cortex of posterior vegetal blastomeres at the position of the CAB strongly and exclusively in 8- to 64-cell stage embryos (Fig. 6D,E). No other membrane or cytoplasmic labelling was detected.

This colocalization of aPKC, PAR-6 and PAR-3 in addition to the sequence conservation in the PDZ and PB1 protein interaction motifs strongly suggest that these three proteins form a complex in the CAB. We hereafter use the term ‘PAR domain’ to refer to the cortical zone containing aPKC, PAR-6 and PAR-3 at the posterior of the ascidian embryo.

## The ascidian CAB is a multilayered structure

In order better to define the structure of the CAB, we analyzed the relationship between the polarized PAR domain, visualized using the aPKC antibody, and mitochondria, ER, microfilaments or microtubules. The cytoplasmic boundary of the CAB can be visualized by labelling mitochondria (Fig. 7A) since the myoplasm closely surrounds but does not penetrate the cER-mRNA domain. Double labelling of B5.2 blastomeres in prophase or metaphase revealed the presence of a 7-10 μm thick space between the myoplasm and the aPKC-rich domain (Fig. 7B,C and Movie 3 in supplementary material). High resolution analysis of embryos double-labelled for actin and aPKC demonstrated that the aPKC-rich domain occupies the same level as the layer of cortical microfilaments underlying the plasma membrane (Fig. 7D). Measurements on confocal images showed that the aPKC-rich layer is 1-2 μm thick, and it remains so throughout the cell cycle. These results show that aPKC protein is present in the cortical-most side of the CAB but absent from the bulk of the CAB which is occupied by the cER-mRNA domain and the electron dense matrix (Hibino et al., 1998; Iseto and Nishida, 1999; Nakamura et al., 2005;

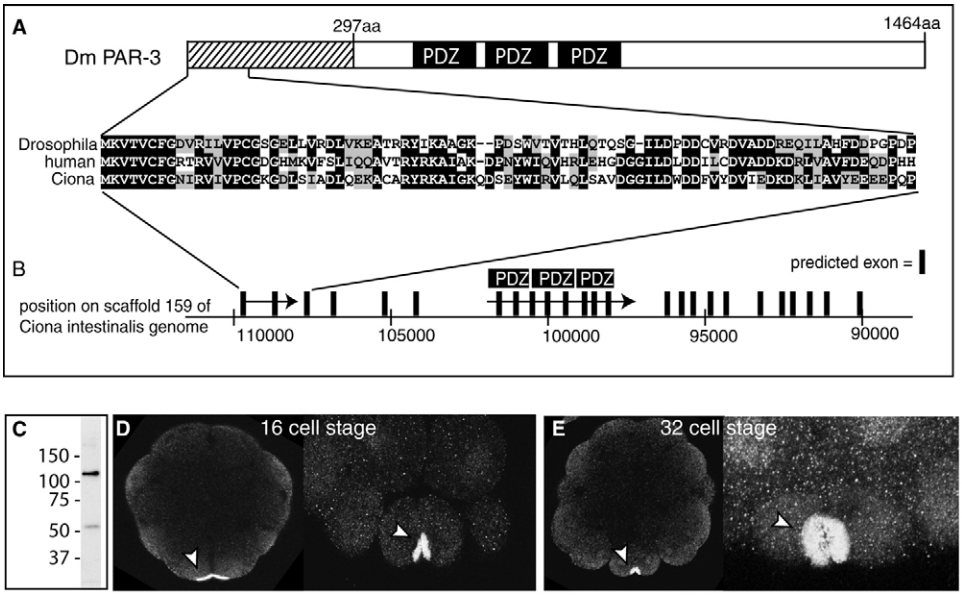


**Fig. 6.** PAR-3 protein localizes to the CAB. (A) Sequence recognized by the PAR-3 antibody. The hatched rectangle represents the region of *Drosophila* PAR-3 protein (Bazooka) used to raise the PAR-3 antibody (Wodarz et al., 1999). The alignment shows high homology between the first 87 amino acids of this domain and an open reading frame in the *Ciona* genome. (B) Structure of PAR-3 gene in *Ciona* genome. The gene covers 20 kb and consists of at least 20 exons, as deduced by comparing conserved coding regions between *Ciona intestinalis* and *Ciona savigny* genomes using Vista software. The first three exons (beginning at position 109598) encode the conserved sequence shown in A. The *Ciona* PAR-3 DNA sequence previously described, which encodes the three PDZ domains (Sasakura et al., 2003) begins 5 kb downstream of the N-terminal homology and reads in the same direction. Numbers below the horizontal line indicate base position on scaffold 159 from version 1.0 of the genome (scaffold 148 in version 1.95). (C) Immunoblot using PAR-3 antibody on protein extract from *Phallusia* eggs. (D,E) Immunolabelling of *Phallusia* embryos with PAR-3 antibody. Arrowhead indicates position of the pair of CABs.

Prodon et al., 2005; Sardet et al., 2003) and reveal for the first time two separate layers in the CAB.

The degree of cortical association of the PAR domain with the plasma membrane was tested by examining isolated cortices labelled for aPKC and ER. Ascidian embryos can be attached to coverslips and the cytoplasm sheared away so that only the plasma membrane and structures which adhere tightly to it remain (Nakamura et al., 2005; Prodon et al., 2005; Sardet et al., 2003). In such cortical preparations made from the posterior vegetal blastomeres of 8-cell stage *Phallusia* embryos, the aPKC-rich zone was retained together with the cER-mRNA domain that characterizes the CAB (Fig. 7E-G), reflecting a tight association of the aPKC-rich layer with the plasma membrane at this location. The aPKC protein retained on cortices is in the form of particles with a size similar to those (approximately 1 μm diameter) observed by immunolabelling of whole embryos (Fig. 4D,I and Movie 2 in supplementary material). When very thin confocal sections acquired at <0.2-μm intervals were examined, the sections closest to the plasma membrane were found to contain mostly aPKC but very little ER (Fig. 7F,G), indicating that the PAR domain is sandwiched between the plasma membrane and the cER-mRNA domain.

Embryos were double labelled for aPKC and tubulin in order to evaluate the relationship of the CAB to centrosomes and microtubules. In B5.2 cells in interphase, the period during which the nucleus migrates towards the cortex (as visible in Fig. 2A,D), a distinct mass of microtubules connecting the nucleus to the CAB was observed (Fig. 7H-J). In some cells the bundle was easily detectable (Fig. 7H); in others a population of long and straight microtubules (arrowheads in Fig. 7I) connecting the centrosome to the aPKC-rich domain could be discerned among a background of astral microtubules emanating in all directions. The duplicated centrosomes cannot always be distinguished since a brightly staining mass of microtubules connects them. In blastomeres where the two centrosomes could be clearly identified, they always aligned between the nucleus and the CAB (Fig. 7J). In the example in Fig. 7J one centrosome can be observed near to the interphase nucleus, and the other is situated up to 10 μm away from the nucleus in the precise direction of the cortical patch of aPKC. These interphase microtubule configurations probably correspond to the thick bundles of microtubules observed to project toward the CAB in extracted *Halocynthia* embryos (Hibino et al., 1998; Nishikata et al., 1999). In blastomeres fixed during prophase and prometaphase, it is rare to observe discrete bundles of microtubules connecting a centrosome to the CAB. Astral microtubules emanating from the centrosome closest to the CAB can be seen to contact the posterior surface of the blastomere at both the aPKC-rich domain and the cell cortex adjacent to the CAB (Fig. 7K,L and Movie 4 in supplementary material). In metaphase and anaphase, one spindle pole appears flattened against the thickened mass of the CAB (Fig. 7M), and few astral microtubules contact the aPKC-rich cortical domain. The eccentric position of the mitotic spindle leads to unequal cleavage, the smaller cell inheriting the CAB with its patch of aPKC, PAR-6 and PAR-3.

## Cytoskeletal dependence of the PAR domain

The dependence of the formation of the PAR domain on cytoskeletal elements was tested by labelling embryos for aPKC, following incubation with agents that depolymerise actin microfilaments (cytochalasin and latrunculin) or microtubules (nocodazole). Two-cell stage embryos were treated with cytoskeletal inhibitors and then fixed after 90 minutes, when control embryos reached the 16-cell stage. In the presence of actin depolymerising agents, no CAB-like domains were formed on the cell surface (Fig. 8A,B right images): aPKC protein was spread uniformly over the cortex or in a few randomly distributed clumps. Nocodazole-arrested

embryos however always contained two brightly staining cortical patches of aPKC protein precisely resembling those associated with normal CABs (Fig. 8C,D, arrowheads). Thus these results show that the formation of the PAR-rich domain requires actin but not microtubules and further demonstrate that in *Phallusia* embryos the CAB can form autonomously in the absence of cell division, as had been observed previously in *Halocynthia* (Nishikata et al., 1999).

In order to determine whether actin microfilaments are also required for the maintenance of the aPKC domain once it has formed, 16-cell stage embryos that had already undergone one asymmetric cleavage were treated with cytoskeletal inhibitors for 30 minutes, then fixed and labelled with the aPKC antibody. In B5.2 cells of embryos treated with cytochalasin or latrunculin for 30 minutes, aPKC was no longer found concentrated in the characteristic posterior moustache pattern in most cases (Fig. 8E,F). Instead aPKC protein was observed to distribute homogeneously throughout the cortex and also to concentrate in small cortical clumps at random positions in all blastomeres. In nocodazole-arrested 16-cell stage embryos, however, aPKC protein remained concentrated in the CAB beneath the myoplasm, as in untreated embryos (Fig. 8G). Similar results were obtained when 8-cell stage embryos were treated with cytoskeletal inhibitors for 30 minutes (not shown). Thus actin is required for the maintenance of the aPKC-rich cortical patch in the position of the CAB. These data indicate that the intimate association between the PAR domain and the actin cortex, suggested by colocalization and retention on isolated cortices (Fig. 7), is functionally significant.

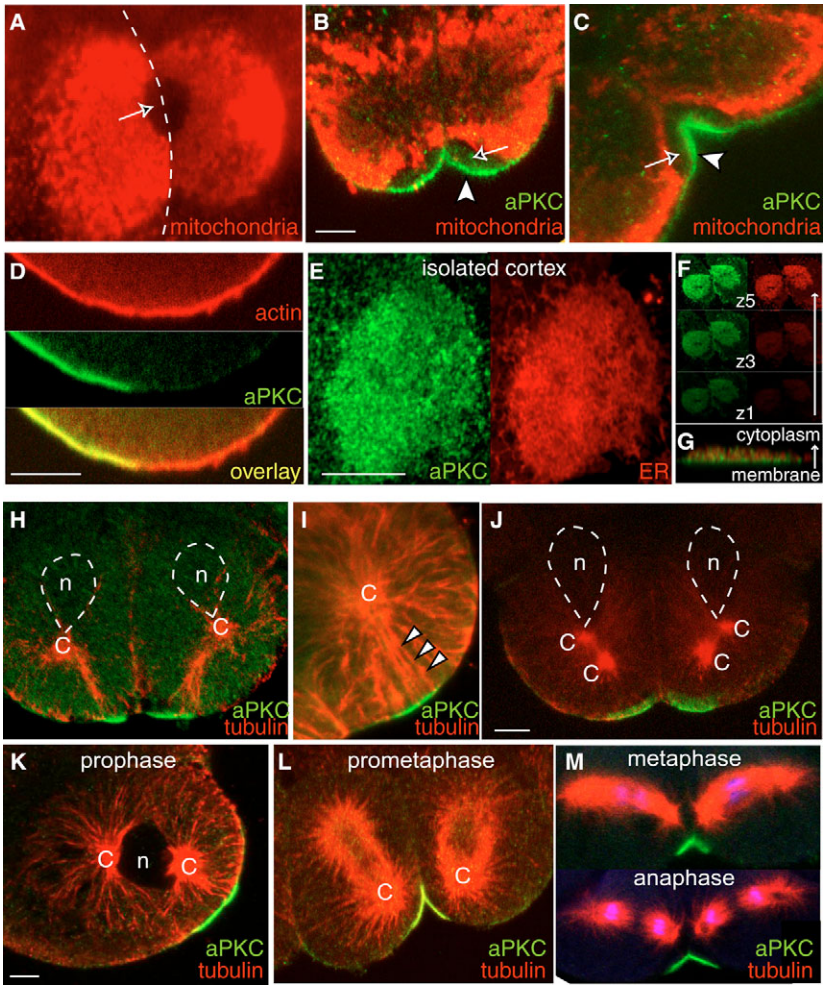


**Fig. 7.** Distribution of mitochondria, microfilaments, microtubules and aPKC protein in CAB-containing blastomeres. (A) B5.2 cells labelled for mitochondria (posterior view). Dotted white line indicates plasma membranes between blastomeres. The dome-shaped CAB is apparent as a mitochondria-free domain (arrow). (B,C) B5.2 cells double labelled for aPKC (green) and mitochondria (red); vegetal views. Arrowhead indicates the cortical face of the CAB; arrow indicates the mitochondria-free space occupied by the cER-mRNA domain and dense granules as in A. Movie 3 in supplementary material displays a series of confocal z sections through a similarly labelled embryo. (D) 8-cell stage embryo double labelled for aPKC (green) and actin microfilaments (red). Posterior vegetal portion of B4.1 cell is shown. (E-G) Isolated cortical fragments from posterior face of 8-cell stage embryo, double-labelled for aPKC (green) and ER (red). (E) One CAB; the aPKC protein (green) is concentrated in particles filling part of the space between ER sheets and tubes (red) which are densely packed in the CAB. (F) Thin confocal sections taken of a pair of CABs, proceeding from the plasma membrane (z1) into the cell (z3, z5), in increments of 0.16 μm, showing that the aPKC-rich layer is closer to the plasma membrane than the ER. (G) Intensity profile of the two signals along the x-z axis; plasma membrane is on the bottom; cytoplasm on top. (H-M) Posterior vegetal blastomeres double-labelled for aPKC (green) and tubulin (red); c, centrosome. (H-J) B5.2 cells in interphase; dotted white lines indicate the position and shape of the nuclei (n) as projected from other focal planes. (K) B4.1 cell in prophase; n, nucleus. Movie 4 in supplementary material shows the complete series of confocal z sections through this cell. (L) B5.2 cells in prometaphase. (M) B5.2 cells in metaphase (above) and anaphase (below). DNA is labelled in blue. Bars, 10 μm.

## Discussion

### Structure and formation of the CAB

Here we have shown that the three members of the conserved polarity cassette, aPKC, PAR-6 and PAR-3, localize together at the CAB during the series of asymmetric divisions that pattern the posterior pole of the ascidian embryo. These results on *Phallusia mammillata* embryos combined with previous studies on *Ciona intestinalis* and *Halocynthia roretzi* (Iseto and Nishida, 1999; Nakamura et al., 2005; Prodon et al., 2005; Sardet et al., 2003) lead us to propose a model for the structure of the CAB, depicted in Fig. 9. The aPKC–PAR-6–PAR-3 complex concentrates into a thin ellipse-shaped patch contiguous with the layer of actin microfilaments, which is sandwiched between the cER-mRNA domain and the plasma membrane. Both the PAR and cER-mRNA layers are widespread during interphase and compact during mitosis (Fig. 9A), when the cER-mRNA domain forms a dome-shaped protrusion into the mitochondria-rich myoplasm. Because of the centrosome-attracting activity of the CAB, the aPKC–PAR-

6–PAR-3 domain is inherited by the smaller daughter cell produced by unequal cleavage, and the entire process is repeated twice.

The localization of the PAR polarity complex to the CAB is probably due to active redistribution of the proteins from an unpolarized protein pool rather than to localized translation, since we observed that mRNAs encoding aPKC and PAR-6 are not localized in the CAB. The cER-mRNA domain, which acquires its posterior position during the first mitotic cell cycle after fertilization, may serve as a spatial landmark providing a localized signal to promote the formation of the polarized PAR domain. Intriguingly, one of the *postplasmic/PEM* RNAs concentrated in the cER-mRNA domain, *PEM2*, encodes the GTP exchange factor specific for CDC42 (Philips et al., 2003; Satou and Satoh, 1997), the G protein which binds to PAR-6 and causes activation of the aPKC–PAR-6–PAR-3 complex (Etienne-Manneville and Hall, 2003; Macara, 2004). Translation of at least some of the *postplasmic/PEM* RNAs is thought to take place before the 8-cell stage (Nishida and Sawada, 2001; Satou, 1999). Thus we hypothesize that translation of *PEM2* RNA localized in the cER-mRNA domain could cause local activation of the CDC42 protein and thus favour the assembly of the aPKC–PAR-6–PAR-3 complex at the proper cortical site.

The punctate distribution of the aPKC protein (Fig. 4I, Fig. 7E and Movie 2 in supplementary material) and the requirement for actin microfilaments (Fig. 8) suggest that accumulation of aPKC in the CAB results from aggregation of aPKC-rich elementary units that are spread beneath the blastomere surface. Similarly, in *Halocynthia* embryos there is evidence for an actin-dependent mechanism that drives posterior accumulation of surface particles to the cortical location of the CAB gradually between the 2-cell and 8-cell stages (Hibino et al., 1998). A recent study has demonstrated that this process is dependent on *POPK-1*, a *postplasmic/PEM* mRNA which encodes a homolog of the SAD kinase implicated in clustering of membrane vesicles (Nakamura et al., 2005). The roles of POPK-1 or of PEM2 in the formation of the PAR domain remain to be tested.

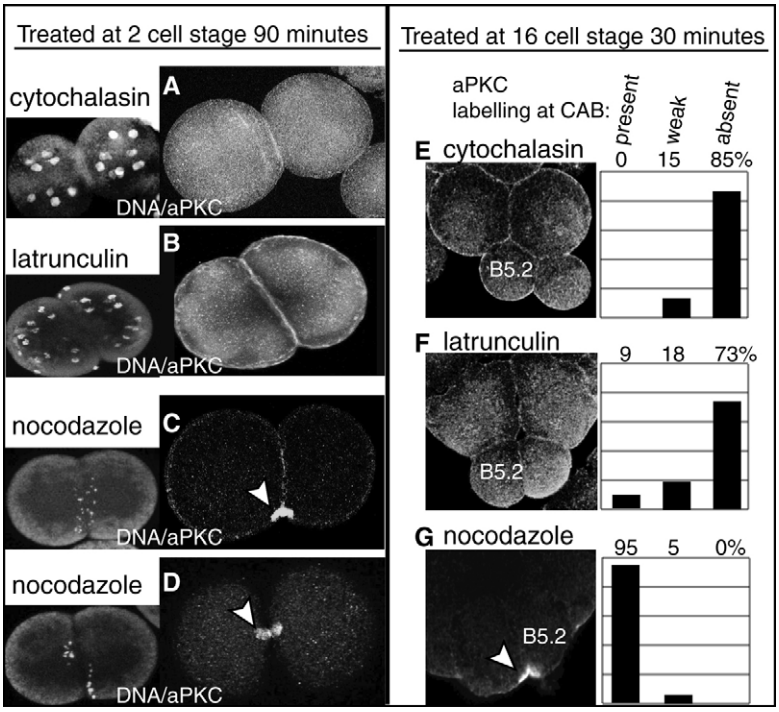


**Fig. 8.** aPKC accumulation at the CAB is dependent on actin microfilaments and independent of microtubules. Embryos were incubated with the indicated inhibitor then fixed, immunolabelled and examined by confocal microscopy. (A-D) Treatment at 2-cell stage for 90 minutes, the time equivalent to three cell cycles. Images on the right show aPKC label (surface views); images on the left show Hoechst label in the same embryos (central planes containing chromatin). In embryos treated with cytochalasin or latrunculin, nuclear cycles continue and each blastomere accumulates 8-16 patches of DNA, which are symmetrically arranged and either decondensed as interphase nuclei (A) or condensed in mitotic configurations (B). In embryos treated with nocodazole (C,D) nuclear cycles arrest; DNA is condensed and dispersed randomly along the cleavage furrow. In C (lateral view of one embryo) and D (posterior view of another embryo), arrowheads indicate CAB-like accumulations of aPKC protein. (E-G) Treatment at 16-cell stage for 30 minutes, the time equivalent to one cell cycle. Images show aPKC labelling in posterior blastomeres (B5.2 cells, surface views) from a typical treated embryo. Arrowhead in G indicates a pair of CABs. Graphs show the percentage of embryos in which a concentrated cortical patch of aPKC protein was either present, absent, or weakly apparent in B5.2 cells; at least 20 embryos were scored for each treatment.

## Possible functions of the PAR domain in the CAB

The principal functions of the CAB correspond to the two features of asymmetric division: eccentric spindle positioning and segregation of localized determinants. It seems unlikely that aPKC, PAR-6 and PAR-3 are involved in the initial localization of mRNAs to the CAB, since the cER-mRNA domain forms well before the PAR domain (this study) (Prodon et al., 2005; Sardet et al., 2003), but they may play a role in the partitioning of mRNA determinants during the series of asymmetric divisions.

The localization of aPKC, PAR-6 and PAR-3 proteins to the CAB occurs prior to and during the series of unequal cleavages. This correlation and the interaction of microtubules with the PAR domain (Fig. 7) suggest that the PAR complex is involved in the mechanism of centrosome attraction. The PAR complex plays a role in controlling spindle position in a number of diverse models including flies, worms and vertebrates (Ahringer, 2003; Betschinger and Knoblich, 2004; Roegiers and Jan, 2004). Studies on polarized mammalian cells are beginning to uncover clear molecular connections between centrosome orientation and localized members of the PAR complex. In astrocytes and neurons it is proposed that phosphorylation of the kinase GSK3 by aPKC leads to a polarized redistribution of the microtubule plus end-binding protein APC (Etienne-Manneville and Hall, 2003; Shi et al., 2004), whereas in epithelial cells it appears that LGN/Pins links the PAR complex to the microtubule-associated protein NuMA (Du and Macara, 2004; Lechler and Fuchs, 2005). It will be interesting to determine whether similar mechanisms operate in the CAB and in other cortical capture sites enriched in aPKC, PAR-6 and PAR-3 proteins.

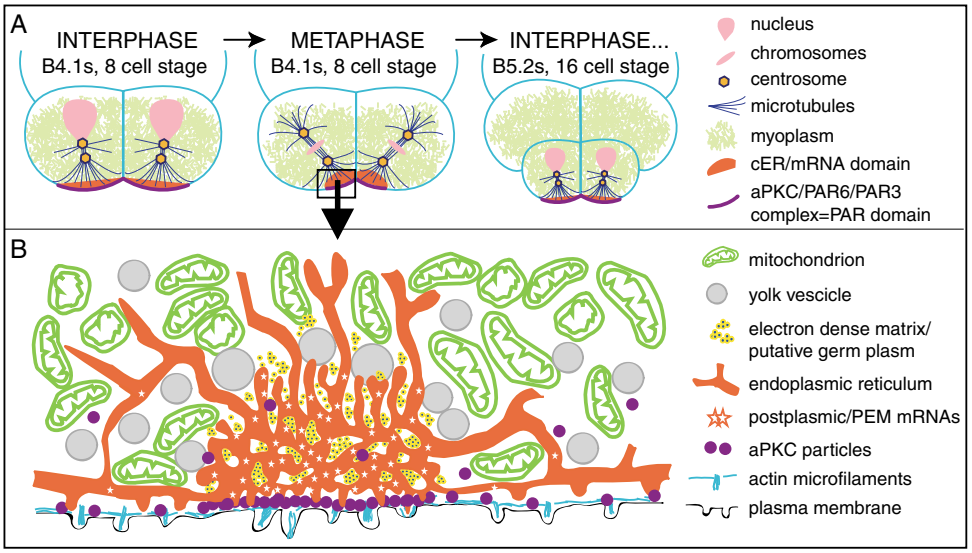


**Fig. 9.** Schematic representation of the structure of the ascidian CAB. The aPKC–PAR-6–PAR-3 domain (PAR domain, purple) is contiguous with the cortical actin microfilament layer (light blue) and sandwiched between the plasma membrane (black) and the cER-mRNA domain (red), all of which is surrounded by the mitochondria-rich myoplasm (green). (A) Dynamic reorganization during asymmetric cleavage. Microtubules (blue) emanating from the proximal centrosome contact the CAB and adjacent cell cortex. During interphase the CAB is widespread and thin as the nucleus migrates towards it. During mitosis the CAB compacts and thickens as the spindle positions asymmetrically. The smaller daughter cell inherits the CAB; centrosome attraction and unequal cleavage are then repeated. (B) Multilayered distribution of the major components of the CAB. Microtubules are not represented since their precise relationship to components of the CAB is not completely understood.

Another potential function of the PAR complex at the CAB is regulation of the actin cytoskeleton, which is one of its roles in *Caenorhabditis elegans* and in mammalian cells (Hurd and Margolis, 2005; Nance, 2005). The membrane rippling and transient protrusion we observed during nuclear migration (Fig. 2C) could be the consequence of a local alteration in contractility or stiffness of the cortical actin layer.

## Centrosome attracting mechanism

The mechanism previously proposed for unequal cleavage in posterior blastomeres of ascidian embryos postulates that microtubule plus ends are captured by the CAB and that one centrosome is pulled via microtubule shortening and/or by microtubule motors concentrated in the CAB (Hibino et al., 1998; Nishikata et al., 1999). Our observations in fixed *Phallusia* embryos are essentially in agreement with this model: some astral microtubules emanating from the proximal centrosome appear to penetrate the CAB whereas others course along the surface of the cER-mRNA domain. When the CAB is in its most compacted state, during metaphase and anaphase, it appears that microtubules are excluded from the cER-mRNA domain (Fig. 7M) but the finding that microtubules remain associated with the CAB in isolated cortices (Prodon et al., 2005) indicates that microtubule plus ends are able to anchor in the cER-mRNA domain. Our observation that many microtubules contact the cell surface adjacent to the CAB (Fig. 7I,K,L and Movie 4 in supplementary material) further suggests that forces distributed throughout the posterior cortex may also contribute to centrosome attraction.

A particularly interesting alignment of duplicated centrosomes was observed in B5.2 cells during interphase (Fig. 7J). One centrosome appeared attached to the nucleus; the other was positioned away from the nucleus in the direction of the CAB, and the two centrosomes were connected to each other by a dense mass of microtubules. Such extreme separation of a centrosome from the nucleus is not common but has been reported in the early *C. elegans* embryo (Keating and White, 1998; Malone et al., 2003) and in mammalian epithelial cells (Reinsch and Karsenti, 1994). This configuration in the ascidian embryo suggests that the nucleus displaces toward the cortex by recapturing an errant centrosome that is tethered to a cortical site. The stretching of the nucleus toward a distant centrosome could also explain our observation in living embryos that the nucleus appears pinched in the direction of the CAB as it migrates toward the cortex (Fig. 2A and Movie 1 in supplementary material).

Specialized cortical sites that can capture plus ends of microtubules and influence spindle positioning exist in many types of polarized cells (Cowan and Hyman, 2004; Kodama et al., 2004). In the two cell *C. elegans* embryo for example, the anterior cortex of the P1 cell contains a structure which is proposed to attract one centrosome by anchoring and pulling on astral microtubules (Hyman, 1989; Hyman and White, 1987; Keating and White, 1998). Similarly, in the one cell *C. elegans* embryo, the alignment of the spindle appears influenced by pulling forces situated along the anterior cortex (Cowan and Hyman, 2004). aPKC, PAR-6, and PAR-3 proteins colocalize along the anterior surface of the zygote and of the P1 cell, although unlike in the CAB, they are distributed over a broader region than just the cortical attraction site. Cortical capture sites anchor the meiotic spindle to the cell surface in many oocytes, prior to the exaggerated case of unequal cleavage which results in the formation of polar bodies. In mouse oocytes, PAR-6 (Maro and Verlhac, 2002; Vinot et al., 2004) and PAR-3 (Duncan et al., 2005) proteins have been found to concentrate at the animal pole, which is the cortical site where the meiotic spindle attaches and which also influences spindle positioning during first mitosis (Plusa et al., 2002). As the CAB is a particularly accessible and well-defined cortical capture site, the ascidian embryo is likely to prove a favourable model in which to address the spatial dynamics and functions of PAR proteins during asymmetric divisions.

## Materials and Methods

### Culture of ascidian embryos and preparation of cortices

All images and blots shown are from embryos of the European ascidian *Phallusia mammillata*. Gametes were collected, dechorionated, fertilized, and cultured as described previously (Prodon et al., 2005; Roegiers et al., 1999) and in methods on our web site http://biodev.obs-vlfr.fr/recherche/biomarcell/. Isolated cortices of 8-cell stage embryos were prepared and labelled as described previously (Prodon et al., 2005; Sardet et al., 2003).

### Imaging of live and fixed embryos

Live embryos were washed several times in calcium-free sea water to flatten them, and mounted in microchambers made of gelatin-formaldehyde-coated coverslips. DIC images were acquired using a Zeiss Axiophot microscope equipped with a Newvicon camera (Lhesa). For vital labelling of mitochondria and endoplasmic reticulum, eggs were treated with 1 μM Mitotracker (Molecular Probes) and injected with a droplet of $DiIC_{16}(3)$ (Molecular Probes) before fertilization. Fluorescent images of live or fixed immunolabelled embryos were acquired with a Leica SP2 confocal microscope and processed using ImageJ (NIH), Photoshop (Adobe) or Metaview/Metamorph (Universal Imaging) software.

### Cloning of aPKC and PAR-6 cDNAs and determination of PAR-3 sequence

Probes were generated by polymerase chain reaction on *Phallusia* egg cDNA using degenerate primers corresponding to conserved domains. A cDNA library synthesized from *Phallusia* egg mRNA in the λZAP vector (Stratagene) was screened to obtain clones encoding the full-length cDNAs of *Phallusia* aPKC (GenBank accession number AY987397) and *Phallusia* PAR-6 (GenBank accession number AY987398). The PAR-3 sequence was obtained by analysis of the unmasked assembly version 1.95 of the *Ciona intestinalis* genome in addition to predicted gene product models 159.16-159.21 and ci0100131546 provided by the Joint Genome Institute (http://genome.jgi-psf.org/ciona4/ciona4.download.ftp.html) and the Satoh Laboratory genome browser (http://hoya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/ci). To aid in exon definition, the *Ciona intestinalis* sequence was compared with the homologous region of the *Ciona savigny* genome (http://www2.bioinformatics.tll.org.sg:8082/Ciona_savignyi/, http://www.broad.mit.edu/annotation/ciona/) using Vista software (Frazer et al., 2004).

### Antibodies

The following primary antibodies were used. Anti-aPKC: rabbit polyclonal C-20 (Santa Cruz Biotechnology SC216) dilution 1:200. Anti-PAR-3: rabbit polyclonal against *Drosophila* Bazooka (Wodarz et al., 1999), dilution 1:200. Anti-mitochondria: mouse monoclonal NN18 (ICN), dilution 1:200. Anti-tubulin: mouse monoclonal DM1A (Sigma), dilution 1:500. Anti-actin: mouse monoclonal N350 (ICN), dilution 1:100. For anti-PAR-6 antibodies, N-terminal (MDKTSSGQRAPSP) and C-terminal (RDSVKAKRSNEPQD) peptides were synthesized and used to immunize rabbits (Covalab). A PAR-6-GST fusion protein was prepared from the vector pGEX4T (Amersham Biosciences) and coupled to Affigel (Bio-Rad). Antisera were loaded onto affinity columns and purified. PAR-6 antibodies were eluted with 0.1 M glycine pH 2.5, neutralized with 1 M Tris pH 8.5, dialyzed and concentrated using Amicon filters (Millipore). Anti-mouse and anti-rabbit secondary antibodies coupled to FITC or to TRITC (Jackson Labs) were used at a dilution of 1:200.

### Immunolabelling

For most antibodies, embryos were fixed in 4% paraformaldehyde in PBS pH 7.5 containing 0.5 M NaCl for 2 hours at room temperature or overnight at 4°C, transferred to 95% ethanol, and stored at –20°C. For labelling with PAR-3 (Bazooka) or tubulin antibodies, embryos were fixed in cold methanol and placed immediately at –20°C. Fixed samples were rehydrated in PBS-Tween (0.1%), blocked in PBS-BSA (1%), and incubated with primary antibodies for 2 hours at room temperature or overnight at 4°C with gentle shaking. Embryos were then washed three times in PBS Tween, incubated with secondary antibodies, washed again in PBS Tween, treated with 5 μg/ml Hoechst 33342 (Sigma) to label DNA, and mounted in Citifluor (Chemlab).

### Immunoblotting

Protein extracts were prepared by boiling dechorionated *Phallusia* eggs or embryos in Laemmli sample buffer. Immunoblots were prepared by standard SDS-PAGE followed by transfer to nitrocellulose membranes (Amersham), and incubated with primary antibodies at dilutions of 1:2000 (anti-aPKC), 1:4000 (anti-PAR-3), or 1:500 (anti-PAR-6). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Jackson Labs) at a dilution of 1:7500 followed by detection on ECL Hyperfilm using chemiluminescent substrates (Amersham).

### Treatment with cytoskeletal inhibitors

Embryos were treated at the 2-cell, 8-cell or 16-cell stages with 10 μg/ml nocodazole, 2 μg/ml cytochalasin B, or 1 μg/ml latrunculin A (Molecular Probes). Since *Phallusia* embryonic cell cycles are not completely synchronous within a population, the phase of the cell cycle in which each embryo was treated could not be determined. All inhibitors caused cell divisions to arrest, and in the presence of actin inhibitors nuclear cycles continue as noted in the legend to Fig. 8. aPKC immunolabelling results were similar for cytochalasin and latrunculin treatment, but only latrunculin caused complete elimination of filamentous actin, as determined by labelling with phalloidin.

### In situ hybridization and northern blot analysis

Embryos were fixed in 4% paraformaldehyde in 0.1 M Mops pH 7.5 containing 0.5 M NaCl. RNA probes were prepared by in vitro transcription in the presence of digoxigenin-UTP (Roche). In situ hybridization was carried out in formamide and signal detection by NBT/BCIP (Roche) were essentially as described by Sardet et al. (Sardet et al., 2003) and on our website. For northern blot analysis, embryos were lysed in 4 M guanine thiocyanate, 0.1 M Tris-HCl, 0.5% sarcosine, 1% β-mercaptoethanol and total RNA was recovered after phenol extraction. RNA was electrophoresed in formaldehyde-containing gels, transferred to nylon membranes (Amersham Biosciences) and hybridized to $^{32}$P-labelled probes according to the standard protocol.

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