**Drosophila Atypical Protein Kinase C Associates with Bazooka and Controls Polarity of Epithelia and Neuroblasts**

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Abstract. The establishment and maintenance of polarity is of fundamental importance for the function of epithelial and neuronal cells. In *Drosophila*, the multi-PDZ domain protein Bazooka (Baz) is required for establishment of apico-basal polarity in epithelia and in neuroblasts, the stem cells of the central nervous system. In the latter, Baz anchors Inscuteable in the apical cytocortex, which is essential for asymmetric localization of cell fate determinants and for proper orientation of the mitotic spindle. Here we show that Baz directly binds to the *Drosophila* atypical isoform of protein kinase C and that both proteins are mutually dependent on each other for correct apical localization. Loss-of-function mutants of the *Drosophila* atypical isoform of PKC show loss of apico-basal polarity, multilayering of epithelia, mislocalization of Inscuteable and abnormal spindle orientation in neuroblasts. Together, these data provide strong evidence for the existence of an evolutionary conserved mechanism that controls apico-basal polarity in epithelia and neuronal stem cells. This study is the first functional analysis of an atypical protein kinase C isoform using a loss-of-function allele in a genetically tractable organism.

Key words: cell polarity • atypical PKC • Bazooka • tight junction • asymmetric cell division

Introduction

Polarity is a common feature of many different cell types and is the prerequisite for various processes including vectorial transport of molecules, directed cell migration, and asymmetric cell division. Epithelial cells, for example, possess apical and basolateral plasma membrane domains that are distinguished from each other by their different lipid and protein composition (Rodriguez-Boulan and Nelson, 1989; Drubin and Nelson, 1996). Similar differences exist between axonal and somatodendritic membrane domains of neurons (Foletti et al., 1999; Winckler and Mellman, 1999). Polarity is not restricted to the plasma membrane but can also be observed in the underlying cytocortex. Examples of cell types with pronounced cortical polarity are the blastomeres of the early *Caenorhabditis elegans* embryo (Bowerman and Shelton, 1999) and *Drosophila* neuroblasts (Lu et al., 1998).

The mechanisms leading to the generation of cell polarity are just beginning to emerge. Membrane proteins and lipids are sorted in the trans Golgi network and are subsequently delivered to distinct regions of the plasma membrane via vesicular transport (Ikonen and Simons, 1998). The cytocortex may acquire polarity by interaction of cortical proteins with certain membrane lipids; e.g., by binding of proteins containing pleckstrin homology domains to phosphatidylinositol (3,4,5) trisphosphate or by binding to the cytoplasmic tails of transmembrane proteins. Vice versa, the correct localization of many transmembrane proteins depends on binding to cortical proteins. So far, it is not known whether membrane polarity can be established in the absence of cortical polarity or whether both processes are strictly dependent on each other. How are the different membrane domains separated from each other in polarized cells? In vertebrate epithelia, the tight junction (TJ) is considered to be the boundary between apical and basolateral plasma membrane domains (Stevenson and Keon, 1998; Tsukita et al., 1999). In addition, TJs create a paracellular seal that prevents the free diffusion of macromolecules in the extracellular space between cells. At the ultrastructural level, TJs are characterized by the fusion of the outer leaflet of the plasma membrane of adjacent cells. The formation of TJs depends on transmembrane proteins of the claudin family (Furuse et al., 1998; Tsukita and Furuse, 1999). Claudins and occludin, another transmembrane protein of the TJ, associate...
with several cortical proteins (ZO-1, ZO-2, ZO-3, and cingulin) that form a link with the actin cytoskeleton (Stevenson and Keon, 1998; Tsukita and Furuse, 1999; Tsukita et al., 1999).

In contrast to vertebrate epithelial cells, neurons do not possess TJ s, but nonetheless establish and maintain separate axonal and somatodendritic plasma membrane domains. The border between these membrane domains is located at the initial axonal segment (Winckler and Mellen, 1999). Measurements of the force required to move transmembrane and GPI-linked proteins in the plane of the membrane revealed that the lateral mobility of both classes of proteins is strongly reduced in the initial segment (Winckler et al., 1999). Treatment with the actin depolymerizing drug latrunculin B disrupts the diffusion barrier in this region (Winckler et al., 1999), suggesting that the submembraneous cytoskeleton plays an important role in maintenance of neuronal cell surface polarity.

Like neurons, most epithelial tissues in arthropods lack TJ s, despite being highly polarized (Tepass and Hartenstein, 1994; Müller, 2000). It has been proposed that the septate junction (SJ) could be the functional equivalent of TJ s in arthropods. This may be true at least in some cases for the sealing of the paracellular space (Skaer et al., 1987; Baumgartner et al., 1996), but the SJ is probably not responsible for the formation of a diffusion barrier in the plasma membrane. In Drosophila, the separation of apical and basolateral membrane domains can already be observed at the time of gastrulation (3 h after egg laying) (Müller and Wieschaus, 1996), while SJs form much later (11 h after egg laying) (Tepass and Hartenstein, 1994). Moreover, the septate junction forms basally of the zona adherens (ZA), a belt-like adhesive junction around the apex of epithelial cells, whereas the border between the apical and the basolateral plasma membrane domain is located at the apical end of the ZA (Tepass, 1996). So how is the separation of apical and basolateral plasma membrane domains achieved in Drosophila epithelia?

Double mutants lacking zygotic expression of the genes stardust (sdt) and bazooka (baz) fail to establish plasma membrane polarity after cellularization of the Drosophila embryo (Müller and Wieschaus, 1996). This phenotype is characterized by expression of the basolateral marker Neurotactin (Nrt) on the whole cell surface and mislocalization of the ZA component Armadillo (Arm). Moreover, in sdt, baz double mutants, the monolayered organization of the blastoderm epithelium is lost and cells acquire irregular shapes. These morphological changes are reminiscent of those seen during epithelial-mesenchymal transitions. Essentially, the same phenotype as in sdt, baz double mutants is observed in baz mutants lacking maternal and zygotic Bazooka (Baz), whereas zygotic sdt and baz single mutants show a weaker phenotype later in development (Tepass and Knust, 1993; Grawe et al., 1996; Müller and Wieschaus, 1996; Kuchinke et al., 1998). These data suggest that baz is absolutely required for establishment of plasma membrane polarity and epithelial morphology, whereas the early function of sdt may be partially redundant with that of baz.

baz is also required for establishment of apico-basal polarity and asymmetric division of neuroblasts in the developing central nervous system (CNS). Neuroblasts delamine from the neuroectodermal epithelium and undergo several rounds of asymmetric cell division, generating a ganglion mother cell and another neuroblast in each division. Before division, the mitotic spindle rotates by 90° and localization of the cell fate determinants Prospero and Numb becomes restricted to the basal cortex of the neuroblast. These events are prerequisites for proper segregation of Prospero and Numb into the ganglion mother cell. From delamination to early anaphase, Baz is localized in the apical cortex of neuroblasts, where it forms a complex with Inscuteable (Insc) (Schober et al., 1999; Wodarz et al., 1999), a protein required for rotation of the mitotic spindle and correct localization of Prospero and Numb (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Kaltschmidt et al., 2000; reviewed in Jan and Jan, 2000). In the absence of Baz, asymmetric cortical localization of Insc is abolished, leading to randomized spindle orientation and mislocalization of cell fate determinants (Schober et al., 1999; Wodarz et al., 1999). These data led to the conclusion that apico-basal polarity in neuroblasts depends on maintenance of apical Baz expression and is thus inherited from the neuroectodermal epithelium.

baz encodes a protein with three PDZ domains which shows significant sequence similarity along its entire length to Par-3 (Caenorhabditis elegans) and ASIP (rat) (Etemad-Moghadam et al., 1995; Izumi et al., 1998; Kuchinke et al., 1998). In the early C. elegans embryo, Par-3 is asymmetrically localized in the anterior cortex of the zygote and of blastomeres that undergo asymmetric cell divisions (Etemad-Moghadam et al., 1995). In these cells, Par-3 controls spindle orientation and asymmetric localization of cell fate determinants (Etemad-Moghadam et al., 1995; reviewed in Rose and Kemphues, 1998). Later on, Par-3 is also expressed in the apical cortex of the embryonic gut epithelium (O. Bossinger, personal communication). Par-3 binds to PKC-3, an atypical protein kinase C (aPKC) isoform (Tabuse et al., 1998; Wu et al., 1998). Both proteins are mutually dependent on each other for correct cortical localization. Moreover, embryos depleted of PKC-3 by RNA interference show a very similar phenotype to par-3 mutant embryos (Tabuse et al., 1998). ASIP was isolated as a binding partner of the mammalian aPKC isoforms, PKCs and PKCζ (Izumi et al., 1998). Intriguingly, ASIP and PKCs colocalize at the TJ in vertebrate epithelial cells (Izumi et al., 1998). These observations suggest that the association of ASIP/Par-3 with aPKCs is functionally important and evolutionarily conserved.

We have identified an aPKC from Drosophila (DaPKC) that shows very high sequence similarity to PKCζ and PKCζ from vertebrates and PKC-3 from C. elegans. DaPKC and Baz coinmunoprecipitate and directly bind to each other in a yeast two-hybrid assay. In embryos, both proteins colocalize in the apical cortex of almost all epithelial tissues and in neuroblasts. Apical localization of DaPKC in epithelia and neuroblasts is abolished in baz mutants, and vice versa, Baz is delocalized in DaPKC mutants. We show that the phenotype of DaPKC loss-of-function mutants resembles that of baz mutants, consistent with a close functional interdependence of both proteins. Together, our data provide the first in vivo evidence for an essential role of an atypical protein kinase C isoform in es-
establishment and maintenance of epithelial and neuronal polarity.

Materials and Methods

Identification of DaPKC

BLAST searches of the Berkeley Drosophila genome database revealed that EST clone HL05754 (obtained from Research Genetices) contains a cDNA insert with high similarity to atypical PKCs from vertebrates and C. elegans. HL05754 was fully sequenced and contains bases 1–1917 of the full length DaPKC cDNA sequence plus additional sequences derived from a transcript unrelated to DaPKC. Additional 3' cDNA sequences including the 3' end of the DaPKC coding region was amplified by 3' RACE from embryonic mRNA with the 5'3' RACE Kit (Roche) using the following primers: 5'-CGCGTTCAATGATATCGTCAGC-3' (forward) and 5'-GTTGATATTGCTGTCATTGC-3' (reverse). The remaining part of the 3' noncoding region has been deduced from the genomic sequence 3' of the region which does not contain a splice donor site but contains a polyadenylation motif (AATAAA).

Fly Stocks and Genetics

Oregon R was used as wild-type stock. Df(2R)1p1 removes the cytological interval 51C3:52F5-9, which contains the complete coding region of DaPKC. The DaPKC51C3 (Bauer et al., 1994) P-element insertion allele was obtained as l(2)k0403 from the Bloomington Drosophila stock center. Revertants of DaPKC51C3 were generated using P[IΔ2-3] (Robertson et al., 1988) as a transposase source. Backcross germ line clones were produced using the FLP/PDSE technique (Chou and Perrimon, 1992; Muller and Wieschaus, 1996). Baz overexpression in embryos was achieved with the UAS-GAL4 system (Brand and Perrimon, 1993) using a UAS-Baz transgene (Kuchinke et al., 1998) and the maternal GAL4 driver mat67G4 (D. St. Johnston and J.P. Vincent, unpublished observations). For analysis of DaPKC localization in insc mutants, insc99 and insc292 (Kraut and Campos-Ortega, 1996) were used in combination with a CyO [P[trz: lacZ] balancer. To generate hemizygous DaPKC51C3 embryos with the wild-type maternal dosage of DaPKC, we crossed females with a compound second chromosome, C(2)v (Merrill et al., 1988), to DaPKC51C3/CyO males. Compound autosomes have both left arms attached to one centromere and both right arms attached to the other, instead of the normal arrangement in which the left and right arms of a given chromosome are attached to the same centromere. C(2)v females produce gametes that contain either two left or two right arms of the attached second chromosome. Thus, one quarter of the progeny from the cross C(2)v × DaPKC51C3/CyO lack zygotic expression of DaPKC. DaPKC mutant embryos were identified by lack of staining with anti-aPKC antibody C20. Staging of embryos was according to Campos-Ortega and Hartenstein (1997).

Immunohistochemistry and In Situ Hybridization

For antibody stainings, embryos were fixed either in 4% formaldehyde, 100 mM phosphate buffer, pH 7.4 (for stainings with antibodies against PKCζ (C20), Insc, β-galactosidase, and Baz in neuroblasts) or according to the heat-methanol fixation procedure described in Muller and Wieschaus (1996). The following primary antibodies were used: rabbit anti–PKCζ (C20) (Santa Cruz Biotechnology, Inc.), 1:5; rat anti-Baz (Wodarz et al., 1999), 1:300; mouse anti-Arm N2-7A1 (Peifer et al., 1994) 1:10; rabbit anti-Insc (Kraut and Campos-Ortega, 1996), 1:5; rat anti-Baz (Wodarz et al., 1999), 1:300; mouse anti-Baz (Wodarz et al., 1999), 1:300; mouse anti-Sex-lethal (Bopp et al., 1991), 1:1,000; mouse anti-Arm N2-7A1 (Peifer et al., 1994) 1:10; rabbit anti-Insc (Kraut and Campos-Ortega, 1996), 1:5; mouse anti-Sex-lethal (Bopp et al., 1991), 1:5; mouse anti-β-galactosidase (Promega), 1:5,000. DNA was stained with YoYo-1 (Molecular Probes). Cy2-, Cy3-, and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1:500. In situ hybridization was performed using an antisense RNA probe of DaPKC labeled with digoxigenin-UTP (Roche) according to standard procedures. Images were taken on a TCSNT confocal microscope (Leica) or on an Axioptip microscope (Carl Zeiss, Inc.) and processed using Photoshop (Adobe) and Canvas (Deneba) software.

Immunoprecipitation and Western Blotting

Immunoprecipitation and western blotting were carried out as described (Willert et al., 1997; Wodarz et al., 1999). For IPs, 3 μl of mouse anti-Baz or 30 μl of monoclonal mouse anti-Nrt were added to a cell lysate containing 500 μg of total protein from S2 cells overexpressing Baz (Wodarz et al., 1999). For Western analysis, rabbit anti-PKCζ C20 was diluted 1:2,000.

Yeast Two-Hybrid Interaction Assays

Full-length DaPKC was cloned in frame with the GAL4 transactivation domain in pACT2 (CLONTECH Laboratories, Inc.). For generation of bait constructs, the regions of Baz shown in Fig. 2 b (below) were cloned in frame with the GAL4 DNA binding domain in pGBT9 (CLONTECH Laboratories, Inc.). Two hybrid assays were performed according to the manufacturers instructions. Details on the generation of two-hybrid constructs will be provided on request.

Results

Molecular Characterization of the DaPKC Gene

To identify an atypical protein kinase C isoform from Drosophila, we screened the Berkeley Drosophila genome database (http://www.fruitfly.org) with sequences from mouse PKCα and C. elegans PKC-3 (Tabuse et al., 1998) using the BLAST algorithm (Altschul et al., 1997). One EST clone (HL05754) showed significant sequence similarity to the NH2 termini of both PKCα and PKC-3. Further sequencing of HL05754 revealed that it contains most of the coding region of DaPKC, except for a few hundred basepairs that are missing at the 3' end. BLAST searches with the HL05754 cDNA fragment showed that the DaPKC gene is located in genomic region 51D on the right arm of chromosome 2, a region that had been sequenced by the Berkeley Drosophila genome project (Adams et al., 2000). Based on sequence similarity to mouse and C. elegans aPKCs and on sequence analysis tools predicting exon–intron boundaries, we identified three additional putative 3' exons that are missing in HL05754. The existence of the predicted transcript was confirmed by 3' RACE analysis of embryonic mRNA. Comparison of the DaPKC cDNA sequence to the genomic sequence of the DaPKC locus revealed the existence of at least 10 exons (Fig. 1 a). Both the first and the last exon are noncoding and the last exon contains a canonical polyadenylation signal (AATAAA). Conceptual translation of the cDNA gives rise to a protein of 606 amino acids with a predicted molecular weight of 69.5 kD. DaPKC shows the highest sequence similarity to mouse PKCα (68% identity), rat PKCζ (63% identity), and C. elegans PKC-3 (58% identity) (Fig. 1 b). In comparison, DaPKC shows significantly lower sequence similarity to two conventional PKC isoforms from Drosophila (Schaeffer et al., 1989), PKC 53E (29% identity) and PKC 98F (36% identity). BLAST searches of the completed genome sequence of Drosophila (Adams et al., 2000) revealed that DaPKC is the only aPKC in Drosophila.

For detection of DaPKC on Western blots and in whole-mount immunofluorescence stainings, we tested an antibody raised against a peptide corresponding to the COOH-terminal 20 amino acids of rat PKCζ (C20) for cross-reactivity with DaPKC. At the very COOH terminus, DaPKC and rat PKCζ show 75% identity and 95% similarity at the amino acid level (Fig. 1 b). Antibody C20 recognizes a protein of ~75 kD in Western Blots of Drosophila embryos and Schneider S2 cells (Fig. 2 a), which is in good agreement with the predicted molecular weight of DaPKC.

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In whole-mount immunofluorescence stainings of embryos, antibody C20 recognizes an epitope that is expressed in the same spatial and temporal pattern as the DaPKC mRNA (see below). The staining intensity is reduced to background level in embryos homozygous for a deficiency that removes the DaPKC locus (data not shown) and in embryos homozygous for the lethal P-element insertion line l(2)k06403, which is located in the third intron of the DaPKC locus (Figs. 1 a, 9 a, below). From these observations, we conclude that antibody C20 specifically recognizes the DaPKC protein.

**DaPKC Binds Directly to Baz**

To test whether DaPKC and Baz are associated in a protein complex, we performed coimmunoprecipitation experiments. Baz was immunoprecipitated from extracts of S2 cells overexpressing Baz (Wodarz et al., 1999). S2 cells express endogenous DaPKC. The immune complex was subjected to SDS-PAGE and Western analysis with anti-aPKC antibody C20 (Fig. 2 a). Anti-Nrt antibody (Hortsch et al., 1990) was used for immunoprecipitation as a negative control. While a significant amount of DaPKC coimmunoprecipitated with Baz, no signal was detected in the Nrt control (Fig. 2 a).
mRNA is present in all cells except for the pole cells (Fig. 3 b). During gastrulation, strong expression of DaPKC is detectable in tissues that undergo morphogenetic movements; e.g., the invaginating mesoderm, the proctodeum, and the cephalic furrow (Fig. 3 c). In embryos at the extended germ band stage, prominent DaPKC expression is detectable in neuroblasts (Fig. 3, d and e). In several epithelial tissues, in particular in the fore- and hindgut and in the Malpighian tubules, DaPKC mRNA is highly enriched in the apical cytokeratin (Fig. 3, d and f), reminiscent of the polarized localization of baz and crumbs (crb) mRNAs (Wodarz et al., 1990; Kuchinke et al., 1998).

The distribution of DaPKC protein was analyzed using anti-PKCζ antibody C20. During cellularization of the embryo, DaPKC becomes localized to the apical cytokeratin of all cells except for the pole cells, which do not contain detectable amounts of DaPKC (Fig. 4, a and b). Already at this stage DaPKC is enriched at apico-lateral cell borders, giving rise to a honeycomb pattern in en face views of the blastoderm (Fig. 4 c). After completion of cellularization, DaPKC is highly concentrated in the apico-lateral cortex (Fig. 4, d and e) and shows little overlap with the basolateral marker Nrt (Fig. 4, d’ and d”) (de la Escalera et al., 1990; Hortsch et al., 1990). Apical localization is maintained throughout embryonic development in most epithelia that are derived from the ectoderm (e.g., epidermis, fore-, and hindgut), Malpighian tubules, and the tracheal system (Fig. 4 f–i). The only ectodermal epithelium devoid of DaPKC expression is the amnioserosa.

Strong expression of DaPKC was also detected in neuroblasts, the stem cells of the embryonic CNS. During delamination of neuroblasts, DaPKC is localized in the apical stalk that is wedged between adjacent cells of the neuroectodermal epithelium (Fig. 5, a–c). In pro- and metaphase, DaPKC forms apical cortical crescents (Fig. 5, d–i). In anaphase, DaPKC staining is strongly diminished and expands over a broader region of the neuroblast cortex, but is clearly excluded from the budding ganglion mother cell (Fig. 5, j–l). Thus, from delamination through pro- and metaphase, localization of DaPKC in neuroblasts is very similar to that of Baz and Insc (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Schober et al., 1999; Wodarz et al., 1999). Simultaneously with DaPKC, cell outlines of epithelial cells and neuroblasts were visualized with the Nrt antibody (Fig. 5, b, e, h, and k). Similar to epithelial cells, Nrt expression is clearly polarized in neuroblasts. Strong staining for Nrt is detectable in the basal and lateral membrane of neuroblasts, whereas staining is strongly reduced in apical regions where DaPKC is expressed (Fig. 5, arrowheads).

To test whether DaPKC and Baz are colocalized, we performed double-label immunofluorescence stainings of embryos (Fig. 6, a–c). DaPKC (Fig. 6 a, red) and Baz (Fig. 6 b, green) are clearly colocalized in the epidermis (Fig. 6 c, yellow) and in neuroblasts (data not shown). To determine the precise subcellular localization of DaPKC and Baz with respect to the ZA, we performed double-label immunofluorescence stainings with antibodies against Arm, a component of the ZA (Fig. 6 d, red) and Baz (Fig. 6 e, green). The merged image (Fig. 6 f) shows that Baz is localized apically to Arm. The same is true for DaPKC (data not shown). At the resolution of the confocal micro-

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**Expression Pattern of DaPKC**

To see where DaPKC is expressed during embryonic development, we analyzed the mRNA distribution by RNA in situ hybridization. DaPKC mRNA is already detectable in freshly laid eggs before the onset of zygotic transcription (Fig. 3 a) and thus must be deposited in the egg during oogenesis. At the cellular blastoderm stage, DaPKC expression is very similar to that of Baz and Insc (Kraut and Campos-Ortega, 1996; Kraut et al., 1996; Schober et al., 1999; Wodarz et al., 1999). Simultaneously with DaPKC, cell outlines of epithelial cells and neuroblasts were visualized with the Nrt antibody (Fig. 5, b, e, h, and k). Similar to epithelial cells, Nrt expression is clearly polarized in neuroblasts. Strong staining for Nrt is detectable in the basal and lateral membrane of neuroblasts, whereas staining is strongly reduced in apical regions where DaPKC is expressed (Fig. 5, arrowheads).

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pressed in neuroblasts (nb, arrows) and in most ectodermally derived epithelia, except for the amnioserosa. Note that DaPKC mRNA is highly enriched in the apical cytocortex of several epithelial tissues, including the primordia of the foregut (fg) and hindgut (hg). Higher magnification of a stage 11 embryo showing strong expression of DaPKC in neuroblasts (arrows). (f) Higher magnification of a stage 11 embryo showing apical localization of DaPKC mRNA in foregut (left) and hindgut (right). The apical cell surfaces of fore- and hindgut are marked by arrows and the basal cell surfaces by arrowheads, respectively. Except for f, which is a dorsal view, all embryos are shown in a lateral view. Dorsal is up and anterior to the left. Bar in a–d = 100 μm; bar in e and f = 50 μm.

It has been shown before that Baz is required for apical localization of Insc in neuroblasts and that Insc is required for stabilization of Baz in neuroblasts after delamination (Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000). We tested whether Baz and Insc are also required for localization of DaPKC in neuroblasts. DaPKC localization was indistinguishable from wild type in neuroblasts of insc<sup>P49</sup>/CyO heterozygous embryos (Fig. 7, a and b), but was neither cortical nor apical in neuroblasts of insc<sup>P49</sup> homozygous mutant embryos (Fig. 7, c and d). In embryos lacking maternal Baz but carrying a paternal wild-type allele of baz (partial paternal rescue), asymmetric cortical localization of DaPKC was detected in most neuroblasts at metaphase (Fig. 7, e and f). However, DaPKC crescents and metaphase plates were often misoriented with respect to the surface of the embryo (Fig. 7, e and f), a phenotype that has also been observed at low penetrance in embryos lacking only zygotic expression of Baz (Kuchinke et al., 1998). In embryos lacking both maternal and zygotic expression of Baz (baz null), DaPKC was completely delocalized in neuroblasts and epithelial tissues (Figs. 6 g and 7, g and h). These results indicate that Baz is absolutely required for apical localization of DaPKC in neuroblasts and epithelial tissues, while Insc is required for localization of DaPKC only in neuroblasts.

**Genetic Analysis of DaPKC Loss-of-Function Mutants**

To study the consequences of DaPKC loss-of-function on epithelial polarity and asymmetric division of neuroblasts, we analyzed the phenotype of mutants in the DaPKC locus. BLAST searches with the genomic sequence of...
the DaPKC gene revealed that the P-element insertion l(2)k06403 is located in the third intron of the DaPKC gene (Fig. 1 a). This insertion line is homozygous lethal, contains a single P-lacW P-element, and does not complement Df(2R)Jp1, which removes the cytological interval 51C3;52F5-9. Staining intensity with anti-PKCζ antibody C20 was reduced to background level in embryos homozygous mutant for l(2)k06403 (Fig. 9 a, below). We mobilized the P-element using the transposase source P[D2-3] (Robertson et al., 1988) and recovered several revertants lacking the w1 marker of the original P-element insertion. Of seven revertant lines we tested, 3 were homozygous viable, demonstrating that insertion of the P-element into the DaPKC locus was the cause of lethality and of the observed phenotype (see below). We thus conclude that l(2)k06403 is an allele of DaPKC and renamed it DaPKCk06403.

Homozygous mutant DaPKCk06403 embryos do not produce any cuticle (Fig. 8 b). The reason for this phenotype is early embryonic lethality before the onset of cuticle secretion. Transheterozygous DaPKCk06403/Df(2R)Jp1 embryos show exactly the same cuticle phenotype (Fig. 8 c), suggesting that DaPKCk06403 is a strong hypomorphic or null allele. The analysis of the DaPKC loss-of-function phenotype was complicated by the fact that DaPKC mutants show maternal haploinsufficiency with incomplete penetrance. The evidence for this conclusion comes from the following observations: we noticed that, in egg collections from the DaPKCk06403/CyO stock, significantly more than 50% of embryos (including CyO/CyO homozygotes)
Figure 5. Expression of DaPKC in neuroblasts. Wild-type embryos at the extended germ band stage (stage 10) were triple labeled for DaPKC (red), Nrt (blue), and DNA (YoYo-1, green). (a–c) During delamination of neuroblasts, DaPKC is expressed in the apical stalk that is wedged between adjacent epithelial cells. In prophase (d–f) and metaphase (g–i), DaPKC forms a crescent in the apical cortex of neuroblasts. In anaphase (j–l), DaPKC staining in neuroblasts is strongly reduced but is still detectable in an enlarged region of the apical cortex. No staining is detectable in budding ganglion mother cells (GMCs). Note that Nrt staining is reduced in regions of the plasma membrane where DaPKC is present in the cortex (regions between arrowheads in b and e). Neuroblasts are marked by asterisks and the budding GMCs in j and k by a circle. Merged images of all three stainings are shown in the right column. Apical is up in all panels. Bar = 10 μm.

Figure 6. Apical localization of DaPKC depends on Baz. (a–c) DaPKC and Baz are colocalized in epithelia. A wild-type embryo at stage 14 was doubly stained for DaPKC (a, red) and Baz (b, green). Both proteins show extensive colocalization (c). (d–f) Baz is localized apically to the ZA marker Arm. A wild-type embryo at stage 14 was doubly stained for Arm (d, red) and Baz (e, green). In the merged image (f) there is very little overlap and Baz is expressed apically to Arm. The indentations of the epidermis in a–f represent segment boundaries. (g–i) DaPKC localization is lost and Nrt localization is not restricted to the basolateral membrane in baz mutant embryos. In a baz<sup>Xi106</sup> null embryo at gastrulation, DaPKC is not localized in the cortex (g) and Nrt is present on the whole-cell surface (h), merged image in i. Note also that the blastoderm epithelium is multilayered and cells show a very irregular shape (compare Fig. 4, d, d', and d''). (j–l) Overexpression of Baz is sufficient for ectopic localization of DaPKC. When overexpressed with the GAL4 system, Baz is not restricted to the apical cortex of epithelial cells and neuroblasts, but becomes localized to ectopic sites in the cortex (k, arrows). Consequently, DaPKC is also detectable at ectopic sites in the cortex (j, arrows), where it colocalizes with Baz (l, arrows). Apical is up in all panels. Bars = 10 μm.
failed to hatch (63.2%, n = 644), which means that even embryos with a zygotic wild-type allele of DaPKC frequently show developmental defects (Figs. 8 e and 9, g and h). This finding was confirmed in crosses of heterozygous DaPKCk06403/CyO females to wild-type males, where we also observed considerable embryonic lethality (25%, n = 244). The reciprocal cross did not show an increase of embryonic lethality compared with wild-type controls. We therefore conclude that the decreased viability of the progeny from DaPKCk06403/CyO mothers is caused by the reduction of the maternal DaPKC level during oogenesis.

To produce embryos with the wild-type maternal contribution of DaPKC, but lacking any zygotic DaPKC expression, we crossed C(2)v females (Merrill et al., 1988) to DaPKCk06403/CyO males and analyzed the cuticle phenotype of their progeny (for details, see Materials and Meth-
ods). One quarter of the embryos derived from that cross completely lacked zygotic expression of DaPKC and produced cuticles with characteristic defects. In most cases, head structures were missing and the ventral cuticle either showed large holes (Fig. 8 f) or was missing altogether (g). These phenotypes are strikingly similar to those of baz mutants. baz null embryos derived from germ-line clones produce very little or no cuticle (Fig. 8 d) and resemble DaPKC<sup>k06403</sup> mutant embryos derived from heterozygous mothers (b and c). baz mutant embryos lacking only the zygotic expression produce cuticles with characteristic head defects and ventral holes (Fig. 8 h) that are very similar to DaPKC<sup>k06403</sup> mutant embryos derived from C(2) maternal mothers (f and g).

**DaPKC Mutants Show Loss of Polarity in Epithelia and Neuroblasts**

To investigate the role of DaPKC in the control of epithelial organization and polarity, we stained DaPKC<sup>k06403</sup> mutant embryos with antibodies against Baz, Nrt, and Arm, the *Drosophila* β-catenin homologue. Most homozygous DaPKC<sup>k06403</sup> embryos from heterozygous mothers arrested very early in development and died before or during cellularization (data not shown). Those that developed further showed dramatic defects in epithelial organization and polarity. The blastoderm epithelium of these embryos was multilayered, cell shapes were extremely irregular and apico-basal polarity of the epithelium was lost (Fig. 9, a–f). Instead of being localized to the apical cortex, Baz was found in randomly scattered aggregates (Fig. 9, c and e). The basolateral marker Nrt was localized on the whole-cell surface in most cells (Fig. 9, d and f; compare Fig. 4 d–f).

A significant fraction of embryos derived from DaPKC<sup>k06403</sup>/CyO heterozygous mothers that possessed at least one zygotic wild-type allele of DaPKC showed characteristic defects in the head region (Fig. 9, g and h). While epithelial structure and distribution of Baz and Nrt was normal in the trunk region of these embryos, the epithelium at the anterior tip of the embryos was multilayered, showed a delocalized distribution of Baz (Fig. 9, g and h) and expression of Nrt on the whole cell surface (h). Thus, the defects observed in the head region of these embryos were very similar to the defects observed in the whole blastoderm epithelium of homozygous DaPKC<sup>k06403</sup> embryos from heterozygous mothers (Fig. 9, a–f). Most likely, these defects reflect an early requirement for DaPKC before the onset of zygotic transcription and are caused by insufficient maternal supply of DaPKC. Consistent with this interpretation, homozygous DaPKC<sup>k06403</sup> embryos with the wild-type maternal contribution of DaPKC (see Materials and Methods) developed further than homozygous mutant embryos derived from heterozygous mothers and did not show obvious defects before germ band extension. At this stage, patches devoid of apical Baz (Fig. 9 i)
PKC isoform in Drosophila and show that DaPKC binds directly to Baz and is colocalized with Baz in epithelia and neuroblasts. Apical localization of DaPKC is lost in baz mutants and vice versa, demonstrating that both proteins are mutually dependent on each other for correct localization. In neuroblasts, localization of DaPKC is not only dependent on Baz, but also on Insc. Baz levels are strongly reduced in neuroblasts of insc mutant embryos, most likely because Insc is required for stabilization of Baz (Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000). Thus, the effect of Insc on DaPKC localization is probably indirect and can be explained by the loss of Baz in insc mutant neuroblasts. Another protein, partner of Insc (Pins), has recently been identified as a binding partner of Insc (Schaefer et al., 2000; Yu et al., 2000). Pins, Baz, and Insc are mutually dependent on each other for correct localization and/or stability (Schaefer et al., 2000; Yu et al., 2000), indicating that loss of any single known component of this complex compromises stability and/or localization of the other components in the apical cortex of neuroblasts.

These findings are reminiscent of the situation in the early C. elegans embryo, where PKC-3, Par-3 and another PDZ domain protein, Par-6, are mutually dependent on each other for correct localization in the anterior cytocortex (Watts et al., 1996; Tabuse et al., 1998; Hung and Kemphues, 1999). Consistent with these results, the phenotype of embryos depleted of PKC-3 by RNA interference is very similar to the phenotype of par-3 and par-6 mutants (Etemad-Moghadam et al., 1995; Watts et al., 1996; Tabuse et al., 1998; Hung and Kemphues, 1999). Interestingly, a Drosophila homologue of par-6 does exist (Tabuse et al., 1998), raising the possibility that the interaction of Par-3/Baz, PKC-3/DaPKC and Par-6 has been evolutionarily conserved.

Another example for a close functional interaction between a PDZ domain protein and protein kinase C has recently been uncovered in Drosophila. The multi-PDZ domain protein InaD binds to the eye-specific, conventional isoform of PKC and is required for its proper localization in photoreceptors (Tsunoda et al., 1997; Xu et al., 1998). InaD contains five PDZ domains and distinct binding partners have been identified for each of them. Intriguingly, all of the proteins that bind to InaD are part of the phototransduction cascade in the Drosophila eye (Tsunoda et al., 1997; Xu et al., 1998). Thus, it has been proposed that InaD provides a scaffold for the assembly of a signaling complex, a so called “transducisome.”

In the case of DaPKC and Baz, the situation is more complicated. Consistent with a function as a scaffold, Baz is required for localization of the signaling protein DaPKC. However, Baz itself is not properly localized in the absence of DaPKC. It is easy to imagine how a structural multi-PDZ domain protein like InaD or Baz can localize a protein kinase, but how can DaPKC be responsible for localization and stabilization of Baz? Baz possesses a PKC consensus phosphorylation site that is conserved between Baz, Par-3, and ASIP. Phosphorylation of this site by DaPKC could be important to regulate binding of Baz to other proteins or to protect Baz from proteolytic degradation. It is also possible that DaPKC binds simultaneously to Baz and another protein that may be required for localization of Baz. A detailed structure–function analysis of both Baz and DaPKC will be necessary to clarify this issue.

Discussion

We have identified the gene encoding the only atypical PKC isoform in Drosophila and show that DaPKC binds directly to Baz and is colocalized with Baz in epithelia and
Analysis of the DaPKC loss-of-function phenotype revealed that DaPKC is already required very early during embryogenesis, before the onset of zygotic transcription. Most homozygous DaPKC<sup>604/605</sup> embryos with a reduced maternal dosage of DaPKC die before cellularization is completed. What could be the reason for this early death? aPKCs have been implicated in the control of apoptotic cell death in vertebrate tissue culture cells. Inhibition of aPKCs induces apoptosis (Diaz-Meco et al., 1996). Treatment of cells with UV irradiation also triggers apoptosis and rapidly inhibits aPKC kinase activity, suggesting that inhibition of aPKCs is an early event in the apoptotic signaling cascade (Berra et al., 1997). In accordance with these data, aPKCs have been implicated in the transduction of survival signals downstream of growth factor receptors. In contrast to conventional and novel PKC isoforms, aPKCs can be activated by phosphatidylinositol(3,4,5)trisphosphate and ceramide, two second messengers that are generated in response to inflammatory cytokines and growth factors (Nakanishi et al., 1993; Lozano et al., 1994; Akimoto et al., 1996). Our observation that DaPKC mutant embryos show premature cell death and strongly increased TUNEL labeling (A. Wodarz, unpublished observations), which is a hallmark of apoptosis, is consistent with a function of DaPKC in the transmission of survival signals. A detailed analysis of the role of DaPKC in the control of cell death in Drosophila is beyond the scope of this work and will be addressed in a future publication.

The loss-of-function phenotype of DaPKC mutants in epithelia is very similar to the phenotypes described for baz null mutants and zygotic sdt, baz double mutants (Müller and Wieschaus, 1996). The most striking abnormalities in these mutants are loss of the monolayered epithelial organization, irregular cell shapes, and loss of plasma membrane polarity. Multilayering of epithelia and abnormal cell shapes are most likely caused by defects in cell adhesion. Indeed, formation of the ZA, a region of intense, cadherin-mediated cell contact, is defective in DaPKC, baz, and sdt mutants (Grawe et al., 1996; Müller and Wieschaus, 1996). Another gene, crb, is also required for correct positioning and maintenance of the ZA (Wodarz et al., 1995; Grawe et al., 1996; Tepass, 1996; reviewed in Müller, 2000). DaPKC, Baz, and Crb are all localized apically of the ZA (Tepass, 1996; this work), so how can they control formation of the ZA? Crb, a transmembrane protein (Tepass et al., 1990) binds via its cytoplasmic tail to the cortical multi-PDZ domain protein Discs Lost (Bhat et al., 1999; Klebes and Knust, 2000), which probably associates with additional proteins. This complex could be involved in the formation of a protein scaffold in the apical cytocortex that prevents ZA components from moving further apically. A similar function can be envisioned for Baz, since it is also a multi-PDZ domain protein with the capacity to interact with several partners at the same time.

How does DaPKC fit into this model? As discussed above, DaPKC is required for localization and stabilization of Baz, but this may not be its only function in ZA formation. Several reports show that PKCs are involved in the assembly of adherens junctions and TJ (Lewis et al., 1994; Citì and Denisenko, 1995; Stuart and Nigam, 1995; van Hengel et al., 1997). The majority of these studies used cultured cell lines and analyzed the effects of different inhibitors and agonists of PKCs on localization and phosphorylation of junctional proteins, cell adhesion, and cell morphology. Although these studies provided compelling evidence for an involvement of PKCs in junction formation, in most cases neither the specific PKC isoforms responsible for the observed phenotypes nor the targets of these PKCs have been unambiguously identified. In one interesting study, inhibition of aPKCs induced epithelial-mesenchymal transformation in quail neural tube explants, while inhibitors of conventional or novel PKCs had little or no effect in this assay (Minichielli et al., 1999).

In addition to their effects on epithelial organization and cell shape, mutations in DaPKC, baz, sdt, and crb also affect plasma membrane polarity (Wodarz et al., 1993, 1995; Müller and Wieschaus, 1996; Klebes and Knust, 2000). As discussed in the Introduction, establishment and maintenance of plasma membrane polarity requires the separation of apical and basal membrane domains by a diffusion barrier in the plane of the membrane. In vertebrate epithelia, this diffusion barrier is created by the TJ. In arthropod epithelia, TJs have not been found by ultrastructural analysis (Tepass and Hartenstein, 1994). We note, however, that the vertebrate homologues of DaPKC and Baz, PKCa, PKCb, and ASIP, are localized at the TJ in epithelial cells (Izumi et al., 1998). Moreover, DaPKC and Baz are localized apically to the ZA in Drosophila epithelia, which corresponds to the position of the TJ in vertebrate epithelia. Thus, based on their localization and their mutant phenotypes, we propose that DaPKC and Baz are components of an evolutionarily conserved protein complex that may serve similar functions as the TJ in vertebrates.

Neuroblasts do not possess elaborate cell junctions but clearly show cortical and, at least to some extent, plasma membrane polarity. DaPKC and Baz are required for anchoring Insc in the apical neuroblast cortex (Schöber et al., 1999; Wodarz et al., 1999; this work) and it is conceivable that DaPKC and Baz may also be involved in the formation of a submembrane protein scaffold analogous to the model we have proposed for epithelia. Consistent with this idea is the finding that Nrt staining is reduced precisely in those regions of the neuroblast plasma membrane where DaPKC and Baz are localized beneath the membrane. Thus, DaPKC and Baz may be generally responsible for the separation of membrane domains by preventing diffusion of basolateral proteins into the apical domain.

From the available data, it is impossible to decide whether the primary function of DaPKC in neuroblasts is the stabilization of Baz or whether DaPKC phosphorylates additional targets involved in asymmetric division of neuroblasts. One candidate for phosphorylation by DaPKC is Miranda, an adaptor protein with six consensus PKc phosphorylation sites that binds to Prospero and Insc (Ikeshima-Kataoka et al., 1997; Shen et al., 1997, 1998; Schuldt et al., 1998). Miranda colocalizes with Insc only briefly in late interphase, and then moves together with Prospero to the basal cortex of the neuroblast during prophase (Shen et al., 1998). It is an attractive possibility that phosphorylation of Miranda by DaPKC regulates binding of Miranda to Insc and its release from the apical complex later in the cell cycle.
In conclusion, we have shown that DaPKC is an essential binding partner of Baz in epithelia and neuroblasts. Surprisingly, Baz does not simply function as a scaffold to anchor DaPKC in the apical cytocortex, but is itself dependent on DaPKC for proper localization and stability. This mutual dependence is indicative of an intimate cross-talk between structural proteins like Baz and the signaling protein DaPKC. The link between signal transduction components and structural components of the cytocortex may be important to allow rapid rearrangement of cellular junctions and cell shape changes such as those occurring during delamination of neuroblasts. To fully understand the role of DaPKC in the generation of cellular asymmetry, it will be essential to identify the physiological activators, inhibitors, and downstream targets of this important protein kinase.

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In our analysis of the zygotic DaPKC loss-of-function phenotype, we reported that embryos homozygous for the DaPKCk06403 allele die during early embryonic stages and fail to properly establish apical–basal polarity in embryonic epithelia and neuroblasts. Subsequent experiments in our lab showed that this early zygotic phenotype was only observed in the genetic background of the original fly stock that we used for our analysis. In a different genetic background, animals homozygous for the DaPKCk06403 allele survive until larval stages due to the maternal supply of DaPKC activity (Kim, S., and A. Wodarz, unpublished data; see Rolls et al., 2003). Upon removal of the maternal and zygotic activity of DaPKC in germ line clones of the DaPKCk06403 allele and of four new EMS alleles of DaPKC, we observed a fully penetrant loss of apical–basal polarity in embryonic epithelia and neuroblasts (Kim, S., B. Moussian, S. Luschnig, and A. Wodarz, unpublished data). We conclude that genetic background effects contributed to the early onset of the zygotic DaPKC loss-of-function phenotype that we originally reported. Nonetheless, our new data clearly show that DaPKC is indeed required for the control of apical–basal polarity in embryonic epithelia and neuroblasts.

We apologize to the readers for any confusion that may have been caused by this inaccuracy in our previous report.

Reference: Rolls, M.M., R. Albertson, H.P. Shih, C.Y. Lee, and C.Q. Doe. 2003. Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia. J. Cell Biol. 163:1089–1098.