Drosophila aPKC regulates cell polarity and cell proliferation in neuroblasts and epithelia

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Cell polarity is essential for generating cell diversity and for the proper function of most differentiated cell types. In many organisms, cell polarity is regulated by the atypical protein kinase C (aPKC), Bazooka (Baz/Par3), and Par6 proteins. Here, we show that Drosophila aPKC zygotic null mutants survive to mid-larval stages, where they exhibit defects in neuroblast and epithelial cell polarity. Mutant neuroblasts lack apical localization of Par6 and Lgl, and fail to exclude Miranda from the apical cortex; yet, they show normal apical crescents of Baz/Par3, Pins, InsCutable, and Discs large and normal spindle orientation. Mutant imaginal disc epithelia have defects in apical/basal cell polarity and tissue morphology. In addition, we show that aPKC mutants show reduced cell proliferation in both neuroblasts and epithelia, the opposite of the lethal giant larvae (lgl) tumor suppressor phenotype, and that reduced aPKC levels strongly suppress most lgl cell polarity and overproliferation phenotypes.

Introduction

Cell polarity is essential for the function of many cell types, as well as for generating cell diversity through asymmetric cell division. Over the past few years, it has become clear that cell polarity in many cell types and organisms is regulated by the evolutionarily conserved protein complex containing Baz/Par3 (Par3 in Caenorhabditis elegans, Bazooka in Drosophila, ASIP in mammals), Par6, and atypical protein kinase C (PKC-3 in C. elegans, aPKC in Drosophila, and PKCζ and λ in mammals; Doe, 2001; Ohno, 2001; Wodarz, 2002). The Baz/Par3 and Par6 proteins contain PDZ protein interaction domains, whereas aPKC is a serine/threonine kinase (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Suzuki et al., 2001). Each of the Baz/Par3, Par6, and aPKC proteins is reported to bind the others in vitro (Tabuse et al., 1998; Joberty et al., 2000; Lin et al., 2000; Wodarz et al., 2000; Suzuki et al., 2001; Betschinger et al., 2003), and the three proteins are reported to be interdependent for their normal localization in C. elegans blastomeres (Etemad-Moghadam et al., 1995; Tabuse et al., 1998; Hung and Kemphues, 1999), mammalian epithelia (Joberty et al., 2000; Suzuki et al., 2001), and Drosophila epithelia and neuroblasts (Wodarz et al., 2000; Petronczki and Knoblich, 2001).

The Baz/Par3–Par6–aPKC complex was initially characterized for its role in the proper partitioning of cell fate determinants during the asymmetric cell division of early blastomeres in C. elegans. In this system, the Par3–Par6–aPKC complex is localized to the anterior cortex of the one-cell embryo and is required to restrict the Par1 and Par2 proteins to the opposite posterior cell cortex (Kemphues, 2000). Analyses of cell polarity in other systems and cell types have shown the Baz/Par3–Par6–aPKC complex to be a widely used and flexible set of proteins that functions in conjunction with many proteins other than Par1 and Par2.

In Drosophila, epithelial cells have an apical/basal polarity that is reflected in the establishment of an apical membrane domain, apical adherens junctions, slightly more basal septate junctions, and a basolateral membrane domain (Tepass et al., 2001). The Baz/Par3–Par6–aPKC protein complex is targeted to the apical membrane domain, and is enriched at the adherens junctions (Tepass et al., 1990; Kuchinke et al., 1998; Wodarz et al., 2000; Bachmann et al., 2001; Hong et al., 2001; Petronczki and Knoblich, 2001).

A modified form of apical/basal polarity is present in mitotic Drosophila neuroblasts, where it is used to generate cellular diversity by asymmetric cell division. Drosophila neuroblasts divide asymmetrically along their apical/basal axis to regenerate an apically positioned neuroblast and bud off a smaller basally positioned ganglion mother cell.

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Abbreviations used in this paper: aPKC, atypical protein kinase C; Dlg, Discs large; Ecad, E-cadherin; GMC, ganglion mother cell; Insc, InsCutable; Lgl, Lethal giant larvae; Scrib, Scribble.

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(GMC). The GMC undergoes one subsequent cell division to generate neurons or glia (Goodman and Doe, 1993). The neuroblast apical domain is derived from the epithelial apical domain during the process of neuroblast delamination, and shows apical localization of the Baz/Par3–Par6–aPKC proteins during mitosis. Unlike epithelial cells, neuroblasts contain the protein Inscuteable (Insc), which is required to maintain apical localization of the Baz/Par3–Par6–aPKC proteins (Schöber et al., 1999; Wodarz et al., 1999, 2000; Petronczki and Knoblich, 2001; Albertson and Doe, 2003). These proteins are required to establish a basal domain in mitosis. Among others, the Miranda protein and its cargo Prospero transcription factor and Staufen RNA-binding protein are localized to this domain (Doe and Bowerman, 2001).

A second group of proteins—Discs large (Dlg), Scribble (Scrib), and Lethal giant larvae (Lgl)—has been shown to regulate apical/basal cell polarity in Drosophila epithelia and neuroblasts (Bilder et al., 2000; Bilder and Perrimon, 2000; Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003). Members of this complex are rich in protein–protein interaction domains. Dlg contains one SH3 and three PDZ domains (Woods and Bryant, 1991), Lgl contains four WD40 repeats (Strand et al., 1994), and Scrib contains four PDZ domains as well as sixteen leucine-rich repeats (Bilder and Perrimon, 2000). Dlg and Scrib interact with a common binding protein called Gukholder (Mathew et al., 2002); Lgl has not been shown to physically interact with any other member of the group. In Drosophila epithelia, Dlg, Lgl, and Scrib are localized to the septate junction and basolateral membrane domain, where they are required for restricting the Baz/Par3–Par6–aPKC complex to the apical membrane domain (Bilder and Perrimon, 2000). In mitotic neuroblasts, Dlg, Scrib, and Lgl proteins localize to the cortex with apical enrichment, and are required for targeting basal proteins to the cortex (Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003). Their functions in addition to basal protein targeting include limiting cell proliferation (Humbert et al., 2003) and promoting spindle asymmetry in neuroblasts (Albertson and Doe, 2003). Recently, a connection has been made between the Baz/Par3–Par6–aPKC complex and the Dlg/Scrib/Lgl group of proteins. In both Drosophila and mammals, it has been shown that aPKC directly binds and phosphorylates Lgl (Betschinger et al., 2003; Yamakata et al., 2003). In mammals, this phosphorylation is proposed to regulate epithelial tight junction formation (Yamanaka et al., 2003), and in Drosophila to regulate neuroblast basal protein targeting (Betschinger et al., 2003). Here, we investigate the role of aPKC in regulating cell polarity and cell proliferation in Drosophila, and the genetic interactions between aPKC and Lgl. We conclude that aPKC is required for proper apical/basal polarity in neuroblasts and epithelia; that Baz/Par3 and aPKC have distinct functions in neuroblasts; and that a major function of aPKC is to suppress Lgl activity.

Results

aPKC zygotic mutant embryos have normal epithelial and neuroblast polarity

It was previously reported that embryos homozygous for the aPKC allele, which contains a P element inserted into the third intron of the aPKC gene, died before gastrulation (Wodarz et al., 2000). We used the same aPKC allele, but found that homozygous mutants survived to mid-larval stages (Table I). We verified the presence of the aPKC allele in single- and double-mutant chromosomes by PCR (Fig. 1 A). To further understand the aPKC mutant allele, we assayed aPKC mRNA and protein levels in homozygous mutant larvae. Northern blots of homozygous mutant second instar larval brain tissue revealed much reduced levels of aPKC mRNA (Fig. 1 B), and
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antibody staining showed no detectable full-length protein in late second instar brain or imaginal disc tissue (Fig. 1C; unpublished data). Thus, the aPKC<sup>k06403</sup> allele is close to a molecular null allele. To determine if aPKC<sup>k06403</sup> behaves as a genetic null allele, we compared aPKC<sup>k06403/aPKC<sup>k06403</sup></sup> to aPKC<sup>k06403/Df(2R)JP1</sup> (a deficiency that removes the entire

Table 1. aPKC<sup>k06403</sup> is a genetic null mutation: aPKC<sup>k06403/aPKC<sup>k06403</sup></sup> and aPKC<sup>k06403/Df(2R)JP1</sup> have indistinguishable phenotypes

| Genotypes<sup>a</sup> | WT | aPKC<sup>k06403/aPKC<sup>k06403</sup></sup> | aPKC<sup>k06403/Df(2R)JP1</sup> |
|----------------|-----|--------------------------------|-------------------------------|
| Metaphase NB (Miranda)<sup>b</sup> | n = 43 | n = 53 | n = 67 |
| basal cortex | 95% | 0% | 0% |
| basal + weak apical | 5% | 21% | 18% |
| uniform cortical | 0% | 79% | 82% |
| Telophase NB (Miranda)<sup>b</sup> | n = 27 | n = 18 | n = 40 |
| basal cortex | 100% | 0% | 0% |
| basal + weak apical | 0% | 44% | 50% |
| uniform cortical | 0% | 56% | 50% |
| Lethal phase | Adult | L2 | L2 |
| Larval locomotion<sup>c</sup> | Normal | Unc | Unc |

<sup>a</sup>Wild type (WT) is yellow white; Df(2R)JP1 removes the entire aPKC locus.
<sup>b</sup>Neuroblast phenotype was assayed in early second instar larval brains.
<sup>c</sup>Locomotion was evaluated 72 h after larval hatching at RT, at which time wild type were third instar larvae and both mutant genotypes were developmentally arrested second instar larvae. Unc, uncoordinated.

Figure 2. aPKC zygotic null mutant larvae have defects in neuroblast cell polarity. Second instar larval neuroblasts; apical cortex to the top; cell cycle stage indicated; insets show double labels for the indicated protein (green) and α-tubulin (red) to document cell cycle stage and spindle orientation. Wild-type larval neuroblasts (first and third rows): aPKC, Pins, Baz/Par3, Lgl, and Par6 are localized to the apical cortex, whereas Miranda is localized to the basal cortex. aPKC mutant neuroblasts (second and fourth rows): aPKC is undetectable; Baz/Par3, Pins, and Dlg are normally localized to the apical cortex; Lgl and Par6 show abnormal cytoplasmic localization (Par6 can form cortical patches at the telophase bud neck); Miranda shows abnormal uniform cortical localization.
aPKC locus) for lethal phase and neuroblast polarity phenotypes. We find indistinguishable phenotypes for both genotypes (Table I), indicating that aPKC<sup>k06403</sup> acts as a genetic null allele.

Stage 15 embryos homozygous for aPKC<sup>k06403</sup> (subsequently called aPKC mutants) have readily detectable maternal aPKC protein (unpublished data) and normal epithelial and neuroblast polarity, as assayed by multiple apical and basal markers (unpublished data). We conclude that zygotic aPKC is dispensable for embryonic epithelial polarity, neuroblast polarity, and embryonic viability.

**aPKC zygotic mutants have defects in larval neuroblast cell polarity**

aPKC zygotic mutants died as late second instar larvae, which allowed us to analyze neuroblast cell polarity in mid-second instar larval brains. In wild-type mitotic neuroblasts, we detected apical cortical localization of Baz/Par3, Pins, Lgl, and Par6 throughout mitosis (Fig. 2). aPKC mutant larvae were identified by the lack of GFP expression from the balancer chromosome. In these mutants, Par6 and Lgl proteins were delocalized into the cytoplasm in all metaphase neuroblasts; the only enrichment of either protein was faint ectopic Par6 at the cleavage furrow in telophase (Fig. 2). In contrast, all mutant neuroblasts showed normal apical localization of Baz/Par3, Pins, and Dlg proteins (Fig. 2). We conclude that aPKC is essential for Lgl and Par6 apical localization, but is not required for Baz/Par3, Insc, Pins, or Dlg apical localization.

Next, we investigated the role of aPKC in basal protein localization in second instar larval neuroblasts. In the wild type, Miranda protein is localized to the basal cortex from prometaphase through telophase, ultimately being partitioned specifically into the basal GMC (Fig. 2). In aPKC mutants at late second instar (when the larvae become uncoordinated), Miranda is localized around the entire cortex from metaphase through telophase in all neuroblasts examined (Fig. 2); earlier in second instar (when larvae are moving normally), about half the neuroblasts still showed weak basal enrichment at telophase (Table I). We conclude that aPKC function is required to exclude Miranda from the apical cortex of the neuroblast, even during telophase when basal localization becomes Baz/Par3-independent (Schober et al., 1999; Peng et al., 2000; Albertson and Doe, 2003; Cai et al., 2003).

It is commonly accepted that the Baz/Par3–Par6–aPKC complex is required for orienting the neuroblast mitotic spindle orthogonal to the overlying ectodermal cell layer, thus reliably placing the GMCs toward the inside of the embryo (Doe and Bowerman, 2001; Jan and Jan, 2001; Ahinger, 2003). We could not assay the role of aPKC in embryonic neuroblast spindle orientation due to the large pool of maternal aPKC protein, but we did assay spindle orientation in aPKC mutant larval neuroblasts. We observed that
the metaphase spindle was always centered on the apical Baz/Par3 crescent in these mutant neuroblasts (Fig. 2, insets). Thus, aPKC is not required for aligning the mitotic spindle along the intrinsic axis of apical/basal polarity within larval neuroblasts, and it is likely that Par6 is also dispensable, as it is completely delocalized in aPKC mutants.

To determine the cell-autonomous aPKC phenotype, and to determine whether a more severe neuroblast phenotype could be observed when neuroblast life was not curtailed by larval death, we generated aPKC mutant clones. The MARCM system (Lee and Luo, 2001) was used to generate positively marked mutant clones in 0–12-h MARCM system (Lee and Luo, 2001) was used to generate by larval death, we generated could be observed when neuroblast life was not curtailed to determine whether a more severe neuroblast phenotype apoptosis mutant eye imaginal discs, which were identified based on the presence of the optic nerve (Fig. 4 C, brackets). Wild-type (aPKC/+) second larval instar eye disc epithelia formed a smoothly folded cellular monolayer, and showed the expected distribution of apical/basal cell polarity markers; aPKC and Discs lost were localized to the apical plasma membrane, E-cadherin (Ecad) was localized to the subapical adherens junctions, and Dlg was localized to the even more basal septate junction and basolateral membrane domain (Fig. 4 A; unpublished data).

In contrast, aPKC mutant second larval instar eye imaginal discs were not organized into an epithelial monolayer (Fig. 4, B–D). There was no detectable aPKC protein, and Ecad/ Dlg staining was less intense than in the wild type (Fig. 4 B), either because the proteins were more widely distributed or because there was less protein present. When the gain was increased to visualize the low levels of Ecad and Dlg proteins, we observed Ecad and Dlg proteins distributed around the cell cortex rather than confined to their normal apical/basal domain (Fig. 4, C and D; unpublished data). Thus, the aPKC mutant eye imaginal disc epithelia appear to have a loss of epithelial polarity characterized by unpolarized cortical distribution of an apical adherens junction marker (Ecad) and a basolateral marker (Dlg).

**aPKC is required for cell proliferation in neuroblasts and epithelia**

During the course of analyzing the aPKC mutant clones, we observed a dramatic decrease in the number of neuronal progeny in the positively marked aPKC mutant clones compared with positively marked wild-type control clones. We focused our analysis on the well-characterized mushroom body neuroblasts: each neuroblast first generates neurons at high gain to visualize the subcellular protein distribution and disc morphology. Dlg and Ecad are delocalized around the cell cortex, although occasional clusters of Ecad were observed. Eye discs (arrowhead) are from late second instar larvae and were identified based on the presence of the optic nerve (brackets) connecting to the brain (arrow). (C and D) aPKC mutant epithelia with extremely small disc size, defects in apical/basal polarity, and loss of epithelial monolayer morphology. Discs are imaged at high gain to visualize the subcellular protein distribution and disc morphology. Dlg and Ecad are delocalized around the cell cortex, although occasional clusters of Ecad were observed. Eye discs (arrowhead) are from late second instar larvae and were identified based on the presence of the optic nerve (brackets) connecting to the brain (arrow). (E and F) Clones of wild-type and aPKC mutant mushroom body neurons. aPKC mutant clones have fewer neurons (cell bodies, arrowheads) than wild-type clones. The calyx (dendritic projections) of each clone is marked by arrows.

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**Figure 4.** aPKC zygotic null mutants have defects in larval epithelial cell polarity. (A and B) aPKC mutant epithelia show decreased levels of apical/basal cell polarity markers. Wild-type (A) and aPKC/aPKC mutant (B) eye imaginal discs 48 h after larval hatching (second instar larvae). Apical/basal polarity is assayed using the apical marker aPKC (green), the apical adherens junctions marker E-cadherin (Ecad; blue), and the septate junction and basolateral membrane marker Dlg (red). Wild-type imaginal disc epithelia show well defined apical/basal polarity, normal disc morphology, and form a smooth monolayer; aPKC mutant imaginal discs show no detectable aPKC and reduced Dlg and Ecad levels. Discs in A and B were stained in parallel and imaged at the same confocal settings to allow protein levels to be compared. (C and D) aPKC mutant epithelia with extremely small disc size, defects in apical/basal polarity, and loss of epithelial monolayer morphology. Discs are imaged at high gain to visualize the subcellular protein distribution and disc morphology. Dlg and Ecad are delocalized around the cell cortex, although occasional clusters of Ecad were observed. Eye discs (arrowhead) are from late second instar larvae and were identified based on the presence of the optic nerve (brackets) connecting to the brain (arrow). (E and F) Clones of wild-type and aPKC mutant mushroom body neurons. aPKC mutant clones have fewer neurons (cell bodies, arrowheads) than wild-type clones. The calyx (dendritic projections) of each clone is marked by arrows.
that send axons into the γ lobe of the adult mushroom body, and subsequently switches to producing neurons that have axon projections into the α and β lobes (Lee et al., 2000). We found that wild-type clones contained 300–400 neurons (n = 2), consisting of early-born γ neurons as well as later-born α and β neurons (Fig. 4 E). In contrast, aPKC clones contained 75–125 neurons (n = 3), and consisted primarily of early-born γ neurons (Fig. 4 F). Because few α and β neurons were present in the mutant clones, the most likely explanation for reduced clone size is the cessation of neuroblast division just after the early-born γ neurons were produced. A similar defect in cell proliferation was observed in aPKC mutant eye imaginal discs: aPKC mutant discs were always much smaller than normal second instar eye discs (Fig. 4, C and D), although the animals remained as second instar larvae for at least twice as long as normal. Thus, aPKC is required to maintain cell proliferation of larval neuroblasts and imaginal disc epithelial cells.

**aPKC and lgl show dosage-sensitive negative genetic interactions**

aPKC binds and phosphorylates Lgl in both mammals and *Drosophila* (Betschinger et al., 2003; Yamanaka et al., 2003), so we investigated possible genetic interaction between aPKC and lgl in regulating cell polarity and cell proliferation. First, we examined whether reduced aPKC could suppress an *lgl* neuroblast cell polarity phenotype in stage 15 embryos. In wild-type neuroblasts, Miranda was localized to the basal cortex, but in *lgl* mutants (which retain low levels of maternal Lgl protein; unpublished data), neuroblasts were observed with Miranda delocalized from the cortex into the cytoplasm and onto the mitotic spindle (Fig. 5, A and B; Table II). In contrast, *lgl* aPKC double mutant embryos showed a clear suppression of the *lgl* polarity phenotype, with more Miranda basal crescents than *lgl* single mutants (Fig. 5 C; Table II).

To test whether reduced aPKC levels could suppress the *lgl* brain tumor phenotype, we examined brain size and neuroblast number (Fig. 5 F; Table II). Thus, reduced aPKC levels suppressed the *lgl* brain tumor phenotype. (D) Wild type, (E) *lgl*/*lgl* aPKC double mutants, there is noticeably more basal Miranda localization than in *lgl*/*lgl* single mutants, although some cytoplasmic/spindle association remains. See Table II for quantitation. (D–F) Reduced aPKC levels suppress the *lgl* brain tumor phenotype. (D) Wild type, (E) *lgl*/*lgl* aPKC double mutants, and (F) *lgl*/*lgl* aPKC. Third instar larval brains 74 h after hatching were stained for the Scrib membrane marker (green) to identify neuroblasts (large cells, arrowheads) and GMCs (smaller cells), and with Elav (red) to mark neurons. In each panel, the optical cross section was taken at the same level, just apical to the mushroom body. See Table II for quantitation. Bar, 25 μm.

Table II. Reduced aPKC suppresses Lgl cell polarity and cell overgrowth phenotypes

| Genotypes* | WT | *lgl*/*lgl* | *lgl*/*lgl* aPKC or *lgl* aPKC/

| NB basal Miranda (E10)* | 94% | 33% | 72%
| NB basal Miranda (E15)* | 94% | 7% | 24%
| Large NB size (E15)* | 100% | 88% | 83%
| NB number (L3)* | 92 ± 6 | 137 ± 10 | 107 ± 4
| Brain size (L3)* | 82 ± 8 | 136 ± 12 | 114 ± 11
| Imaginal disc size (L3) | Normal | Enlarged | Normal
| Imaginal disc monolayer (L2/3) | Yes | Never | Yes

*The wild-type (WT) stock were *w* (rows 1–3), *lgl*/*lgl* aPKC*+/+* (rows 4 and 7), and *lgl*/*lgl* (rows 5 and 6). The *lgl*/*lgl* mutants were *lgl*/*lgl* (rows 1–3), *lgl*/*lgl* (row 5), and *lgl*/*lgl* (rows 4, 6, and 7). The *lgl* aPKC double mutants were *lgl*/*lgl* aPKC*+/+* (rows 1–3), *lgl*/*lgl* aPKC*+/+* (rows 5 and 6, and 7).

*Number of neuroblasts (NB) scored is >41 for all genotypes.

*NB/GMC cell size ratio of ≥0.8 at telophase (Albertson and Doe, 2003).

*Neuroblast number per hemisphere; n = 10 for each genotype.

*Brain size in volume (mm^3 × 10^-3) per hemisphere; n = 10 for each genotype.
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instar leg and wing imaginal discs have a well-defined morphology (Fig. 6 A) with a smooth monolayer of columnar cells that have a clear apical/basal polarity (Fig. 6, D and D'). In contrast, lgl+/lgl homozygous third instar imaginal discs are highly disorganized and often fused with each other or nearby tissues (Fig. 6 B); at the cellular level, mutant epithelial cells are round instead of columnar, with an expanded apical membrane domain (Fig. 6, E and E'). Strikingly, lgl+/lgl mutants that are heterozygous for an aPKC mutation have a nearly normal late third instar leg and wing imaginal disc morphology (Fig. 6 C), including columnar cell shape and an approximately normal apical membrane domain (Fig. 6, F and F'); Table II). In addition, we characterized an earlier stage of imaginal disc development (early third instar; 74 h after larval hatching at 25°C). We find that lgl mutants already show morphological disorganization of imaginal discs (Fig. 6 H), but both wild-type and lgl aPKC/lgl+ mutants show essentially normal leg and wing imaginal disc morphology (Fig. 6, G and I; unpublished data). We conclude that reduced aPKC levels can suppress the lgl epithelial cell polarity phenotype.

Are all lgl phenotypes suppressed by reduced aPKC levels? Recently, we have shown that ~12% of the neuroblasts in lgl homozygotes have a reduced apical cortical domain and apical spindle poles, leading to an inverted asymmetric division and a smaller neuroblast size (Albertson and Doe, 2003). This lgl phenotype was not suppressed by reducing aPKC levels, and in fact may have been slightly enhanced (Table II, row 3). The inability of aPKC mutation to suppress the lgl "small neuroblast" or "inverted division" phenotype is consistent with a recent observation that aPKC positively regulates neuroblast cell size (Cai et al., 2003). Thus, aPKC and Lgl appear to cooperate to positively regulate neuroblast cell size, despite their negative interaction in regulating other aspects of cell polarity.

Discussion

It was previously reported that aPKCmutant embryos died before gastrulation and that all epithelial and neuroblast polarity was lost (Wodarz et al., 2000). We find a much later onset phenotype: survival of aPKC homozygotes to second larval instar with no significant embryonic phenotype. Although surprising, we feel our results reflect the authenticzygotic aPKC mutant phenotype for several reasons. First, recent work from the Wodarz lab confirms that aPKC homozygotes survive to at least late embryonic stages without significant epithelial or neuroblast defects (Wodarz, A., personal communication), presumably due to the absence of deleterious second site mutations that were on the original aPKC chromosome. Second, we used PCR to verify the presence of the aPKC allele in all of our stocks, and used dominantly marked balancer chromosomes to independently confirm the identity of every aPKC homozygote that we analyzed. Third, we showed that aPKC homozygous second instar larvae had very low levels of aPKC mRNA and no detectable full-length protein; thus, we analyzed a very strong or null aPKC mutant phenotype. Fourth, we showed that aPKC and aPKC/Df(2R)P1 larvae have indistinguishable lethal phases and quantitative neuroblast phenotypes; thus, aPKC behaves as a genetic null allele. Finally, we showed that aPKC homozygous embryos had persistent maternal aPKC protein, explaining the lack of an embryonic phenotype in these mutants.

One of our more unexpected findings is that aPKC mutant neuroblasts show normal Baz/Par3 apical localization. The Baz/Par3aPKC complex has been suggested to form a functional unit that is interdependent for localization in C. elegans, mammals, and Drosophila (Doe and Bowerman, 2001; Ohno, 2001). We find that Baz/Par3 shows normal apical localization in aPKC mutant neuroblasts,
showing that normal Baz/Par3 localization can occur without being part of the Par3–Par6–aPKC complex. In addition, we show that neuroblasts lacking apical aPKC and Par6 still form a molecularly defined apical cortical domain containing Baz/Par3, Insc, Pins, and Dlg. Our results allow us to propose a hierarchy for apical protein localization in neuroblasts: Baz/Par3–Insc–Pins–Dlg→aPKC→Par6–Lgl (Schober et al., 1999; Wodarz et al., 1999; Albertson and Doe, 2003). Our hierarchy is consistent with recent biochemical analyses in which a protein complex was isolated containing Par6–aPKC–Lgl, but not Baz/Par3 (Betschinger et al., 2003; Yamanaka et al., 2003). We suggest that aPKC may be required to anchor the Par6–aPKC–Lgl complex at the apical cortex of the neuroblast.

Both aPKC and Lgl are required for Miranda basal localization in neuroblasts, and all available data support a model in which Lgl is required for targeting Miranda to the neuroblast cortex, whereas aPKC blocks Lgl function on the apical side of the neuroblast. First, lgl mutants have little or no Miranda at the cortex (Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003). Second, aPKC mutants show uniform cortical Miranda localization (this paper). Third, a weak lgl phenotype can be suppressed by reducing aPKC levels, showing that aPKC activity antagonizes Lgl activity (this paper). Fourth, aPKC and Lgl physically interact (Betschinger et al., 2003). Finally, an overexpressed nonphosphorylatable Lgl protein is uniformly cortical and able to induce uniform cortical Miranda localization, whereas phospho-Lgl is preferentially released from the cell cortex (Kalmes et al., 1996; Betschinger et al., 2003). This has led to a model in which Lgl acts as an anchor for Miranda at the basal cortex, but is absent from the apical cortex due to aPKC-mediated phosphorylation (Betschinger et al., 2003). Although this simple model is attractive, we note that Lgl has never been observed colocalized with Miranda in a basal cortical crescent (Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003; Betschinger et al., 2003), and a role for cytoplasmic Lgl in Miranda localization has not been definitively ruled out.

The Baz/Par3–Par6–aPKC complex has a well-characterized role in regulating neuroblast spindle orientation (Doer and Bowerman, 2001; Jan and Jan, 2001; Knoblich, 2001; Ahhringer, 2003). Spindle orientation can be measured relative to extrinsic landmarks around the neuroblast (e.g., perpendicular to the overlying ectoderm) or relative to intrinsic cues within each neuroblast (e.g., perpendicular to the Baz/Par3–Par6–aPKC apical crescent). Mutations in baz or par6 genes randomize embryonic neuroblast spindle orientation relative to the overlying ectoderm (Kuchinke et al., 1998; Wodarz et al., 1999; Petronczki and Knoblich, 2001), but it is not clear whether these phenotypes are due to disruption of the ectodermal layer or to a cell-autonomous defect in the neuroblast. We cannot assay the function of aPKC in embryonic neuroblast spindle orientation due to high levels of maternal aPKC protein present in aPKC zygotic mutant embryos. However, we do show that aPKC is not required for intrinsic spindle orientation in larval neuroblasts; the mitotic spindle is always perpendicular to the Baz/Par3–Insc–Pins apical crescent.

The Baz/Par3 and Par6 proteins are required to establish epithelial polarity in Drosophila (Muller and Wieschaus, 1996; Schober et al., 1999; Wodarz et al., 1999; Petronczki and Knoblich, 2001), and we show here that aPKC is also required for normal apical/basal epithelial cell polarity. The similar phenotype in baz, par6, and aPKC mutants may indicate that these proteins function together as a complex to provide a single function in epithelia, despite the evidence that they have independent functions in neuroblasts. One primary function may be the inhibition of Lgl activity because we find that the lgl epithelial polarity defects can be strongly suppressed by reducing aPKC levels. Lgl–Dlg–Scrib activity can also antagonize Baz/Par3–Par6–aPKC activity (Bilder et al., 2003), and it is tempting to speculate that aPKC inactivates Lgl by phosphorylation, whereas Lgl can inactivate aPKC by sequestering it into an Lgl–Par6–aPKC complex and out of the Baz/Par3–Par6–aPKC complex (Betschinger et al., 2003; Yamanaka et al., 2003).

The role of aPKC in cell proliferation has not been previously investigated in Drosophila. There are three lines of evidence showing that aPKC promotes cell proliferation in neuroblasts and epithelia. We find that the number of cells in aPKC mutant mushroom body neuroblast clones is significantly lower than the number in wild-type clones. There appears to be a normal number of early-born γ neurons in these clones, followed by only a few later-born α and β neurons. The normal number of early-born γ neurons suggests that loss of aPKC does not lead to cell death in this population; moreover, we see no decrease in the number of neuroblasts per brain lobe in aPKC mutant larvae (unpublished data). We conclude that cell death is not contributing to the reduction in neurons observed in the clones, but rather, that the neuroblast stops dividing near the time the neuroblast switches over to generating α and β neurons. The neuroblast may become arrested at some point in the cell cycle, or it may undergo a terminal division to generate a pair of GMCs (perhaps due to both daughter cells inheriting Miranda and Prospero GMC determinants). A second indication that aPKC promotes cell proliferation is that we observe far fewer epithelial cells in aPKC mutant eye imaginal discs compared with the wild type, even with an additional day of growth as second instar larvae. Finally, we observe that a 50% reduction in aPKC levels (aPKC+/−) can strongly suppress the epithelial and brain overproliferation phenotypes of lgl mutants. Together, our data show that aPKC positively regulates cell proliferation in epithelia and neuroblasts. Interestingly, reduction in the function of the mammalian atypical PKCγ (using overexpression of a dominant-negative kinase) can suppress Rac1/cdc42-induced overproliferation (Qiu et al., 2000). Thus, aPKC may have an evolutionarily conserved role in promoting cell proliferation, as well as in the establishment of cell polarity.

Although aPKC and Lgl act antagonistically to regulate many aspects of epithelial and neuroblast cell polarity and cell proliferation, they may share a common positive function in regulating neuroblast apical cell size. Previously, we showed that lgl zygotic mutants have some embryonic telophase neuroblasts with an abnormally small apical cortical domain, apical spindle pole, and neuroblast size (Albertson and Doe, 2003). These defects are not suppressed by reduc-
ing aPKC levels, and in fact may be enhanced (Table II). Thus, we propose that Lgl and aPKC both act positively to promote large apical cell and spindle pole size. It has been reported that Baz/Par3–Par6–aPKC and PIns–GoII act in parallel pathways to promote large apical cell size and apical spindle size (Cai et al., 2003); Lgl could be acting as part of the PIns–GoII pathway, or as a third input promoting apical cell and spindle size.

Materials and methods
Fly genetics and morphological analysis
The aPKC(G304A), lgl4, lgl2, and Df(2R)P1 stocks were obtained from the Bloomington Stock Center (Bloomington, IN), and rebalanced over CyO actin::GFP. All alleles are described in Flybase (http://flybase.bio.indiana.edu/82/).

Analytic PCR
Genomic DNA was isolated as described by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/about/methods/inverse.pcr.html). PCR primers were designed to detect the presence of the Pplac transposon in the third intron of aPKC as it had been described previously (Wodarz et al., 2000). One primer was complementary to the 3' end of the transposon (5'-CACTCGCATTTACCAGC-3'), and the other was complementary to the third exon of aPKC (5'-TGCCCATCGCAAGACATCGCCG-3'). PCR was performed using the Expand system (Roche).

Northern blot
RNA was isolated from second larval instar brains 48 h after hatching. Brains were dissected in Schneider’s medium and immediately transferred to TRizol® reagent (Invitrogen); RNA was isolated according to the manufacturer’s directions. RNA was separated on a formaldehyde gel and transferred to zetabind membrane (Cuno, Inc.). Random-primed probe was prepared using the entire aPKC coding region, and the SV40 poly(A)+ region from pUAST, as a template.

Antibody production, staining, and imaging
The Par6 antibody was generated in rats against aa 130-255. The protein fragment was isolated with a His tag that was subsequently removed. The Lgl antibody was generated against an Lgl COOH-terminal 21-aa peptide (Albertson and Doe, 2003); using our fixation protocol, this antisera detects only apical cortical Lgl protein, and not the uniform cortical or cytoplasmic protein reported for other Lgl antisera (Oshiro et al., 2000; Peng et al., 2000). Lge was reared on standard food. Brains and imaginal discs were dissected in Schneider’s Medium (Sigma-Aldrich), fixed for 20 min in 4% formaldehyde, and stained as described previously (Cox et al., 1996). Em-}

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