Direct association of Bazooka/PAR-3 with the lipid phosphatase PTEN reveals a link between the PAR/aPKC complex and phosphoinositide signaling

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Summary

Cell polarity in *Drosophila* epithelia, oocytes and neuroblasts is controlled by the evolutionarily conserved PAR/aPKC complex, which consists of the serine-threonine protein kinase aPKC and the PDZ-domain proteins Bazooka (Baz) and PAR-6. The PAR/aPKC complex is required for the separation of apical and basolateral plasma membrane domains, for the asymmetric localization of cell fate determinants and for the proper orientation of the mitotic spindle. How the complex exerts these different functions is not known. We show that the lipid phosphatase PTEN directly binds to Baz in vitro and in vivo, and colocalizes with Baz in the apical cortex of epithelia and neuroblasts. PTEN is an important regulator of phosphoinositide turnover that antagonizes the activity of PI3-kinase. We show that *Pten* mutant ovaries and embryos lacking maternal and zygotic *Pten* function display phenotypes consistent with a function for PTEN in the organization of the actin cytoskeleton. In freshly laid eggs, the germ plasm determinants *oskar* mRNA and Vasa are not localized properly to the posterior cytocortex and pole cells do not form. In addition, the actin-dependent posterior movement of nuclei during early cleavage divisions does not occur and the synchrony of nuclear divisions at syncytial blastoderm stages is lost. *Pten* mutant embryos also show severe defects during cellularization. Our data provide evidence for a link between the PAR/aPKC complex, the actin cytoskeleton and PI3-kinase signaling mediated by PTEN.

Key words: Cell polarity, PAR proteins, PTEN, Actin cytoskeleton, *Drosophila*

Introduction

The establishment and maintenance of cell polarity in many different cell types of animals relies on the function of the evolutionarily conserved PAR/aPKC complex (Ohno, 2001; Wodarz, 2002). The PAR/aPKC complex consists of an atypical isoform of protein kinase C (aPKC), the two PDZ (Postsynaptic density 95; Discs Large; Zonula occludens 1) domain containing proteins PAR-3/Baz and PAR-6, and the small GTPase Cdc42 (Ohno, 2001; Wodarz, 2002). In mammalian epithelial cells, the PAR/aPKC complex is required for the formation of tight junctions, which separate the apical from the basolateral plasma membrane domains (Suzuki et al., 2001; Hirose et al., 2002). In mutants for components of the PAR/aPKC complex in *Drosophila*, apicobasal polarity of epithelia is lost, concomitant with the failure to establish a zonula adherens (ZA) (Müller and Wieschaus, 1996; Wodarz et al., 2000; Petronczki and Knoblich, 2001; Bilder et al., 2003; Rolls et al., 2003; Tanentzapf and Tepass, 2003; Hutterer et al., 2004). The PAR/aPKC complex also controls the polarity of cells that lack intercellular junctions, e.g. the zygote of the nematode *C. elegans*, the *Drosophila* oocyte and *Drosophila* neuroblasts (Kemphues et al., 1988; Schober et al., 1999; Wodarz et al., 1999; Huynh et al., 2001) (reviewed by Kemphues, 2000; Doe and Bowerman, 2001; Pellettieri and Seydoux, 2002; Wodarz, 2002; Wodarz and Huttner, 2003).

The architecture of these different cell types is very diverse, raising the question of how the PAR/aPKC complex communicates with downstream effector molecules that specifically control different aspects of cell polarity, including cell shape, the type and position of intercellular junctions and the asymmetric localization of cortical and transmembrane proteins. One attractive possibility would be that the PAR/aPKC complex affects the organization of the submembrane cytoskeleton, which could have an impact on every aspect of polarity mentioned above. Indeed, it has been shown that, depending on the cell type, both actin and microtubules are involved in the control of cell polarity (Broadus and Doe, 1997; Clark et al., 1997; Wallenfang and Seydoux, 2000). However, except for the fact that the small GTPase Cdc42 is a potent regulator of the actin cytoskeleton, no direct link between components of the PAR/aPKC complex and cytoskeletal regulators has been uncovered so far.

In order to find molecules that bind to Baz/PAR-3 and that

may provide a link between the PAR/aPKC complex and the cortical cytoskeleton, we have performed a yeast two-hybrid screen using the three PDZ domains of Baz as bait. We isolated three independent clones of the lipid phosphatase PTEN that specifically bound to Baz. PTEN catalyzes the dephosphorylation of phosphoinositide lipids at the D3 position of the inositol ring (Leslie and Downes, 2002). One substrate of particular importance is the lipid phosphatidylinositol (3,4,5) trisphosphate [PtdIns(3,4,5)P_3], which is converted by the activity of PTEN to phosphatidylinositol (4,5) bisphosphate [PtdIns(4,5)P_2]. PtdIns(3,4,5)P_3 is produced by activation of phosphatidylinositol 3-kinase (PI3-kinase) in response to stimulation by a multitude of growth factors and cytokines. Interestingly, PtdIns(3,4,5)P_3 locally activates Cdc42 by recruitment of guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP for GTP specifically on Cdc42 (Zheng, 2001). Moreover, in mammalian cells PtdIns(3,4,5)P_3 recruits phosphoinositide dependent kinase 1 (PDK1; Pk61C – FlyBase) which activates aPKC by direct phosphorylation of a conserved threonine residue in the activation loop of the kinase (Le Good et al., 1998). Thus, PtdIns(3,4,5)P_3 is likely to activate two components of the PAR/aPKC complex, Cdc42 and aPKC. Because PTEN is predicted to antagonize the activation of both Cdc42 (Liliental et al., 2000) and aPKC by lowering the level of PtdIns(3,4,5)P_3 in the plasma membrane, the association of PTEN with Baz may have a significant impact on the activity of these two key components of the PAR/aPKC complex.

Recently, PI3-kinase signaling and PTEN have been implicated in the polarization of *Dictyostelium* amoebae in response to a source of chemoattractant (Funamoto et al., 2002; Iijima and Devreotes, 2002). PI3-kinase and PTEN are localized to the leading edge and uropod, respectively, in a very dynamic fashion. PI3-kinase signaling also appears to be required for directed migration of leukocytes (Servant et al., 2000; Wang et al., 2002). In both cases, PI3-kinase and PTEN are thought to participate in a self-sustaining loop that intracellularly amplifies the shallow concentration gradient of the chemoattractant. PI3-kinase and PTEN also affect the polarization of hippocampal neurons in culture and, more specifically, the localization of PAR-3 and aPKC to the tip of the neurite that is going to become the axon (Shi et al., 2003; Jiang et al., 2005). Thus, there is increasing evidence that PTEN and the PAR/aPKC complex may cooperate in the control of cell polarity.

In *Drosophila*, the function of PTEN has mainly been studied with respect to its role in the regulation of growth and proliferation in larval and adult tissues (Stocker and Hafen, 2000). *Pten* mutant cells have elevated PtdIns(3,4,5)P_3 levels and are larger than wild-type cells owing to increased growth (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000; Stocker et al., 2002). Clones of *Pten* mutant cells in imaginal discs also show subtle defects in the organization the actin cytoskeleton (Goberdhan et al., 1999). *Pten* interacts genetically with components of the insulin signaling pathway including the insulin receptor, the insulin receptor substrate IRS-1/Chico, PI3-kinase and protein kinase B (PKB) (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000; Oldham et al., 2002; Stocker et al., 2002). These findings provided solid evidence for an antagonistic relationship between PTEN and PI3-kinase and showed that the regulation of phosphoinositide levels is the main vital function of PTEN.

Here, we show that PTEN directly binds to Baz/PAR-3 and colocalizes with Baz in the apical cortex of epithelia and neuroblasts. *Pten* mutant embryos lacking maternal and zygotic *Pten* function show defects during early embryonic development that point to a function for *Pten* in the organization of the actin cytoskeleton. Removal of *Pten* function from the germline in ovaries causes abnormal actin organization in nurse cells and in the oocyte. We propose that recruitment of PTEN by Baz may contribute to the polarization of the actin cytoskeleton, most likely by creating local differences in the balance between PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 in the plasma membrane. Moreover, PTEN may affect the activity of two key components of the PAR/aPKC complex, aPKC and Cdc42. The binding of PTEN to Baz provides the first molecular link between the PAR/aPKC complex, the actin cytoskeleton and phosphoinositide signaling.

Materials and methods

Yeast two hybrid screen

A fragment of the Baz cDNA corresponding to amino acids 291-737 that contains all three PDZ domains was cloned into the GAL4 DNA-binding domain vector pGBT9 (Clontech).This bait was used to screen 10 million colonies of an 0-24 h embryonic cDNA library in the GAL4 transcriptional activation domain vector pACT2 (Clontech). Interacting clones were selected for activity of the HIS3 and *lacZ* reporter genes.

Fly stocks, antibodies and immunohistochemistry

We used the amorphic allele $Pten^{DJ189}$ (Gao et al., 2000) and the phosphatase dead allele $Pten^{c494}$ (Huang et al., 1999). Germ-line clones of both alleles were produced in females of the genotype *y w P*[*ry*$^+$ *FLP*]^{12}; *Pten P*[*hs neo ry*$^+$ *FRT*]$^{2L\text{-}40A}$/*P*[*mini w*$^+$ *ovo*^{D1}]$^{2L-13X13}$ *P*[*hs neo ry*$^+$ *FRT*]$^{2L\text{-}40A}$. To mark germ-line clones in ovaries by absence of GFP expression, *P*[w^{+mC} Ubi-GFP(S65T)nls}2L *P*[*hs neo ry*$^+$ *FRT*]$^{2L\text{-}40A}$ was used. Expression of PTEN in embryos with the UAS GAL4 system (Brand and Perrimon, 1993) was done using UAS PTEN2 (Huang et al., 1999) and UAS PTEN3 (Goberdhan et al., 1999). UAS PLCδ-PH-GFP flies were a gift from L. Cooley. P{w^{+mC}=matalpha4-GAL-VP16}V67 and P{w^{+mC}=Act5C-GAL4}17bF01 were used as drivers. Oregon R was used as wild type.

To generate specific antibodies against PTEN, rabbits and rats were immunized with a GST fusion protein containing amino acids 316-511 of PTEN2. The rabbit antibody was affinity purified against the immobilized GST fusion protein. Additional antibodies used were rabbit and rat anti-Baz (Wodarz et al., 1999; Wodarz et al., 2000), mouse anti-Neurotactin BP106 (Developmental Studies Hybridoma Bank), rat anti-Vasa (Tomancak et al., 1998), rabbit anti-Staufen (St Johnston et al., 1991), goat anti-PKC zeta C20 (Santa Cruz) and mouse anti-alpha tubulin (SIGMA). Actin was visualized with AlexaFluor 568-phalloidin (Molecular Probes) and DNA was stained with YOYO-1 (Molecular Probes). For whole-mount immunohistochemistry of embryos with PTEN antibodies, embryos were fixed according to the heat-methanol procedure described by Müller and Wieschaus (Müller and Wieschaus, 1996). For actin and tubulin staining, embryos were fixed in 37% formaldehyde/heptane. Secondary antibodies conjugated to Cy2, Cy3 or Cy5 were obtained from Jackson Laboratories. Images were taken on a Leica TCSNT confocal microscope or on a Zeiss Axioplan 2 fluorescence microscope and processed using Photoshop (Adobe) and Canvas (Deneba) software.

Western blots and immunoprecipitation

Western blotting was done according to standard procedures. Rat anti-PTEN was used at 1:500, rat anti-Baz (Wodarz et al., 1999) and goat anti-PKC zeta C20 (Santa Cruz) were used at 1:1000. For immunoprecipitation, 8 μl of affinity purified rabbit anti PTEN or 2 μl of rabbit anti-Baz serum were added to embryonic or S2 cell extracts containing 2 mg of total protein in TNT (1% Triton X-100, 150 mM NaCl, 50 mM Tris-Cl pH 7,5) supplemented with protease inhibitors. Immune complexes were harvested using protein A-conjugated agarose (Roche), washed four times in TNT and boiled in 1×SDS sample buffer before SDS-PAGE and western blot.

Results

Bazooka binds to PTEN

In order to identify binding partners of Baz that may participate in the control of cell polarity and asymmetric division of neuroblasts, we performed a yeast two-hybrid screen using the central region of Baz containing the three PDZ domains as bait. We isolated three independent clones that encode the C-terminal region of the lipid phosphatase PTEN (Fig. 1A). All three clones contain a canonical PDZ-binding motif at the very C terminus (STYL) (Songyang et al., 1997) and correspond to splice form 2 of PTEN (PTEN2) (Smith et al., 1999). The PDZ-binding motif is essential for interaction with the PDZ domains of Baz. Neither a deletion mutant of PTEN lacking the 3 C-terminal amino acids (PTEN2 ΔTYL) nor PTEN isoform 3 (PTEN3), which lacks the PDZ binding motif at the C-terminus, bound to Baz in the yeast two-hybrid assay (Fig. 1A). To determine which region of Baz is sufficient for binding to PTEN2, we performed two-hybrid assays with a series of smaller bait fragments encoding individual PDZ domains or pairs of PDZ domains, as well as the N- and C-terminus of Baz (Fig. 1B). PTEN2 bound to the bait protein containing all three PDZ domains and to a smaller bait protein consisting only of PDZ domains 2 and 3 (Fig. 1B). Bait proteins containing single PDZ domains or PDZ domains 1 and 2 together did not bind to PTEN2, nor did the bait proteins comprising the N- or C-terminal regions of Baz (Fig. 1B).

To test whether the interaction between Baz and PTEN that we observed in the yeast two-hybrid system does also occur in vivo in *Drosophila* embryos and cultured cells, we raised polyclonal antibodies against the *Drosophila* PTEN protein. In extracts of untransfected *Drosophila* S2 cells our antibodies recognized a weak band of ~65 kDa, which is slightly larger than the predicted molecular mass of 59 kDa (Fig. 2A). In addition, the antibody recognizes a larger band of ~85 kDa, which is probably a cross-reacting protein unrelated to PTEN (Fig. 2A, asterisk). The 65 kDa band was strongly increased in S2 cells transfected with PTEN2 alone or with PTEN2 and Baz together (Fig. 2A). PTEN2 produced by in vitro translation in a reticulocyte lysate system was also recognized by our antibody and migrated at the same size as the band that was increased in transfected S2 cells, demonstrating that this band corresponds to PTEN (Fig. 2A).

The PTEN antibody recognized the same two bands in extracts of wild-type embryos (Fig. 2B). In co-immunoprecipitation (co-IP) experiments using an antibody directed against Baz for IP, only the 65 kDa band corresponding to PTEN specifically co-precipitated with Baz (Fig. 2B), demonstrating the association of endogenous PTEN and Baz in vivo.

To test whether the association of PTEN with Baz occurred only with the PTEN2 isoform, as predicted from the yeast two-hybrid assays, we performed co-immunoprecipitation experiments with extracts of S2 cells co-transfected either with PTEN2 or PTEN3 and Baz together. In extracts of untransfected S2 cells, neither the preimmune nor the anti-PTEN immune serum precipitated detectable amounts of Baz (Fig. 2C). This finding can be explained with the very low level of endogenous Baz expression in S2 cells (Fig. 2C, top panel). In S2 cells co-transfected with PTEN2 and Baz together, Baz was co-immunoprecipitated with the PTEN antibody, but not with the preimmune serum, demonstrating the association of Baz and PTEN2 in a protein complex (Fig. 2C). By contrast, Baz did not co-immunoprecipitate with PTEN3 in co-

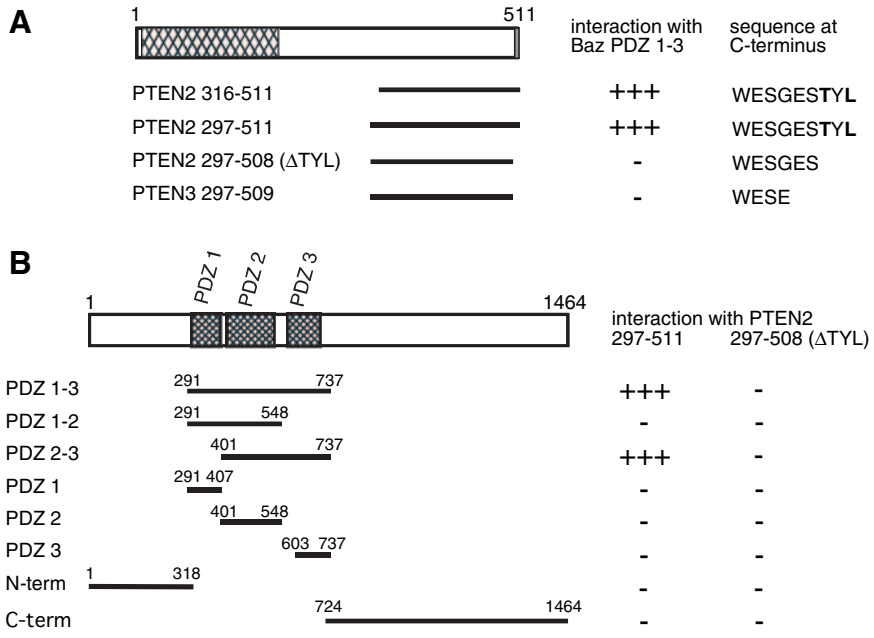

Fig. 1. PDZ domains 2 and 3 of Baz bind directly to the C-terminal PDZ-binding motif of PTEN2 in the yeast 2-hybrid system. (A) In the yeast 2-hybrid screen using the three PDZ domains of Baz as bait, we isolated three independent clones corresponding to the C terminus of PTEN2. One clone contains amino acids 316-511 and two clones contain amino acids 297-511 of PTEN2. A deletion mutant lacking the three C-terminal amino acid residues of PTEN2 did not bind to the PDZ domains of Baz, nor did PTEN isoform 3, which differs only at the C terminus from PTEN2 and lacks the PDZ-binding motif. (B) Different regions of Baz were tested for interaction with the C terminus of PTEN2 and with the C-terminal deletion mutant of PTEN2 lacking the PDZ-binding motif. The smallest fragment that interacted with PTEN2, but not with the mutant lacking the PDZ-binding motif, contained PDZ domains 2 and 3.

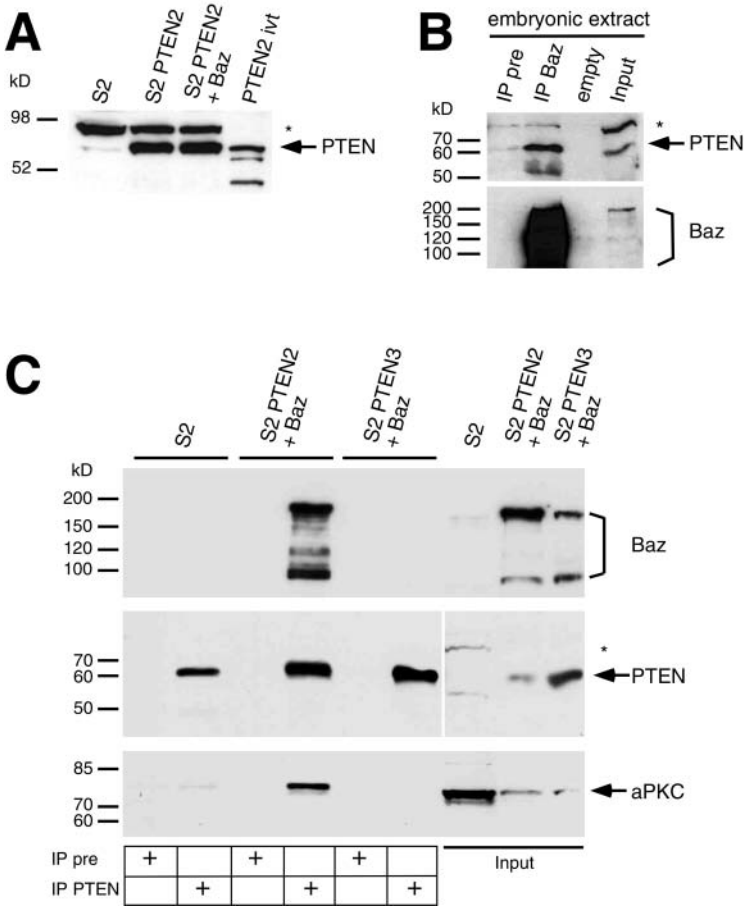

Fig. 2. PTEN2, Baz and aPKC are associated in a protein complex. (A) Extracts of untransfected S2 cells and S2 cells transfected with PTEN2 alone or with PTEN2 and Baz together were subjected to western blot analysis with an antibody directed against the C terminus of PTEN2. The antibody recognizes a 65 kDa protein that is strongly increased in S2 cells transfected with PTEN2. A band of the same size is detected when PTEN2 is translated in vitro in a reticulocyte lysate system (PTEN2 ivt). An additional band of 85 kDa (asterisk) is probably a crossreacting protein unrelated to PTEN. (B) Extracts from wild-type embryos were subjected to immunoprecipitation with an antibody directed against Baz or with the corresponding preimmune serum. Immunoprecipitates were probed on western blots with antibodies against PTEN (top panel) and Baz (bottom panel). (C) Extracts of untransfected S2 cells and S2 cells co-transfected with PTEN2 or PTEN3 together with Baz were subjected to immunoprecipitation with either PTEN antibody or with the corresponding pre-immune serum. Immunoprecipitates were analyzed by western blot with Baz antibody (top panel), PTEN antibody (middle panel) and aPKC antibody (bottom panel). Specific co-immunoprecipitation of Baz was detected only with PTEN2 but not with PTEN3. Endogenous aPKC co-immunoprecipitates with PTEN2 but not with PTEN3 in the presence of Baz. Western blots of the cell extracts used for immunoprecipitation are shown on the right (Input).

transfected cells (Fig. 2C), confirming that Baz binds specifically to the PTEN2 isoform, which contains the PDZ-binding motif at its C terminus. To test whether an additional component of the PAR/aPKC complex is present in the complex of PTEN2 with Baz, we probed the immunoprecipitates for the presence of aPKC. aPKC co-immunoprecipitated with PTEN2 and Baz, but not with PTEN3 and Baz (Fig. 2C, bottom panel), indicating that Baz may function as a scaffold that links PTEN2 with aPKC.

In conclusion, we have shown that PTEN2 binds to the region containing PDZ domains 2 and 3 of Baz and that this interaction depends on the presence of the PDZ-binding motif at the very C terminus of PTEN2. Consistent with these in vitro binding data, PTEN2 and Baz form a complex in wild-type embryos and transfected S2 cells that also contains aPKC.

Bazooka colocalizes with PTEN in epithelia and neuroblasts

We next analyzed the subcellular localization of PTEN in embryonic epithelia and neuroblasts. PTEN mRNA is ubiquitously expressed both maternally and zygotically, as determined by RNA in situ hybridization (data not shown). We could not detect endogenous PTEN protein with our antibody, presumably because of low expression levels or insufficient sensitivity of the antibody. However, when we expressed PTEN with the UAS-GAL4 system (Brand and Perrimon, 1993), we could readily detect the protein in embryonic tissues (Fig. 3). In embryos at the extended germ band stage (stage 10), PTEN2 was strongly enriched in the apical cortex of the neuroectodermal epithelium and in the apical cortex of neuroblasts (Fig. 3A). In both cell types, PTEN2 colocalized with endogenous Baz (Fig. 3A). At later stages of embryogenesis, the apical enrichment and colocalization of PTEN2 with Baz was even more pronounced (Fig. 3B). Intriguingly, apical enrichment of PTEN2 depends on the PDZ-binding motif, as the PTEN3 protein, which lacks the PDZ binding motif but is otherwise identical to PTEN2 (Smith et al., 1999), was present on the whole plasma membrane and in the cytoplasm (Fig. 3C). In *baz* mutant embryos lacking maternal and zygotic Baz, PTEN2 showed diffuse cytoplasmic localization and was not enriched in the apical cortex (data not shown). However, we cannot conclude with certainty that the mislocalization of PTEN2 is a primary consequence of the absence of Baz function, because *baz* mutant embryos are already undergoing massive degeneration at the time when PTEN2 expression driven with the GAL4 system was strong enough to be detected by immunohistochemical staining (Müller and Wieschaus, 1996; Schober et al., 1999; Wodarz et al., 1999; Wodarz et al., 2000). Together, we have shown that PTEN2 precisely colocalizes with Baz in epithelia and neuroblasts. Colocalization depends on the presence of the PDZ-binding motif of PTEN2, consistent with recruitment of PTEN2 by PDZ domains 2 and 3 of Baz.

PtdIns(4,5)P_2 enrichment coincides with Bazooka and PTEN localization

What could be the consequences of apical localization of PTEN? PTEN dephosphorylates phosphoinositides at the D3 position. Of special interest is the dephosphorylation of PtdIns(3,4,5)P_3, which leads to generation of PtdIns(4,5)P_2. Both phosphoinositides are important signaling molecules and are implicated in the control of the actin cytoskeleton (Czech, 2000; Caroni, 2001; Yin and Janmey, 2003). To visualize the

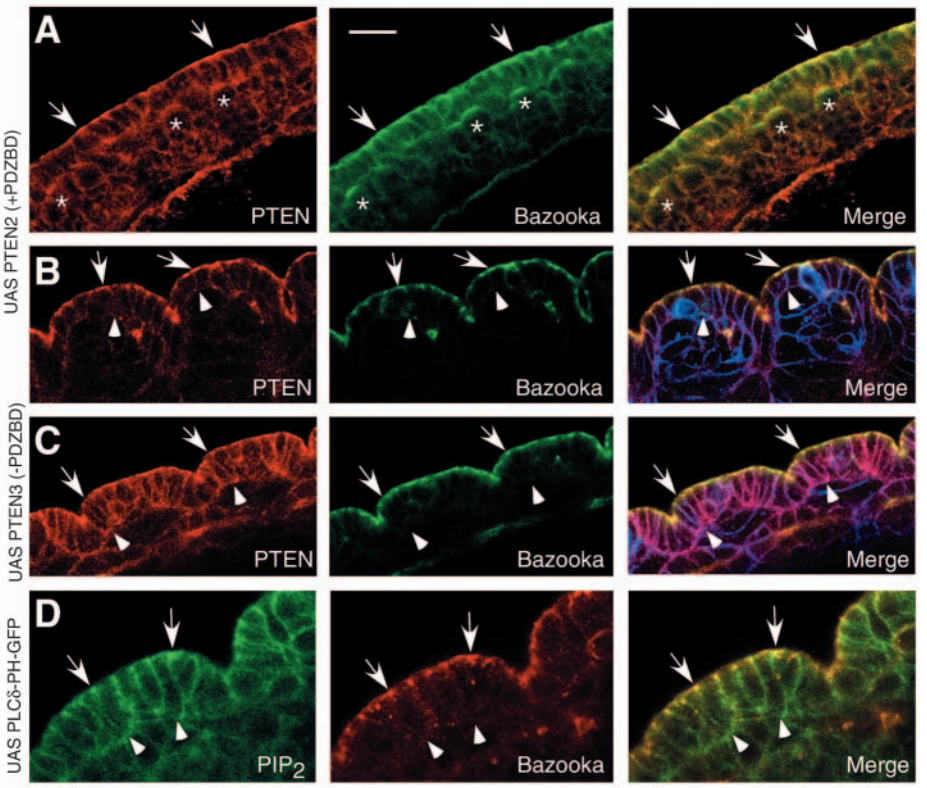

Fig. 3. Colocalization of PTEN2 and Baz in embryonic epithelia and neuroblasts is dependent on the PDZ-binding motif of PTEN2. (A) An embryo expressing PTEN2 ubiquitously under control of a maternal GAL4 driver was stained with antibodies against PTEN and Baz. Both proteins colocalize in the apical cortex of the epidermis (arrows) and in the apical cortex of neuroblasts (asterisks). (B) At later developmental stages, apical colocalization of PTEN2 and Baz in epithelia is even more pronounced. Neurotactin (blue, right panel) was used as a marker for the basolateral membrane. No overlap between PTEN and Neurotactin is visible. (C) The PTEN3 isoform, which lacks the PDZ-binding motif at the C terminus but is otherwise identical to PTEN2 is localized on the whole plasma membrane and in the cytoplasm. PTEN3 staining clearly overlaps with Neurotactin on the basolateral membrane (purple, right panel). Apical localization of Baz is unaffected by overexpression of PTEN3. (D) PtdIns(4,5)P_2 colocalizes with Baz in the embryonic epidermis. We expressed a PLCδ-PH-GFP fusion protein under control of a ubiquitous maternal promoter and stained these embryos with an antibody against Baz. The PLCδ-PH-GFP fusion protein is present on the whole plasma membrane except for the free apical surface and is strongly enriched in the most apical region of the lateral plasma membrane where it colocalizes with Baz. The embryos shown are at stage 10 (A) and at stage 13 (B-D) (Campos-Ortega and Hartenstein, 1997). Genotypes are: *P{w $^{+mC}$=matalpha4-GAL-VP16}V67;UAS Pten2* (A); *P{w $^{+mC}$=Act5C-GAL4}17bF01;UAS Pten2* (B); *P{w $^{+mC}$=Act5C-GAL4}17bF01/UAS Pten3* (C); and *P{w $^{+mC}$=matalpha4-GAL-VP16}V67/UAS PLCδ-PH-GFP* (D). Scale bar: 20 μm.

subcellular localization of PtdIns(4,5)P_2 in embryonic tissues, we expressed a fusion protein of the pleckstrin homology (PH) domain of phospholipase Cδ with GFP, which binds specifically to PtdIns(4,5)P_2 (Varnai and Balla, 1998). Intriguingly, we found strong enrichment of PtdIns(4,5)P_2 in the most apical region of the lateral plasma membrane of epithelia, exactly where PTEN and Baz are colocalized (Fig. 3D). We also attempted to visualize the subcellular localization of PtdIns(3,4,5)P_3 in embryos by expressing a fusion protein of the pleckstrin homology domain of GRP1 with GFP (Gray et al., 1999). The GRP1-GFP fusion protein was localized to the plasma membrane in transfected Schneider S2 cells grown in medium supplemented with serum (data not shown), but the GFP fluorescence of this fusion protein in embryos was too weak to be visualized by confocal microscopy.

PTEN mutant embryos show defects in early embryonic development

Embryos lacking zygotic *Pten* function die late in embryogenesis or early larval stages and do not show any obvious defects in embryonic development (Goberdhan et al., 1999; Huang et al., 1999) (data not shown). By contrast, embryos derived from *Pten* germ-line clones lacking maternal and zygotic *Pten* function (*Pten^{mat,zyg}*) showed severe developmental abnormalities already in freshly laid eggs. Eggs derived from *Pten* germ-line clones were generally smaller and more roundish than wild-type eggs (Fig. 6C,D), and many did not show any development. In those *Pten^{mat,zyg}* embryos that initiated development, we only rarely observed the formation of pole cells (Fig. 4G, Fig. 5F,H) (see Movie 2 in the supplementary material). In the few cases where pole cells were formed, their number was very low, typically two or three, when compared with an average of 35 in wild type (Campos-Ortega and Hartenstein, 1997). The lack of pole cells pointed to a potential defect in the assembly or maintenance of the germ plasm (Rongo and Lehmann, 1996). In *Pten^{mat,zyg}* embryos, the mRNA of the germ plasm determinant *oskar* was either diffusely localized to the posterior pole or was completely undetectable (Fig. 4B,C). In wild type, the germ cell determinant Vasa becomes localized to the posterior of the oocyte during oogenesis and is incorporated into the pole cells during early embryonic development (Fig. 4D,F,H) (Hay et al., 1988; Lasko and Ashburner, 1990). In early *Pten^{mat,zyg}* embryos, Vasa staining at the posterior pole was strongly reduced and was undetectable at later stages, consistent with the failure to form germ cells (Fig. 4E,G,I).

In wild-type embryos, the first three cleavage divisions of nuclei occur in the anterior center of the egg. In cycles 4-7, nuclei spread out along the long axis of the embryo in a migration termed axial expansion (Fig. 4D) (Hatanaka and Okada, 1991; von Dassow and Schubiger, 1994). During the remaining preblastoderm divisions, the nuclei move towards the cortex until they reach the surface at cycle 10 (Campos-Ortega and Hartenstein, 1997). Cleavage divisions of cortical nuclei in

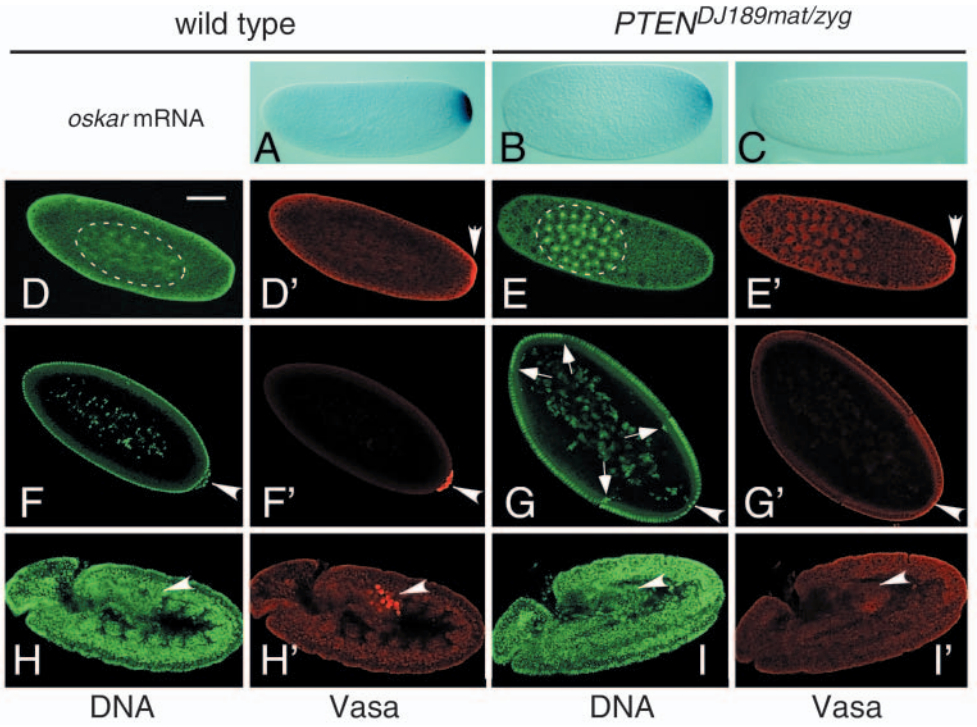

Fig. 4. *Pten* mutant embryos fail to localize germ plasm determinants and do not form pole cells. (A) In freshly laid wild-type eggs, *oskar* mRNA is strongly enriched at the posterior pole. (B,C) in eggs derived from *Pten* germline clones, *oskar* mRNA is detectable at the posterior pole only at a low level (B) or not at all (C). (D,D′) In wild-type embryos at nuclear division cycle 6 (64 nuclei), the nuclei have spread into an ellipsoid cloud in the yolk (dashed ellipse) and the germ plasm component Vasa is enriched at the posterior pole (arrowhead). (E,E′) In *Pten* mutant embryos derived from germ-line clones at nuclear division cycle 6 the nuclei stay together in an almost circular cloud (broken circle) and Vasa is only slightly enriched at the posterior pole. (F,F′) Vasa staining is restricted to pole cells (arrowhead) in wild-type embryos at late syncytial blastoderm stage. (G,G′) In *Pten* mutants, pole cells do not form and Vasa staining is absent from the posterior pole (arrowhead). Arrows indicate borders between nuclei in different stages of the cell cycle. (H,H′) In a wild-type embryo at the extended germ band stage, Vasa staining is restricted to germ cells (arrowhead). (I,I′) In a *Pten* mutant embryo derived from a germ-line clone, germ cells are absent and no Vasa staining can be detected (arrowheads indicate the position where germ cells are found in wild type). DNA was stained with YOYO-1. In all images, anterior is towards the left. Scale bar: 100 μm.

wild type occur in a wave-like nearly synchronous fashion, with the mitotic waves starting simultaneously at the anterior and posterior poles and moving towards the center (see Movie 1 in the supplementary material) (Foe and Alberts, 1983).

By contrast, in $Pten^{mat,zyg}$ embryos, axial expansion did not occur, resulting in a spherical rather than ellipsoid arrangement of nuclei prior to cortical migration (Fig. 4E) and an abnormally low nuclear density in the posterior region of the embryo at the syncytial blastoderm stage (Fig. 5B). In addition, the synchrony of the cell cycle during cleavage divisions was lost (Fig. 5B,D). The pattern of mitoses was not completely randomized but can be described as occurring in waves that always started at the posterior of the embryo (see Movie 2 in the supplementary material). Consequently, mitotic figures representing all stages of the cell cycle could be seen next to each other in $Pten^{mat,zyg}$ embryos (Fig. 5D). Thus, at the beginning of cellularization in cycle 14, the blastoderm nuclei showed different morphology and different condensation states of the chromatin in distinct regions of the embryo (Fig. 5F). These regional differences were reflected by the behavior of the actin network at the cellularization front, which advanced much more rapidly in the anterior region of the embryo than in the posterior region (Fig. 5F,H; see Movie 2 in the supplementary material). The delay of cellularization at the posterior pole was often accompanied by severe defects in the morphogenetic movements of gastrulation, which frequently led to rapid degeneration of $Pten^{mat,zyg}$ embryos shortly after the onset of gastrulation movements (data not shown).

However, a significant number of $Pten^{mat,zyg}$ embryos recovered surprisingly well from these early developmental defects and completed embryogenesis without any gross morphological abnormalities. The survival beyond gastrulation was independent of whether the embryos received a wild-type allele of *Pten* from their father, demonstrating that only the maternal supply of PTEN is crucial for proper development of the early embryo. Immunohistochemical staining of embryos that continued development beyond gastrulation with antibodies against proteins that localize specifically to the apical or basal pole of epithelial cells and neuroblasts, including Baz, Neurotactin, Inscuteable and Miranda, did not reveal any obvious defect in polarization of both cell types (data not shown).

PTEN controls the organization of the actin cytoskeleton in the female germline

The small size and aberrant shape of eggs derived from *Pten* germ-line clones (Fig. 6A,C,D) and the failure to localize the posterior determinants *oskar* mRNA and Vasa protein in early $Pten^{mat,zyg}$ embryos (Fig. 4) point to a function for *Pten* during oogenesis. Small egg size and roundish egg shape have been reported for mutants in which the actin cytoskeleton is disorganized, leading to inefficient transport of material from the nurse cells into the oocyte (Robinson and Cooley, 1997). In wild-type egg chambers at stage 10 (Spradling, 1993), actin localizes along the cell borders of the nurse cells and is enriched in ring canals, which stabilize the cytoplasmic bridges between nurse cells and the oocyte (Fig. 6E′). In addition, actin is prominently enriched in the cortex underlying the plasma membrane of the oocyte (Fig. 6E′).

By contrast, in egg chambers at stage 10 in which the germline was mutant for *Pten*, the actin cytoskeleton had a

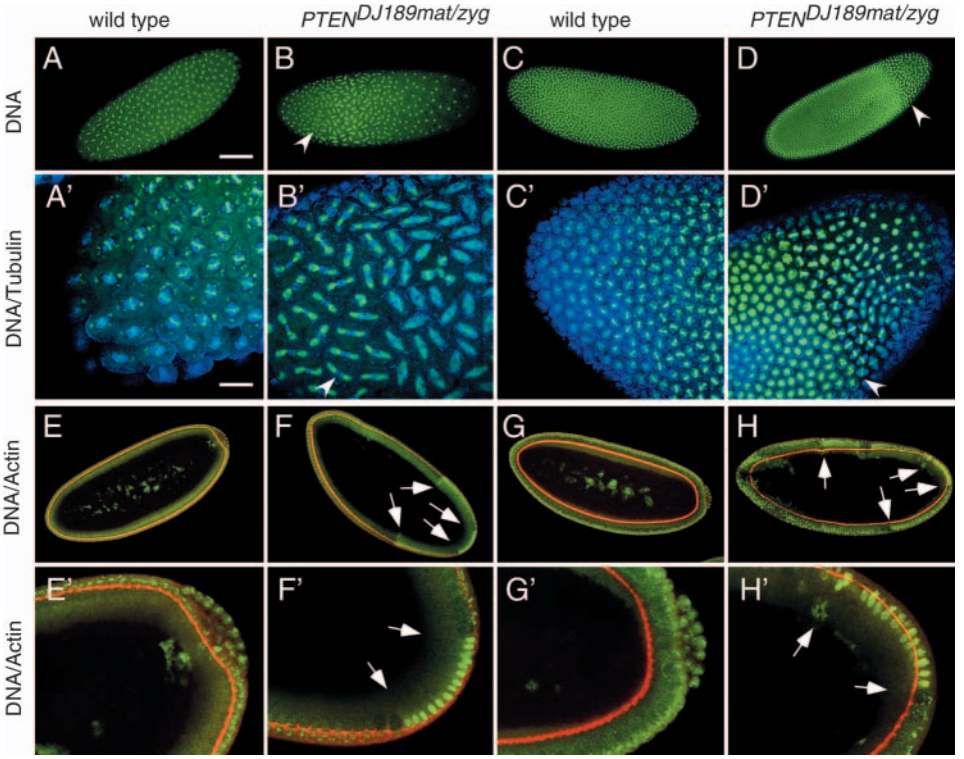

Fig. 5. *Pten* mutant embryos show defects in nuclear migration, cell cycle regulation and in organization of the actin cytoskeleton during cellularization. (A,A′,C,C′) Cortical nuclei in wild-type embryos are evenly spaced and divide in a nearly synchronous pattern. (B,B′) In *Pten* mutant embryos, nuclear density in the posterior region of the embryo is much lower than in the anterior region because of a defect in nuclear migration in pre-blastoderm stages. (D,D′) At syncytial blastoderm, nuclei are still unevenly spaced and divide asynchronously. (A-D) Whole embryos stained for DNA; (A′-D′) higher-magnification images of the same embryos as shown in A-D, stained for DNA (green) and α-tubulin (blue). The arrowheads in B,B′,D,D′ are in corresponding positions to illustrate which region of the embryo is shown at higher magnification. (E,E′,G,G′) During cellularization of wild-type embryos, a regular network of actin filaments (red) forms at the front of the ingrowing plasma membrane. (F,F′,H,H′) In *Pten* mutant embryos, the cellularization front is very uneven. Cellularization is particularly slow at the posterior pole where the pole cells have failed to form (F′,H′). Arrows in F,F′,H,H′ indicate borders between nuclei at different cell cycle stages. In all images, anterior is towards the left. Scale bar: 100 μm in A-H; 20 μm in A′-H′.

very disorganized structure and filled the nurse cell cytoplasm instead of localizing to cell borders (Fig. 6F′). The enrichment of actin in the oocyte cortex was strongly reduced in *Pten* germ-line clones (Fig. 6F′). In addition, we frequently observed fusion of nurse cells and mispositioned nurse cell nuclei that appeared to have moved into the oocyte in *Pten* germline clones (Fig. 6H′). We suppose that these severely abnormal oocytes give rise to the very small roundish eggs, such as the one shown in Fig. 6D. The vast majority of these very small eggs did not initiate embryonic development. Despite of the severe misorganization of the actin cytoskeleton, most *Pten* mutant oocytes showed normal localization of the polarity determinants Staufen (Fig. 6F″), *oskar* mRNA and Vasa protein (data not shown). Thus, we conclude that the failure to localize *oskar* mRNA and Vasa protein properly in early $Pten^{mat,zyg}$ embryos points to a function for *Pten* in maintenance rather than establishment of posterior determinant localization. The subcellular localization of Baz was also unaffected in germ-line clones and somatic clones mutant for *Pten* (data not shown), demonstrating again that *Pten* is not required for establishment of polarity in oocytes and follicle cells. We also attempted to determine the subcellular localization of endogenous PTEN in wild-type and *baz* mutant ovaries but could not detect any specific staining above background, as in embryos. Thus, the question of whether Baz is required for the correct subcellular localization of PTEN in ovaries cannot be answered at this point.

Discussion

PTEN activity may be regulated by its subcellular localization

We show here that Baz/PAR-3 and PTEN directly bind to each other and colocalize in the apical cortex of neuroblasts and epithelia. What could be the physiological meaning of this interaction? Evidence for a functional link between the PAR/aPKC complex and PI3-kinase signaling comes from a recent study that showed that both pathways are required for polarization of cultured hippocampal neurons (Shi et al., 2003). In this system, the PAR/aPKC complex localizes to the tip of the outgrowing axon and its localization is abolished upon overexpression of PTEN (Shi et al., 2003). However, no information is available on the mechanism of how PTEN interacts with the PAR/aPKC complex in this system.

Polarized localization of PTEN to the rear of the cell has been reported for *Dictyostelium* cells migrating towards a source of chemoattractant (Funamoto et al., 2002; Iijima and Devreotes, 2002). This localization was complementary to the localization of PtdIns(3,4,5)P_3 at the leading edge, consistent with a function for PTEN in lowering the local concentration of PtdIns(3,4,5)P_3 in the plasma membrane. Moreover, both loss of *Pten* function and PTEN overexpression led to the loss of cell polarity in *Dictyostelium* and strongly impaired the movement of the cells towards the source of the chemoattractant (Funamoto et al., 2002; Iijima and Devreotes, 2002). How PTEN is targeted to the rear of the cell in migrating cells is unknown.

Mammalian PTEN contains a canonical PDZ-binding motif

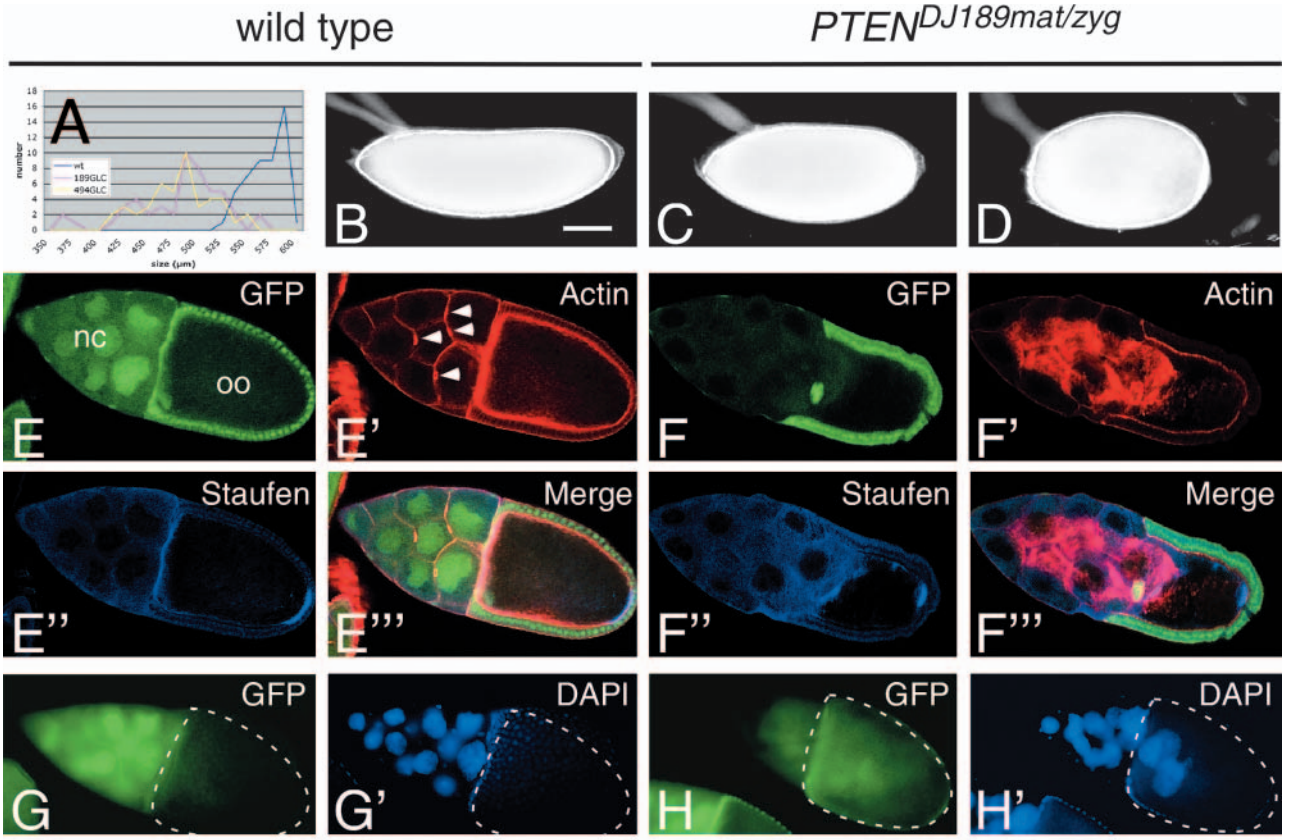

Fig. 6. PTEN is required for organization of the actin cytoskeleton during oogenesis. (A) Eggs derived from *Pten* germ-line clones are generally smaller than wild-type eggs. The number of eggs counted for each genotype was plotted against egg size (in µm). Fifty eggs were counted for each genotype. (B) Wild-type eggs are long and slender, whereas eggs derived from *Pten* germ-line clones (C,D) are shorter and more rounded. (E) In a wild-type egg chamber at stage 10, actin (E′) is localized along the cell borders of the nurse cells, in ring canals (arrowheads) and underlying the plasma membrane of the oocyte. The Staufen protein (E″) is localized to the anterior and posterior pole of the oocyte. (F) In an egg chamber at stage 10 in which the germline is mutant for *Pten*, actin (F′) is disorganized but the localization of Staufen (F″) is normal. (G) In a wild-type egg chamber at stage 10, the nurse cell nuclei (G′, visualized with DAPI) do not penetrate into the oocyte. (H) By contrast, in many stage 10 egg chambers with *Pten* germ-line clones, nurse cell nuclei are found within the oocyte. Clones of homozygous *Pten* mutant cells were marked by absence of GFP fluorescence. The weak green fluorescence signal in H is bleed through from the follicle cells, as images in G and H were taken with a conventional epifluorescence microscope in order to visualize DAPI. The oocyte is circled with a broken white line in G and H. In all panels, anterior is towards the left. nc, nurse cell; oo, oocyte. Scale bar: 100 µm.

at its C terminus, and this motif has been reported to interact with the multi-PDZ proteins MAGI-2 and MAGI-3 (Wu et al., 2000a; Wu et al., 2000b). Both PDZ proteins localize to tight junctions in mammalian epithelia and cooperate with PTEN to control the activity of the downstream kinase PKB/Akt (Wu et al., 2000a; Wu et al., 2000b; Kotelevets et al., 2005), indicating that subcellular targeting of PTEN may be important for its biological activity. This hypothesis is supported by studies of a deletion mutant of PTEN lacking the PDZ-binding motif. Although this mutant retained lipid phosphatase activity, its activity differed from the full-length wild-type form of PTEN in several biological assays (Leslie et al., 2000; Leslie et al., 2001). Together, these observations demonstrate that targeting of PTEN to a specific subcellular location may be essential for its proper function in the control of cell polarity. Our data show for the first time that PTEN is specifically recruited to the apical plasma membrane of epithelia and neuroblasts by direct association with Baz/PAR-3, a key regulator of cell polarity.

PTEN activity is required for the control of several actin dependent processes in *Drosophila*

In order to address the issue of whether *Pten* activity is required for the control of cell polarity in *Drosophila*, we analyzed the phenotype of mutant ovaries and embryos lacking maternal and zygotic *Pten* activity. The organization of the actin cytoskeleton in nurse cells and in the oocyte of *Pten* germ-line clones becomes abnormal from stage 9 onwards, resulting in the production of smaller, misshapen eggs. $Pten^{mat,zyg}$ embryos show defects in the axial expansion of nuclei during nuclear division cycles 4-7 and fail to synchronize the cell cycle in syncytial blastoderm nuclei. In addition, pole cells are strongly reduced in number or are missing altogether, which is accompanied by the failure to maintain *oskar* mRNA and Vasa protein localization at the posterior pole. Very similar phenotypes have been reported for embryos treated with the actin depolymerizing drug cytochalasin D and for mutants in genes that are required for the organization of the actin cytoskeleton (Hatanaka and Okada, 1991; Erdelyi et al., 1995;

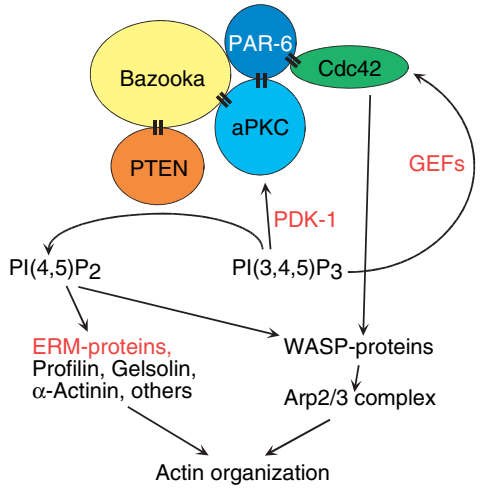

Fig. 7. A model for the activities of PTEN after recruitment to the PAR/aPKC complex. Recruitment of PTEN by Baz probably leads to local reduction of PtdIns(3,4,5)P_3 and a local increase of PtdIns(4,5)P_2 in the plasma membrane at the site where recruitment occurs. This should result in downregulation of the activities of aPKC and Cdc42, because PDK1, the kinase that activates aPKC, and guanine nucleotide exchange factors (GEFs) that activate Cdc42 are recruited to the plasma membrane via their PH domains by PtdIns(3,4,5)P_3. How exactly recruitment of PTEN to the PAR/aPKC complex would affect actin organization is difficult to predict, as both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are important effectors of actin dynamics. Proteins that bind to phosphoinositide lipids via PH domains are highlighted in red. Direct protein-protein interactions within the PAR/aPKC complex are indicated by double bars. ERM, ezrin, radixin, moesin; WASP, Wiskott-Aldrich syndrome protein; Arp2/3, actin related protein 2/3 (for reviews, see Pollard et al., 2000; Millard et al., 2004).

Wheatley et al., 1995; Tetzlaff et al., 1996; Lantz et al., 1999). Mutations in the gene *shackleton* also show defects in axial expansion and lack pole cells, but the posterior localization of *oskar* mRNA is normal, indicating that defects in axial expansion alone are sufficient to cause the lack of pole cells (Yohn et al., 2003). Interestingly, although germ plasm determinants were mislocalized or absent in early *Pten*^{mat,zyg} embryos, they were still localized normally during oogenesis, pointing to a function for *Pten* in maintenance, rather than establishment, of germ plasm determinant localization. Studies on ovaries and embryos mutant for the actin-binding protein tropomyosin II gave essentially the same results (Erdelyi et al., 1995; Tetzlaff et al., 1996). Thus, all of the phenotypes of *Pten*^{mat,zyg} mutant ovaries and embryos described here can be related to a function for PTEN in actin-dependent processes.

The links between PTEN and actin are obviously the substrate and the product of the enzymatic activity of PTEN, PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2. Both phosphoinositide lipids are important regulators of the actin cytoskeleton. PtdIns(4,5)P_2 acts mostly by direct binding to actin-associated proteins that link the actin cytoskeleton to the plasma membrane or by binding to proteins that are involved in the initiation of de novo actin polymerization, e.g. profilin and WASP (Fig. 7) (Yin and Janmey, 2003). PtdIns(3,4,5)P_3 in turn acts on the actin cytoskeleton via recruitment of guanine nucleotide exchange factors (GEFs) for the small GTPases Rac1, Rho and Cdc42 (Zheng, 2001), which can activate WASP proteins and the Arp2/3 complex (Fig. 7). Because we do not know the subcellular localization of endogenous PTEN, we cannot predict at present how exactly PTEN may affect the organization of the actin cytoskeleton during early embryonic development. However, the fact that overexpressed PTEN2 colocalizes with PtdIns(4,5)P_2 to the junctional region of epithelial cells indicates that PTEN may locally alter the balance between PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in the plasma membrane, leading to a modification of the actin cytoskeleton in defined regions of the cytocortex. Studies of PTEN knockout cells and *Pten* mutants in *Drosophila* have indeed shown that loss of *Pten* leads to a significant increase in the amount of PtdIns(3,4,5)P_3 in the plasma membrane (Stambolic et al., 1998; Oldham et al., 2002).

Surprisingly, PTEN does not appear to be required for the control of apicobasal polarity of neuroblasts and epithelia, despite its apical colocalization with Baz in these two cell types. The asymmetric localization of cell fate determinants to the basal cortex of mitotic neuroblasts requires both an intact actin cytoskeleton and the activity of the PAR/aPKC complex (Broadus and Doe, 1997; Knoblich et al., 1997) (reviewed by Wodarz and Huttner, 2003). Thus, the PAR/aPKC complex must be communicating with the actin cytoskeleton, but how this occurs is unknown. Our finding that mutations in *Pten* lead to severe defects in several actin dependent processes during oogenesis and early embryonic development support the hypothesis that PTEN may provide a link between the PAR/aPKC complex and the actin cytoskeleton in neuroblasts and epithelia. However, this link may not be essential in these cell types because of functional redundancy in the system that controls the levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 at the plasma membrane. Functional redundancy has recently been uncovered for the pathways that control the different cell size of neuroblasts and ganglion mother cells during asymmetric neuroblast division. Here, the activity of either the PAR/aPKC complex or the Pins/Gαi complex alone is sufficient to generate two daughter cells of different size. Only the simultaneous inactivation of both complexes leads to loss of cell size asymmetry (Cai et al., 2003). Alternatively, even if the balance between PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 at the plasma membrane were altered in neuroblasts and epithelia of *Pten*^{mat,zyg} embryos, alterations in the biological activity of downstream components of the system may compensate for this imbalance. In support of this interpretation, the loss of *Pten* function that we describe here affects only a subset of actin-dependent processes in oogenesis and early embryogenesis, while a participation of PTEN in other actin-dependent processes may be masked by the redundant activities of additional actin effectors.

A potential function for PTEN in regulation of aPKC and Cdc42

Besides its function in the regulation of actin, PTEN may regulate the catalytic activity of aPKC, a core component of the PAR/aPKC complex that directly binds to Baz (Wodarz et al., 2000) and associates in a protein complex with PTEN2 (Fig. 2C; Fig. 7). The mammalian homologs of aPKC, the atypical PKC isoforms λ and ζ, require phosphorylation by the upstream kinase PDK1 in order to become fully active (Chou et al., 1998; Le Good et al., 1998; Standaert et al., 2001). PDK1

is recruited to the plasma membrane by direct binding of its pleckstrin homology (PH) domain to PtdIns(3,4,5)P_3. PDK1, PTEN and several downstream effectors of the PI3-kinase signaling pathway in *Drosophila* show strong genetic interactions and are crucial for the regulation of cell growth and proliferation (Goberdhan et al., 1999; Huang et al., 1999; Gao et al., 2000; Scanga et al., 2000; Oldham et al., 2002; Radimerski et al., 2002; Stocker et al., 2002). We have obtained biochemical evidence that aPKC is a substrate for PDK1 (A.G. and A.W., unpublished) and propose that aPKC is phosphorylated in response to elevated PtdIns(3,4,5)P_3 levels. According to this hypothesis, PTEN would be a negative regulator of the kinase activity of aPKC.

In addition to PDK1, PtdIns(3,4,5)P_3 recruits GEFs that activate the small GTPases Cdc42 and Rac1 (Fig. 7) (Zheng, 2001). Intriguingly, active GTP-bound Cdc42 is also a component of the PAR/aPKC complex in mammalian cells and in *Drosophila* (Joberty et al., 2000; Johansson et al., 2000; Lin et al., 2000; Qiu et al., 2000; Hutterer et al., 2004) (D. Egger and A.W., unpublished). GTP-bound Cdc42 binds directly to the CRIB domain of PAR-6 and this interaction could elevate the kinase activity of aPKC, as has been shown in mammalian cells (Yamanaka et al., 2001). Thus, PtdIns(3,4,5)P_3 could activate aPKC both by recruitment of PDK1, which directly phosphorylates aPKC, and by recruitment of GEFs, which activate aPKC via Cdc42 and PAR6. Studies on PTEN knockout cells have indeed shown that PTEN inhibits Rac1 and Cdc42 (Liliental et al., 2000). The presence of PTEN in one complex together with aPKC, Cdc42 and PAR-6 should therefore lead to inhibition of both pathways that activate aPKCs, revealing a novel way to control the activity of a key component of the PAR/aPKC complex.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/7/1675/DC1

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