Assembly of Bazooka polarity landmarks through a multifaceted membrane-association mechanism

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Accepted 18 October 2011

Journal of Cell Science 125, 1–14

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doi: 10.1242/jcs.091884

Summary

Epithelial cell polarity is essential for animal development. The scaffold protein Bazooka (Baz/PAR-3) forms apical polarity landmarks to organize epithelial cells. However, it is unclear how Baz is recruited to the plasma membrane and how this is coupled with downstream effects. Baz contains an oligomerization domain, three PDZ domains, and binding regions for the protein kinase aPKC and phosphoinositide lipids. With a structure–function approach, we dissected the roles of these domains in the localization and function of Baz in the Drosophila embryonic ectoderm. We found that a multifaceted membrane association mechanism localizes Baz to the apical circumference. Although none of the Baz protein domains are essential for cortical localization, we determined that each contributes to cortical anchorage in a specific manner. We propose that the redundancies involved might provide plasticity and robustness to Baz polarity landmarks. We also identified specific downstream effects, including the promotion of epithelial structure, a positive-feedback loop that recruits aPKC, PAR-6 and Crumbs, and a negative-feedback loop that regulates Baz.

Key words: Bazooka/PAR-3, Drosophila, PAR complex, Epithelial cell polarity

Introduction

Establishing and maintaining epithelial cell polarity is essential for animal development and physiology. Epithelia are sheets of adherent cells that form our skin and line our organs. Each side of an epithelium has distinct molecular properties to engage the extracellular environments on either side of the sheet. Similarly to all polarized cells, apical–basal epithelial cell polarity is established and maintained by cortical landmarks (reviewed by Nelson, 2003; Suzuki and Ohno, 2006; Goldstein and Macara, 2007; St Johnston and Ahringer, 2010). Defining how these landmarks are positioned and how they organize the cell is essential for understanding epithelial cell polarity.

Bazooka (Baz/PAR-3) forms apical polarity landmarks in epithelial cells. In MDCK cells, PAR-3 is important for assembling and maintaining tight junctions and adherens junctions (Chen and Macara, 2005; Ooshio et al., 2007). In C. elegans, PAR-3 has been shown to localize to the apical domain above Baz and adherens junctions in Drosophila (Harris and Peifer, 2005), C. elegans (Totong et al., 2007) and MDCK cells (Martin-Belmonte et al., 2007). In Drosophila, Baz is prevented from stably interacting with PAR-6 and aPKC in two ways. First, the apical transmembrane protein Crumbs (Crb) competes with Baz for binding to PAR-6 (Morais-de-Sa et al., 2010). Second, aPKC dissociates from Baz after it phosphorylates its binding site (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). Phosphorylation of Baz by aPKC also inhibits its interaction with Stardust, a Crb-interacting protein (Krahn et al., 2010b). Excluding Baz from the basolateral and apical surface domains restricts it to the apical circumference, but mechanisms anchoring Baz around the apical circumference are unknown.

Baz/PAR-3 is a scaffold protein. It contains an N-terminal oligomerization domain (OD), three PDZ domains, an aPKC binding region and a phosphoinositide lipid (PIP) binding region (Fig. 1A). The OD promotes the cortical localization of Baz/PAR-3 in Drosophila, C. elegans and mammalian cells (Benton and St Johnston, 2003b; Mizuno et al., 2003; Li, B. et al., 2010). Thus, Baz/PAR-3 oligomerization is central to landmark assembly, but the other domains probably link it to the cell cortex. PDZ domains bind C-terminal protein tails, other protein motifs and lipids (Nourry et al., 2003). In Drosophila, PDZ domains in Baz can bind the C-terminal tails of the adherens-junction-associated transmembrane protein Echinoid (Ed), the
adherens junction component Armadillo (Arm) (Wei et al., 2005), the lipid phosphatase PTEN2 (von Stein et al., 2005) and PAR-6 (Morais-de-Sa et al., 2010). However, removing all three PDZ domains has no apparent effect on localization of Baz in Drosophila epithelia (Krahn et al., 2010a). The aPKC binding region can also be removed with no apparent effect of Baz localization (Krahn et al., 2010a). Similarly, removal of a recently identified PIP binding region had no apparent effect on localization of Baz (Krahn et al., 2010a). Two models could explain these results. Either a key single localization site has yet to be identified, or localization of Baz involves multiple redundant activities.

Many proteins are recruited to the plasma membrane by one upstream interaction, such as the recruitment of adaptor proteins to adhesion or signaling receptors (reviewed by Scott and Pawson, 2009; Harris and Tepass, 2010). Other proteins, such as the scaffold proteins Scribble (Albertson et al., 2004; Zeitler et al., 2004) and Discs Large (Hough et al., 1997; Thomas et al., 2000), use a ‘two-step’ mechanism in which one upstream interaction recruits the proteins to the plasma membrane and secondary interactions fine-tune their positioning. Our results argue that Baz is positioned by a distinct mechanism. We find that no single domain is essential for cortical recruitment of Baz. Instead, multiple redundant interactions position Baz. Our results define a multifaceted membrane association mechanism that localizes Baz to the apical circumference where it functions as a polarity landmark.

Results
Baz PDZ domains affect epithelial development to different extents
To assess how the different protein domains of Bazooka affect its function and localization, we first focused on the PDZ domains. We generated a set of deletion constructs removing the domains singly, in pairs or all together (Fig. 1A). The transgenes were all inserted at the same chromosomal site (attp2; confirmed by PCR) and expressed using the Gal4–UAS system. The proteins were tagged C-terminally with GFP.

To test the function of the constructs during embryogenesis, we assessed their ability to rescue the embryonic lethality of zygotic baz mutants – the maternal supply of Baz is undetectable in these mutants by stage 12 (Tanentzapf and Tepass, 2003) (our observations). The transgenes were expressed zygotically in the mutants using the maternal-a4tubulin-GAL4-VP16 driver (Fig. 1B). Without construct expression, the offspring of heterozygous baz mutants had 28.34±3.08% (n=5 experiments, 300 embryos each) lethality, as expected for full hemizygous baz mutant lethality (Fig. 1C). Expression of full-length Baz reduced embryonic lethality to 4.21±2.07% (n=5 experiments, 300 embryos each) (Fig. 1C), and rescued baz mutants to adulthood, indicating that the construct is fully functional. The embryonic rescue abilities of BazΔPDZ1 and BazΔPDZ3 were indistinguishable from the full-length protein (Fig. 1C), indicating that PDZ1 and PDZ3 are dispensable for this stage of development. By contrast, the rescue ability of
BazPDZ2 was significantly reduced (Fig. 1C), suggesting a key role for PDZ2. BazΔPDZ1–2, BazΔPDZ2–3 and BazΔPDZ1–3 also had reduced rescue abilities (Fig 1C), consistent with an important role for PDZ2. Notably, BazΔPDZ1+3 had reduced rescue ability (Fig 1C), indicating that PDZ1 and PDZ3 do contribute to Baz function to some extent. Importantly, every construct except BazΔPDZ2–3 had some degree of rescue ability compared with the absence of construct expression (Fig 1C). Remarkably, we found that all but one of the constructs could also rescue baz mutants to adulthood, although these rescue abilities varied and generally matched the ability of each construct to rescue embryos (supplementary material Table S1). To test for dominant-negative effects, we expressed the constructs zygotically with the stronger actin5cGAL4 driver in a wild-type (WT) background, but we observed no lethality. To further assess the rescue abilities of each construct, we analyzed the cuticle phenotypes of the offspring that failed embryogenesis. The cuticle is secreted from the apical domain of epidermal cells and provides a read-out of epithelial structure and morphogenesis. Without construct expression, the zygotic baz mutant cuticles had multiple holes (head, dorsal and/or ventral) or the holes fused resulting in a sheet of residual cuticle (Fig. 1D,E). Remarkably, all of the constructs shifted this distribution to cuticles with single holes or no apparent defects (Fig. 1D,E). Overall, these data indicate that Baz is partially functional without its PDZ domains; that PDZ2 plays an important role in Baz function during embryogenesis; and that the roles PDZ1 and PDZ3 are dispensable.

**Baz PDZ domains are not essential for its cortical localization**

To test how the PDZ domains affect the cortical localization of Baz, we imaged the set of PDZ domain deletions live in the dorsal ectoderm during dorsal closure (stage 14) (unless noted, all imaging was done in this manner). With zygotic expression driven by maternal-α4tubulin-GAL4-VP16, all of the constructs accumulated around the apical circumference of the epithelial cells (Fig. 2A). To test whether this was due to the presence of endogenous Baz, we expressed them in the same way in the zygotic baz<sup>Xi106</sup> mutant background (all baz mutant analyses were performed with zygotically driven transgene expression by maternal-α4tubulin-GAL4-VP16). Again, all of the constructs accumulated around the apical circumference of the epithelial cells (Fig. 2B), suggesting that they localize independently of endogenous Baz. To test whether their localization is affected by elevated expression levels, we drove expression with the stronger actin5c-GAL4 driver (all actin5c-GAL4 experiments were in WT). All of the constructs remained localized around the apical

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**Fig. 2. Baz PDZ domains are not essential for its cortical localization.** (A–E) Live imaging of stage 14 embryo dorsal ectoderm. For A–D, x-y and z sections are shown. (A) maternal-α4tubulin-GAL4-VP16-expressed Baz constructs. (B) maternal-α4tubulin-GAL4-VP16-expressed Baz constructs in the baz zygotic mutant. (C) actin5c-GAL4-expressed Baz constructs. (D) actin5c-GAL4-expressed BazΔPDZ1–3. Arrowheads indicate the position of the y-z frame with ectopic apical surface puncta. (E) Time-lapse imaging of BazΔPDZ1–3 showing relatively stationary ectopic puncta (arrows).
circumference except for BazΔPDZ1–3, which additionally accumulated in puncta (Fig. 2C). These puncta localized to the apical surface of the cells (Fig. 2D), and were retained there for 3 minutes or more (Fig. 2E). Thus, at lower levels Baz does not need its PDZ domains to localize around the apical circumference. However, elevated levels of BazΔPDZ1–3 appear to saturate available circumferential binding partners.

**Baz PDZ domains contribute to its cortical localization**

To assess whether other regions of Baz compensate for the loss of the PDZ domains, we made a specific deletion of the OD (Fig. 3A). As discussed above, the OD has been shown to promote the apical localization of Baz in *Drosophila* follicular epithelial cells (Benton and St Johnston, 2003b). Expressed with actin5c-GAL4, BazΔOD localized around the apical circumference of epithelial cells (Fig. 3C), although more weakly than full-length Baz (Fig. 3B) or BazΔPDZ1–3 (Fig. 3D) (insets show data collected and adjusted identically) [it also localized around the apical circumference in the baz mutant background (supplementary material Fig. S1B)]. To test for redundant localization mechanisms involving the OD and the PDZ domains, we deleted both regions. BazΔOD+ΔPDZ1–3 showed no membrane localization after expression with actin5c-GAL4, and the mosaic expression pattern of the construct revealed cytoplasmic accumulation [Fig. 3E; it was also non-cortical in the baz mutant background (supplementary material Fig. S1C)]. Fixing and staining embryos expressing BazΔOD+ΔPDZ1–3 with antibodies against GFP revealed low-level cortical localization (Fig. 3F), presumably through cytoplasmic BazΔOD+ΔPDZ1–3 extraction and enhanced GFP detection. Thus, the OD is important for the cortical localization of BazΔPDZ1–3, and the PDZ domains are important for the cortical localization of BazΔOD. With both regions deleted, cortical localization is very low. To further assess the ability of the PDZ domains to mediate cortical binding, we generated a construct comprised of only the PDZ domains tagged to GFP (Fig. 3G). This construct was undetectable with live imaging, but fixation and staining revealed weak localization around the apical circumference (Fig. 3G). Thus, the PDZ domains contribute to localization of Baz.

**Each Baz PDZ domain promotes cortical localization to a different extent**

To determine which PDZ domains are responsible for recruiting Baz to the cortex in the absence of the OD, we analyzed double and single PDZ domain deletions with the OD removed (Fig. 3H). Expressed with actin5c-GAL4, BazΔOD+ΔPDZ1–2 localized primarily to the apical circumference (Fig. 3I), whereas BazΔOD+ΔPDZ2–3 localized to the apical circumference but was also diffusely localized over the apical surface (Fig. 3J) (it also localized around the apical circumference in the baz mutant background (supplementary material Fig. S1D,F)). Thus, PDZ1 and PDZ3 can independently contribute to Baz cortical localization. By contrast, BazΔOD+ΔPDZ1+3 was detected only cytoplasmically with live imaging [Fig. 3J; it was also non-cortical in the baz mutant background (supplementary material Fig. S1E)]. However, fixation and staining for GFP revealed a low-level association along the length of the lateral membrane (Fig. 3K). Thus, PDZ2 appears to mediate weak, non-polarized plasma membrane association. Constructs with each single PDZ domain deleted with the OD also removed localized around the apical circumference (supplementary material Fig. S2A–C), as expected from the double PDZ domain deletions. These results indicate that each PDZ domain contributes to cortical localization of Baz, but at different strengths (PDZ1>PDZ3). Also, the localization mechanisms of PDZ1 and PDZ3 appear to differ.

To probe the localization mechanisms of PDZ1 and PDZ3, we mutated the predicted base of their peptide-binding pockets. For PDZ1, we mutated five amino acids (321–325) to alanine in PDZ1 of the BazΔOD+ΔPDZ2–3 construct. For PDZ3, we mutated five amino acids (364–368) to alanine in PDZ3 of the BazΔOD+ΔPDZ1–2 construct. Expressed with actin5c-GAL4, the distribution of BazΔOD+ΔPDZ1–2, PDZ3(5A) was altered versus BazΔOD+ΔPDZ1–2, with substantial apical surface localization, in addition to weak apical circumferential localization (Fig. 3M). By contrast, the distribution of BazΔOD+ΔPDZ1(5A), ΔPDZ2–3 was indistinguishable from BazΔOD+ΔPDZ2–3, with both apical circumferential and apical surface localization (Fig. 3N). Thus, PDZ1 appears to mediate apical surface and circumferential localization independently of its peptide-binding pocket, whereas PDZ3 has two cortical localization mechanisms: an apical circumferential localization mechanism dependent on its peptide-binding pocket, and an apical surface and circumferential localization mechanism that is independent of the pocket.

**Each Baz PDZ domain helps to restrict Baz to the apical circumference**

As discussed, we found that BazΔPDZ1–3 mislocalizes to the apical surface when overexpressed with the strong actin5c-GAL4 driver (Fig. 2D). This also implicated a role for the PDZ domains in the localization of full-length Baz. In fact, we found that addition of any single PDZ domain, or any pair of PDZ domains, into BazΔPDZ1–3 eliminated the apical surface puncta and restricted the Baz constructs around the apical circumference (Fig. 2C). To assess whether BazΔPDZ1–3 loses association with circumferential proteins to form the ectopic apical surface puncta, we compared its distribution with DE-cad, Arm and Ed, which are all adherens junction proteins with which Baz is known to form complexes (Harris and Peifer, 2005; Wei et al., 2005). Immunostaining of these proteins in embryos overexpressing BazΔPDZ1–3 with actin5c-GAL4 revealed that they were retained around the apical circumference, as in the WT, and had minimal or undetectable localization at the ectopic BazΔPDZ1–3 apical surface puncta (Fig. 4B–G). Thus, at high levels, BazΔPDZ1–3 might lose association with circumferential binding partners and drift apically.

To dissect how each PDZ domain restricts Baz to the apical circumference, we disrupted their peptide-binding pockets. For PDZ1 and PDZ3 we mutated the same sites as above, but in BazΔPDZ2–3 and BazΔPDZ1–2, respectively. For PDZ2, we mutated four amino acids (453–456) to alanine in the BazΔPDZ1+3 construct (Fig. 4A). Remarkably, mutation of PDZ1 and PDZ3 led to apical surface puncta and apical circumference localization that was similar to that of BazΔPDZ1–3 (Fig. 4H). Mutation of PDZ2 led to a cytoplasmic localization that was detectable only with GFP staining (Fig. 4I), suggesting destabilization of the protein. Thus, the peptide-binding pockets of PDZ1 and PDZ3 help to restrict Baz to the apical circumference. For PDZ3, the results are consistent with its abilities to promote cortical localization when the OD was also deleted (Fig. 3M). For PDZ1, the results appeared to contrast with those obtained when the OD was
Fig. 3. Baz PDZ domains promote cortical localization to different extents. (A) Design of constructs used in B–F. (B–E,J and L–N) Live imaging of actin\textsuperscript{5c}GAL4-expressed constructs in the stage 14 embryo dorsal epidermis. (F,G,K) Fixed and immunostained embryos. Baz\text{OD} localizes to the apical circumference (C) but at lower levels than full-length Baz (B) (insets show data collected and adjusted with same settings). (D) Baz\text{ΔPDZ1–3} at apical surface puncta and apical circumference. (E) Deletion of the OD and PDZ domains eliminates live cortical detection. (F) GFP antibody staining shows low-level apical localization (arrows; DE-cad shows apical circumference in \textit{x-y} and \textit{z} sections). (G) Baz 290-736 and GFP staining showing weak apical circumferential localization of the PDZ domains alone. (H) Constructs used in I–N. (I) Addition of PDZ3 to Baz\text{OD+ΔPDZ1–3} restores apical circumferential localization (compared with E). (J) Addition of PDZ2 to Baz\text{OD+ΔPDZ1–3} does not restore live cortical detection. (K) GFP staining shows low level, non-polarized membrane binding. (L) Addition of PDZ1 to Baz\text{OD+ΔPDZ1–3} restores apical circumferential localization (compared with E), but the protein also localizes to the apical surface. (M) Mutation of the peptide-binding pocket of PDZ3 of Baz\text{OD+ΔPDZ1–2} causes apical surface localization in addition to apical circumferential localization (compare with I). (N) Mutation of the peptide-binding pocket of PDZ1 of Baz\text{OD+ΔPDZ2–3} has no detectable effect (compared with L).
Our results show that the Baz PDZ domains contribute to its cortical localization. However, other cortical localization mechanisms must exist because BazΔPDZ1–3 can be recruited to the apical circumference. Because elevated BazΔPDZ1–3 expression led to...
ectopic apical surface puncta, we pursued their cortical localization mechanism. First, we investigated whether the apical surface proteins aPKC, PAR-6 and Crb were involved. In WT embryos, aPKC, PAR-6 and Crb were largely restricted to the marginal zone (Fig. 6A–C). In embryos overexpressing BazΔPDZ1–3, each protein accumulated in proximity to the BazΔPDZ1–3 puncta (Fig. 6A–C); expression of BazΔPDZ1–3 significantly increased the number of apical surface clusters and the intensity of these clusters (Fig. 6D), indicating that BazΔPDZ1–3 recruits aPKC, PAR-6 and Crb to apical surface subdomains.

The ability of BazΔPDZ1–3 to associate with aPKC, PAR-6 and Crb was somewhat surprising, because Baz normally segregates from these proteins (Harris and Peifer, 2005). Moreover, the construct lacks the binding site for PAR-6.
and contains the aPKC binding site that aPKC normally phosphorylates and dissociates from (Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). We considered two possible explanations. BazPDZ1–3 might recruit aPKC, PAR-6 and Crb but the proteins then segregate locally. Alternatively, aPKC might be inactive in the complex, remain bound to BazPDZ1–3, and thus anchor the protein at the apical surface. We distinguished these possibilities in two ways. First, we examined the colocalization of BazPDZ1–3 with aPKC, PAR-6, Crb and Arm apical surface spots in WT embryos versus those expressing BazPDZ1–3 (n=5 embryos with five cells each). (E–I) Deconvolved images. aPKC (E), PAR-6 (F), and Crb (G) locally segregated from BazPDZ1–3 apical surface puncta. (I) Labeling of BazPDZ1–3 apical surface puncta with anti-phospho-Baz980 which only labels the apical circumference in WT (H). Arm shows apical circumference. (I) pBaz staining and BazΔPDZ1–3 overlap. (J) Constructs in K–O. (K–N) Live imaging of actin5cGAL4-expressed constructs in the stage 14 embryo dorsal ectoderm (x-y and z sections shown). aPKC binding region deletion has minimal effect on cortical levels (L) compared with distribution of full-length Baz (K). Insets show data collected and adjusted with same settings. (M) BazΔPDZ1–3 at apical surface puncta and apical circumference. (N) Deletion of both the aPKC binding region and the PDZ domains eliminates live cortical detection. (O) GFP staining shows low-level apical localization. DE-cad shows apical circumference.

Second, we tested whether BazΔPDZ1–3 is phosphorylated at the aPKC binding site using a phosphorylation-specific antibody (Morais-de-Sa et al., 2010). The antibody labeled the BazΔPDZ1–3 apical surface puncta, and the ratio of the antibody staining intensity between the puncta and the circumference was similar to that of total BazΔPDZ1–3 protein (Fig. 6H–I). Thus, although BazΔPDZ1–3 recruits aPKC, PAR-6 and Crb, it is effectively phosphorylated by aPKC and locally segregates from the apical proteins. Because BazΔPDZ1–3 segregates from aPKC, PAR-6 and Crb, it is unlikely that they persistently anchor BazΔPDZ1–3 to the plasma membrane. However, dynamic interactions could help...
localize Baz. To test this idea, we deleted the aPKC binding region of Baz (Fig. 6J). Expressed with actin5c-GAL4, BazΔaPKC localized around the apical circumference (Fig. 6K,L), as reported by others (Krahn et al., 2010a) [it also localized around the apical circumference in the baz mutant background (supplementary material Fig. S1G)]. By contrast, deletion of the aPKC binding region from BazΔPDZ1–3 eliminated live detection of its apical surface and circumference localization [Fig. 6M,N; it was also non-cortical in the baz mutant background (supplementary material Fig. S1H)]. Fixation and staining for GFP revealed weak apical circumferential localization of the construct (Fig. 6O). Thus, aPKC binding appears to be important for BazΔPDZ1–3 localization to the apical surface and the apical circumference. Together, these results suggest that continuous cycles of aPKC– Baz binding and release contribute to the localization of each protein.

To test the relationship of BazΔPDZ1–3 and aPKC around the apical circumference, we examined their associations in baz zygotic mutants. In contrast to controls (supplementary material Fig. S5A), aPKC was depleted from the cortex of baz zygotic mutant epithelial cells at dorsal closure (supplementary material Fig. S5B) (Morais-de-Sa et al., 2010). Zygotic expression of full-length Baz with the maternal-α-tubulin-GAL4-VP16 driver rescued aPKC localization to the apical circumference (supplementary material Fig. S5D), but expression of BazΔPDZ1–3 had marginal or no effect (supplementary material Fig. S5F,G). Similarly, BazΔaPKC was unable to rescue cortical aPKC in the baz zygotic mutants (supplementary material Fig. S5J), suggesting that both the PDZ domains and the aPKC binding region contribute to localization of aPKC. The inability of BazΔPDZ1–3 to affect aPKC in this context might be due to its lower expression level versus expression with actin5c-GAL4. Nonetheless, residual aPKC segregated above BazΔPDZ1–3 in the baz mutant background (supplementary material Fig. S5H), as did rescued aPKC above full-length Baz (supplementary material Fig. S5E), which is consistent with a dynamic role for aPKC in the localization of both proteins.

Phosphoinositide lipids contribute to cortical localization of Baz

To test additional localization mechanisms, we examined a PIP binding region identified in Baz (Krahn et al., 2010a). First, we tested whether PIPs could contribute to the localization of BazΔPDZ1–3 by localizing the pleckstrin homology domain of phospholipase C delta 1 fused to Cerulean (a cyan fluorescent protein), as a probe for PtdIns(4,5)P2 (Mavrikis et al., 2009). Strikingly, the BazΔPDZ1–3 apical surface puncta colocalized with patches of the probe (Fig. 7A; seen in 6/6 embryos). The probe also labeled the lateral membrane of the cells (Fig. 7A). In cells without BazΔPDZ1–3, the probe labeled the lateral membrane and apical surface patches, but more rarely (Fig. 7A; apical surface patches seen in 1/6 embryos). Thus, the BazΔPDZ1–3 puncta closely associate with local patches of PtdIns(4,5)P2 and might promote their formation.

To test the role of PIP binding in Baz localization, we deleted the PIP binding region (Fig. 7B). Expressed with actin5c-GAL4, BazΔPIP localized around the apical circumference (Fig. 7C,D), as seen previously (Krahn et al., 2010a) [it also localized around the apical circumference in the baz mutant background (supplementary material Fig. S1J)]. When deleted from BazΔPDZ1–3, live detection of localization at the apical surface and circumference was lost [Fig. 7E,F; it was also non-cortical in the baz mutant background (supplementary material Fig. S1J)]. Fixation and staining for GFP revealed weak apical circumferential localization of the construct (Fig. 7G). Thus, PIP binding also appears to be important for localization of BazΔPDZ1–3 to the apical surface and the apical circumference.

Discussion

Our results show that Baz functions as a polarity landmark by linking multiple cortical anchorage mechanisms to multiple downstream effects (Fig. 8). Baz is anchored by redundant mechanisms involving each of its known interaction domains. Downstream effects include the promotion of epithelial structure, the recruitment of other polarity regulators, and negative feedback on Baz.

The roles of Baz PDZ domains

We and others have found that the PDZ domains of Baz are dispensable for its localization (Krahn et al., 2010a) (Fig. 2). Our results now show that this is due to redundant mechanisms in other parts of the protein. In fact, each PDZ domain plays a unique role in Baz positioning and activity in the Drosophila embryonic ectoderm. We identified the following main roles for the PDZ domains: PDZ1 and PDZ3 recruit Baz to the apical domain, PDZ2 mediates downstream effects on epithelial structure and PDZ1 promotes the turnover of Baz. Each domain also has minor effects that might result from distinct activities or secondary effects of their main activities: PDZ1 and PDZ3 have non-essential but detectable effects on epithelial structure and PDZ2 promotes weak membrane binding.

PDZ1 and PDZ3 activities involve at least two sub-regions of the domains. PDZ domains typically use their peptide-binding pocket to bind the C-termini of their protein partners, but regions outside of these pockets can also mediate interactions (reviewed by Nourry et al., 2003). We found that PDZ1 promotes apical surface and circumferential localization independently of its peptide-binding pocket, and that its peptide-binding pocket plays a distinct role in promoting Baz turnover. These opposing activities might form a negative-feedback loop that regulates localization of Baz (Fig. 8). By contrast, PDZ3 appears to solely promote Baz localization. It can promote apical surface and circumferential localization independently of its peptide-binding pocket, whereas its peptide-binding pocket specifically promotes circumferential anchorage (Fig. 8). The binding partners that engage these sites are unknown. However, in vitro studies have shown that the C-termini of Arm and Ed can bind Baz PDZ1–3 in tandem (Wei et al., 2005), and that the C-terminus of PTEN can bind Baz PDZ2–3 (von Stein et al., 2005). Binding partners for regions outside the peptide-binding pockets have not been identified for Baz PDZ domains, but the binding of rat PAR-3 PDZ2 to PIPs involves outside regions (Wu et al., 2007), as does the binding of C. elegans PAR-3 PDZ1 to PAR-6 (Li, J. et al., 2010).

A major function of Baz PDZ domains is to maintain the protein around the apical circumference. PDZ1 and PDZ3 use peptide-binding-pocket-independent mechanisms to generally localize Baz to the apical domain, but Baz is focused around the apical circumference through mechanisms involving the peptide-binding pockets of these domains. Without these pockets,
Baz can saturate its remaining apical anchors and mislocalizes in puncta over the apical surface. PDZ1 appears to prevent this mislocalization by reducing protein levels below saturation, but PDZ2 and PDZ3 might directly bind circumferential proteins or promote an active redistribution of Baz. This activity is weaker for PDZ2 versus PDZ3 (with the former requiring oligomerization and the latter not) and it is possible that the localization activity of PDZ2 is a by-product of its binding to downstream effectors localized to the apical circumference. Thus, we propose that PDZ3 has the most direct role in anchoring Baz around the apical circumference (Fig. 8).

Other localization mechanisms
Because Baz can localize to the apical membrane without its PDZ domains, other localization mechanisms are also involved. Our results clarify the importance of two additional mechanisms. The first involves dynamic interactions with apical polarity proteins. Baz has been shown to recruit aPKC to the apical domain as epithelial polarity is first established (Harris and Peifer, 2005) and to maintain aPKC during later stages (Morais-de-Sa et al., 2010). However, aPKC normally localizes at the apical surface with PAR-6 and the Crb complex above Baz and adherens junctions (Harris and Peifer, 2005). When BazΔPDZ1–3 forms puncta over the apical surface domain it recruits aPKC, PAR-6 and Crb, but the proteins then segregate locally, mimicking their associations around the apical circumference. Indeed, BazΔPDZ1–3 might separate from the apical polarity proteins by two known mechanisms: the release of aPKC after it phosphorylates its binding site on Baz, and the loss of PAR-6 binding as a result of competition with Crb (Morais-de-Sa et al.,

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**Fig. 7. Phosphoinositide lipids recruit Baz to the cortex.**
(A) Live imaging shows PLCD1[PH]::Cerulean colocalizing with BazΔPDZ1–3::GFP apical surface puncta (arrows).
(B) Constructs in C–G. (C–F) Live imaging of actin5cGAL4-expressed constructs in stage 14 embryos (x-y and z sections shown). PIP binding region deletion has minimal effect on cortical levels (D) versus distribution of full-length Baz (C). Insets show data collected and adjusted with same settings. (E) BazΔPDZ1–3 at apical surface puncta and apical circumference. (F) Deletion of both the PIP binding region and the PDZ domains eliminates live cortical detection. (G) GFP staining shows low-level apical localization. DE-cad shows apical circumference.
The segregation of aPKC, PAR-6 and Crb from BazD PDZ1–3 suggests that they would not form a stable anchorage site for Baz. However, removal of the aPKC binding region from BazD PDZ1–3 (but not full-length Baz) severely weakens its cortical localization. This suggests that a positive-feedback loop exists between Baz and aPKC to maintain localization of each protein (Fig. 8). We propose that the proteins undergo continuous cycles of attraction and local repulsion to maintain their close but non-overlapping positioning around the apical domain.

An additional Baz localization mechanism involves PIPs. A conserved region of the C-terminal tail of Baz has been shown to bind PIPs (Krahn et al., 2010a), and we found that the apical surface puncta of BazΔPDZ1–3 colocalize with plasma membrane domains enriched with PIPs. Although deletion of the PIP binding region had no effect on full-length Baz (Krahn et al., 2010a; Fig. 7), deleting it and the PDZ domains together strongly disrupts plasma membrane binding. Thus, the aPKC binding region and the PIP binding region might both mediate apical localization of Baz in the absence of the PDZ domains. These anchorage mechanisms are also dependent on the oligomerization of Baz, because BazΔOD+ΔPDZ1–3 shows minimal cortical localization. Moreover, the mechanisms appear to support each other because the aPKC binding region cannot compensate for the loss of the PIP binding region from BazΔPDZ1–3 and vice versa. Also, membrane binding is

Fig. 8. Interactions upstream and downstream of a Baz polarity landmark. (A) Summary of localization of Baz constructs expressed using actin5cGAL4. (B) Model. Non-peptide-binding pocket interactions of PDZ1 and PDZ3 can localize Baz to the apical surface and circumference (green arrows; in A, compare ΔOD+PDZ1 5A, ΔPDZ2–3 and ΔOD+ΔPDZ1–2, PDZ3 5A with ΔOD+ΔPDZ1–3). The aPKC or PIP binding regions can localize Baz to the apical surface and circumference (green arrows; in A, compare ΔPDZ1–3 with ΔPDZ1–3+aPKC and ΔPDZ1–3+ΔPIP). The peptide-binding pockets of PDZ1 and PDZ3 restrict Baz to the apical circumference (in A, compare ΔPDZ2–3 with PDZ1 5A, ΔPDZ2–3 and ΔPDZ1–2 with ΔPDZ1–2; PDZ3 5A and ΔOD+ΔPDZ1–2 with ΔOD+ΔPDZ1–2, PDZ3 5A). The PDZ3 peptide-binding pocket might restrict Baz to the apical circumference by direct anchorage (red arrow). The PDZ1 peptide-binding pocket affects localization by reducing total Baz levels (black arrow). PDZ2 is key for maintaining polarity (blue arrow). The aPKC binding region recruits aPKC to form a positive-feedback loop with Baz to impact polarity (blue arrows).
localized normally in most ectodermal cells of amnioserosa (Laplante and Nilson, 2006). However, this might involve multiple mechanisms, but typically there is a primary mechanism that localizes the scaffold to the membrane and secondary mechanisms that focus localization to a particular site. For example, the leucine-rich repeats of Scribble are crucial for its cortical localization in Drosophila epithelia, whereas its second PDZ domain promotes septate junction localization (Albertson et al., 2004; Zeitler et al., 2004). In Drosophila, the Hook domain of Discs Large is crucial for plasma membrane targeting, whereas particular PDZ domains promote septate junction localization in epithelia and synapse localization in neurons (Hough et al., 1997; Thomas et al., 2000). Similar ‘two-step’ localization mechanisms have been described for C. elegans and mammalian Discs large (Wu et al., 1998; Lockwood et al., 2008), mammalian PSD-95 (Arnold and Clapham, 1999; Craven et al., 1999; Nonaka et al., 2006; Sturgill et al., 2009), Drosophila InsCut and Pins (Knoblich et al., 1999; Yu et al., 2002) and Drosophila Stardust (Bulgakov et al., 2008). Contrasting these mechanisms, no single site in Baz is essential for membrane recruitment in ectodermal cells. However, any of these mechanisms could be context dependent. Scaffolds shown to localize through a two-step mechanism in one context might use a multifaceted membrane-association mechanism in another, and Baz could localize by one-step or two-step mechanisms in other cell types or developmental stages.

We have identified a multifaceted membrane-association mechanism that localizes Baz to the apical circumference in epithelial cells. This mechanism integrates with downstream pathways, involving both negative- and positive-feedback loops, which regulate Baz and epithelial polarity. It is important to define the partners for the interaction sites involved, and to dissect how these interactions are controlled.

The importance of a multifaceted membrane-association mechanism

Our results indicate that there are at least five sites in Baz, in addition to its OD, that are involved in membrane localization. No single site is essential, and different combinations of interactions are sufficient for anchorage. This suggests that the individual anchorage mechanisms are relatively weak, as has been shown for the PIP binding region (Krahn et al., 2010a). Cortical localization through multiple weak interactions might provide plasticity and robustness for the role of Baz/PAR-3 as a multifunctional polarity landmark.

The membrane-association mechanism of Baz would allow fine regulation of protein positioning. For example, Baz becomes planar polarized around the apical domain to regulate germ band extension in the Drosophila embryo (Zallen and Wieschaus, 2004). Rho kinase has been shown to reduce Baz at anterior and posterior cell edges by phosphorylating the Baz C-terminus and inhibiting PIP binding (Simoes Sde et al., 2010). However, Baz is not fully lost from these edges. Thus, planar polarity might arise from a partial set of membrane-association mechanisms acting along anterior–posterior edges and a more complete set acting at dorsal–ventral edges. Apical localization of Baz is also altered in amnioserosa cells to regulate apical constriction during dorsal closure (David et al., 2010). Here, Baz forms apical surface puncta in addition to its circumferential localization. Although the mechanisms for this redistribution are unclear, our work suggests that it might involve weakening of PDZ domain activities. Intriguingly, Ed, an in vitro binding partner of the PDZ domains (Wei et al., 2005), is specifically absent in the amnioserosa (Laplante and Nilson, 2006). However, this might not fully explain the redistribution because Baz appears to be localized normally in most ectodermal cells of ed mutants (Laplante and Nilson, 2011). A more dramatic cellular reorganization occurs as neuroblasts delaminate from the epithelium. As this occurs, adherens junctions and Crb are lost from the cells, but Baz is retained apically and engages with new partners to direct asymmetric cell division after delamination (reviewed by Prehoda, 2009). The mechanisms regulating Baz during this transition are unknown, but its multifaceted membrane-association mechanism might ensure robust apical localization as Baz exchanges molecular interaction networks.

Redundancies in Baz/PAR-3 scaffold activity might also have permitted co-evolution with polarity networks to organize eggs, single-cell embryos, epithelial cells, neurons and stem cells (reviewed by Wiggins et al., 2005; Goldstein and Macara, 2007; Wodarz and Nathke, 2007; St Johnston and Ahringer, 2010). Indeed, roles for Baz/PAR-3 PDZ domains appear to have diverged. In C. elegans, PDZ2, but not PDZ1 or PDZ3, is essential for embryogenesis, as in Drosophila, but PDZ2 of C. elegans PAR-3 was also shown to be required for proper localization (Li, B. et al., 2010), in contrast to Baz PDZ2. Also, mammalian PDZ2 has also been shown to mediate membrane binding through PIPs, but key residues involved in the interaction are not conserved in Drosophila or C. elegans (Wu et al., 2007).

Comparisons with other scaffold proteins

The Baz localization mechanism appears to be unique among characterized polarity scaffold proteins. Other scaffolds also involve multiple mechanisms, but typically there is a primary mechanism that localizes the scaffold to the membrane and secondary mechanisms that focus localization to a particular site. For example, the leucine-rich repeats of Scribble are crucial for its cortical localization in Drosophila epithelia, whereas its second PDZ domain promotes septate junction localization (Albertson et al., 2004; Zeitler et al., 2004). In Drosophila, the Hook domain of Discs Large is crucial for plasma membrane targeting, whereas particular PDZ domains promote septate junction localization in epithelia and synapse localization in neurons (Hough et al., 1997; Thomas et al., 2000). Similar ‘two-step’ localization mechanisms have been described for C. elegans and mammalian Discs large (Wu et al., 1998; Lockwood et al., 2008), mammalian PSD-95 (Arnold and Clapham, 1999; Craven et al., 1999; Nonaka et al., 2006; Sturgill et al., 2009), Drosophila InsCut and Pins (Knoblich et al., 1999; Yu et al., 2002) and Drosophila Stardust (Bulgakov et al., 2008). Contrasting these mechanisms, no single site in Baz is essential for membrane recruitment in ectodermal cells. However, any of these mechanisms could be context dependent. Scaffolds shown to localize through a two-step mechanism in one context might use a multifaceted membrane-association mechanism in another, and Baz could localize by one-step or two-step mechanisms in other cell types or developmental stages.

We have identified a multifaceted membrane-association mechanism that localizes Baz to the apical circumference in epithelial cells. This mechanism integrates with downstream pathways, involving both negative- and positive-feedback loops, which regulate Baz and epithelial polarity. It is important to define the partners for the interaction sites involved, and to dissect how these interactions are controlled.

Materials and Methods

Fly stocks and genetics

Fly stocks used were: UAS-PLCD1-1PH::GFP (Bloomingston Drosophila Stock Center (BDSC), #30895); UAS-baz::mcherry (McGill et al., 2009); actin-SC-GAL4 (BDSC, #3954); maternal-n4-tubulin-GAL4-VP16 (a gift from Mark Peifer, University of North Carolina at Chapel Hill, NC); and baz mutants (a gift from Andreas Wodarz, University of Gottingen, Germany). WT was yellow white. A recombinant chromosome containing maternal-n4-tubulin-GAL4-VP16 and the baz allele allowed correct expression in baz mutants. For genotyping, the chromosome was balanced over FM7 [two-GAL4, UAS-GFP (BDSC, #6873) and females were crossed to males with Red-Stinger (BDSC, #6873) on the X chromosome and UAS-Baz constructs on the third chromosome.

Constructs and transgenics

The baz cDNA (a gift from Andreas Wodarz) was amplified by PCR and cloned into the pENTR vector using EcoRI and EcoRV. All single deletions were created in this vector. For the OD, PDZ domains, aPKC binding region, PIP binding region and C-terminus, we created HindIII sites flanking the deletion fragment by PCR-mediated site-directed mutagenesis, and for the N-terminus we created flanked 5′ sites. Templates were from E. coli C7510 cells and parental strands were destroyed with Dpn I. Following mutagenesis, the deletion was made by HindIII-mediated excision and re-ligation. Peptide binding pocket point mutations were created by PCR mutagenesis (two or three rounds). All constructs were fully...
sequenced. See supplementary material Table S3 for primers, parental templates and sequencing results.

For double deletions involving the OD, the OD and surrounding regions from the desired single deletion constructs were excised using HpaI and SacII, and replaced with the corresponding Hpal-SacII fragment from the ΔOD construct. For double deletions involving the PDZ domains and the aPKC binding region or the PIP binding region, the PDZ domains and surrounding regions were excised from the single deletion constructs using SacII and AfeI and replaced with the corresponding SacII-AfeI fragment from the ΔPDZ1-3 construct.

PDZ domain positions were predicted using InterProScan Sequence Search (European Bioinformatics Institute, UK). Deletions were within the predicted domain boundaries. OD deletion was based upon the Par-3 OD structure and sequence alignment with Baz (Feng et al., 2007). Peptide-binding-pocket sequences were based on structures of PAR-3 PDZ2 (Wu et al., 2007) and PAR-3 PDZ3 (Feng et al., 2008). Secondary structure predictions and sequence alignments predicted the PDZ1 pocket. Similar binding pocket mutations were observed in PDZ3 (Feng et al., 2008). Secondary structure predictions and sequence alignments predicted the PDZ1 pocket. Similar binding pocket mutations were observed in PDZ3 (Feng et al., 2008).

Gateway cloning (Invitrogen) recombined all constructs into pPWG for C-terminal EGFP tagging and placement downstream of the UASp promoter. An attP recombination site was cloned into the pPWG Nxl site. Vectors were targeted to the attP2 recombination site on chromosome 3 for transgenic flies (Genetic; see supplementary material Table S3 for primers, parental templates and sequencing results.

Embryonic lethality rates and cuticle preparations

Embryonic lethality rates, flies laid eggs for up to 24 hours at 25°C. 300 eggs were collected and incubated for 48 hours. The percentage of unhatched embryos was determined versus the total number of embryos (unfertilized eggs excluded). For cuticle preparations, all unhatched eggs were collected, washed, dechorionated with 50% bleach, mounted on slides with Hoyer’s mountant and lactic acid (1:1), and baked at 60°C overnight.

Embryo staining

Embryos were washed with 0.1% Triton X-100; dechorionated with 50% bleach, washed, fixed for 20 minutes in 1:1 3.7% formaldehyde in PBS:heptane, de-vascularized in methanol then blocked and stained with PBS containing 1% goat serum, 0.1% Triton X-100 and 1% sodium azide. Antibodies used were: mouse anti-Arm (N27A1; 1:100; Developmental Studies Hyridomaha Bank (DSHB)), anti-Crb (1:350; DSHB); rabbit anti-aPKC (1:100); Santa Cruz Biotechnology), anti-Baz (1:200; raised in our lab against GST-Baz-311),-anti-Echinoid (1:5000; gift from Laura Nilson, McGill University, Montreal, Canada), anti-GFP (1:500; Abcam), anti-phosphorylated Ser980 Baz (1:100; gift from Daniel St. John), University of Cambridge, Cambridge, UK); rat anti-DE-cad (1:100; DSHB) and anti-PAR-6 (1:100; gift from Chris Doe, University of Oregon, Eugene, OR). Secondary antibodies were conjugated to Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 647 (Invitrogen).

Imaging

Dechorionated live embryos were mounted in halocarbon oil (series 700; Halocarbon Products) on petriPERM dishes (Sigma). Fixed embryos were mounted in Aquapho (Polysciences). Images were collected by spinning-disk confocal system (Quorum Technologies) at room temperature using a 63 x Plan Apochromat NA 1.4 objective (Carl Zeiss) with a piezo top plate or EM CCD camera (Hamamatsu Photonics). Images were analyzed with Voctology software (PerkinElmer). Z-stacks had 300 nm step sizes.

Post-acquisition image analysis and manipulation

For total cortical fluorescence measurements, 3D datasets collected with identical settings were analyzed with Imaris software (version 6.2; Bitplane). Similar total volumes around the apical circumferential were selected for each construct (one set of standardized settings could not account for the range of intensities among the constructs, but for each construct, identical settings were used for different embryos). For each sample, total fluorescence within the selection for the field of view was recalculated per cell. To determine Baz cluster volumes, all individual clusters were selected around the apical circumference, excluding low-level fluorescence of intervening membranes (one set of standardized settings could not account for the range of signal intensities among the constructs, but for each construct, identical settings were used for different embryos). If a selection extended beyond a single cell boundary it was ‘cut’ at the boundary into two clusters.

For fluorescence intensity measurements of apical surface polarity proteins in Fig. 6D, puncta were identified above background, intensities were measured in a box and intensities of equally sized neighbouring areas were subtracted (ImageJ). Image deconvolution and maximum intensity projections done with Volocity, and images were resized by bicubic interpolation (Adobe Photoshop).

Immunoblotting

Dechorionated embryo pellets of equal volume mixed 1:10 (v/v) with 2 × SDS-PAGE sample buffer, homogenized, (0.5 ml glass homogenizer, Kontes Glass Company), boiled for 5 minutes, separated by 6% SDS-PAGE, and blotted. Blots were blocked (TBS-T with 5% milk powder), washed (TBS-T) and probed. Antibodies used were rabbit anti-Baz (1:8000), rabbit anti-GFP (1:1000; Abcam), mouse anti-β-tubulin (E7; 1:800; DSHB), and HRP-conjugated secondary antibodies (Thermo Fisher Scientific). HRP detection reagents (Thermo Fisher Scientific) and film (Bioflex) detected signals.

Statistics

Comparisons were done using Student’s t-tests. Means are shown with s.d.

Acknowledgements

We thank U. Tepass and R. Winklbauer for critiques and suggestions. We thank D. Christendat and G. Fulice for advice and M. Peifer, A. Wodarz, L. Nilson, D. St Johnston and C. Doe for reagents.

Funding

This work was supported by a Canadian Institutes of Health Research operating grant. R.F.A.M. was supported by an Ontario Graduate Scholarship in Science and Technology. T.J.C.H. holds a Tier 2 Canada Research Chair.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl?doi:10.1242/jcs.091884/-/DC1

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