Formation of a Bazooka–Stardust complex is essential for plasma membrane polarity in epithelia

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Introduction

In Drosophila melanogaster, epithelial differentiation begins with the formation of spot adherens junctions during celluarization (Knust and Bossinger, 2002). By the coalescence of spot adherens junctions, the zonula adherens (ZA) is formed in early gastrulation as a junctional belt in the apical part of the lateral plasma membrane (Tepass and Hartenstein, 1994; McGill et al., 2009). Several proteins essential for the control of apical–basal polarity are localized in the apical plasma membrane domain and are enriched just apical to the ZA (Müller and Bossinger, 2003). The establishment of epithelial polarity and the assembly of the ZA in the ectodermal epithelium are regulated by a complex hierarchy of interacting proteins (Bilder et al., 2003; Johnson and Wodarz, 2003; Müller and Bossinger, 2003; Tanentzapf and Tepass, 2003; Harris and Peifer, 2004, 2005). The first protein complex relevant in this context is the PAR-3 (partitioning defective 3)–PAR-6–atypical protein kinase C (aPKC) complex (Macara, 2004; Suzuki and Ohno, 2006). Its core component PAR-3 (Bazooka [Baz] in Drosophila) serves as a scaffold for aPKC and its regulator PAR-6 (Wodarz et al., 2000; Petronczki and Knoblich, 2001; Macara, 2004; Suzuki and Ohno, 2006). A recent study revealed that Baz localizes to the ZA, whereas PAR-6 and aPKC segregate from Baz and localize slightly more apical (Harris and Peifer, 2005). In the second protein complex required for the establishment of epithelial polarity, the cytoplasmic domain of the transmembrane protein Crumbs (Crb) binds to the Postsynaptic density 95/Discs large/Zonula occludens 1 (PDZ) domain of Sdt and a region of Baz that contains a phosphorylation site for aPKC. Phosphorylation of Baz causes the dissociation of the Baz–Sdt complex. Overexpression of a nonphosphorylatable version of Baz blocks the dissociation of Sdt from Baz, causing phenotypes very similar to those of crb and sdt mutations. Our findings provide a molecular mechanism for the phosphorylation-dependent interaction between the Baz–PAR-3 and Crb complexes during the establishment of epithelial polarity.

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Abbreviations used in this paper: aPKC, atypical PKC; Baz, Bazooka; Crb, Crumbs; DE-cadherin, Drosophila epithelial cadherin; Dlg, Discs large; Lgl, Lethal giant larvae; PATJ, Pals1-associated tight junction protein; PDZ, Postsynaptic density 95/Discs large/Zonula occludens 1; Scrib, Scribble; Sdt, Stardust; STED, stimulated emission depletion; ZA, zonula adherens.
the apical membrane domain (Bilder et al., 2003; Johnson and Wodarz, 2003; Tanentzapf and Tepass, 2003).

Several molecular interactions between the Crb–Sdt and the PAR-3 (Baz)–PAR-6–aPKC complexes have been uncovered: PAR-6 can bind directly to Crb and to Sdt, although up to now no particular function or mechanism for this binding has been described (Wang et al., 2004; Kempekens et al., 2006). In addition, the cytoplasmic tail of Crb can be phosphorylated by aPKC at two conserved threonine residues, which is required for its proper localization and function (Sotillos et al., 2004).

In this study, we demonstrate a new and functionally important link between both complexes, the transient formation of a complex between Baz and Sdt. The stability of this complex is regulated through phosphorylation of Baz by aPKC, which triggers the dissociation of the Baz–Sdt complex and thus allows the formation of the Crb–Sdt complex. Our results provide mechanistic insight into the molecular interactions between Baz, aPKC, Sdt, and Crb during the establishment of plasma membrane polarity. Because all of the proteins we analyzed in this study are evolutionarily conserved in all higher animals regarding both their structure and function, we expect that this is also true for the mechanisms regulating their interactions that we uncovered in this work.

Results and discussion

Phosphorylation of S980 is required for proper subcellular localization of Baz

In mammalian epithelial cells, the overexpression of a version of PAR-3 that cannot be phosphorylated by aPKC-λζ (PAR-3S827A) causes defects in the formation of tight junctions and in the establishment of apical–basal cell polarity after calcium switch (Nagai-Tamai et al., 2002). S980 of Drosophila Baz, which corresponds to S827 of PAR-3, is also phosphorylated by aPKC (Kim et al., 2009), but no particular function has been described for this phosphorylation event so far. Therefore, we investigated whether phosphorylation of Baz by aPKC at S980 might be required for the proper subcellular localization and function of Baz. Using stimulated emission depletion (STED) microscopy, we have been able to determine the exact subcellular localization of Baz, Crb, and Sdt relative to each other with a resolution <50 nm, in contrast to the resolution limit of ~200 nm set by conventional confocal microscopy (Hell, 2009). Consistent with published data (Harris and Peifer, 2005), endogenous Baz as well as GFP-Baz (Fig. 1 a) always localized slightly basal to Crb (Fig. 1 b) and Sdt (not depicted), with a mean distance between the peaks of GFP-Baz and Crb of 268 ± 69 nm (n = 17). GFP-BazS980E (Fig. 1 a), which mimics constitutive phosphorylation of S980 of Baz, showed the same localization basal to Crb as wild-type Baz and GFP-Baz (Fig. S1 g). Staining with a phosphospecific antibody raised against a Baz peptide phosphorylated at S980 (Kim et al., 2009; Krahn et al., 2009) showed that this phosphorylated form of Baz only partially colocalized with the bulk of Baz and was concentrated in the most apical part of the region where Baz is localized (Fig. 1, f and g). In contrast, GFP-BazS980A (Fig. 1 a) did not have a defined localization with respect to Crb and Sdt and could frequently be found colocalized with or even apical of Crb and Sdt (Fig. 1 c). Collectively, these data indicate that phosphorylation of Baz at S980 is essential for the segregation of Baz at the ZA from the Crb–Sdt complex in the apical plasma membrane. This is consistent with our observation that GFP-Baz and GFP-BazS980E but not GFP-BazS980A rescued the lethality of embryos lacking maternal and zygotic baz expression.

Overexpression of nonphosphorylatable Baz phenocopies mutations in crb and sdt

To determine whether the failure of Baz to segregate from the Crb–Sdt complex affects embryonic development, we overexpressed GFP-BazS980A with the UAS-GAL4 system (Brand and Perrimon, 1993). Strong overexpression of GFP-BazS980A triggered the formation of mislocalized aggregates that contained all proteins of the apical junctional complexes that we investigated (Drosophila epithelial cadherin [DE-cadherin], Armadillo, α-catenin, PAR-6, aPKC, Crb, Sdt, PATJ, and Lin-7; Fig. 1 e, Fig. S1, and not depicted). In contrast, Dlg as a marker for the lateral plasma membrane domain was excluded from these aggregates and localized normally at the cortex (unpublished data). We do not think that the formation of aggregates upon GFP-BazS980A overexpression is caused by nonspecific segregation of apical components because we observed these aggregates only in epithelia that express Sdt and Crb and not in neuroblasts and oocytes, although in these cell types some apical components are present, including aPKC and PAR-6. Moreover, we did not observe the formation of aggregates in embryos overexpressing GFP-Baz or GFP-BazS980E, which are both fully functional and rescue baz loss of function mutations. Upon GFP-BazS980A overexpression, the morphology of the epithelial monolayer was disrupted (Fig. 2 a), the cells rounded up, and most of the cells died by apoptosis in late embryogenesis (Fig. 2 b and compare Video 1 with Video 2). These dominant-negative effects of GFP-BazS980A overexpression were cell autonomous because upon overexpression in stripes using the en::GAL4 driver, only cells within the stripes showed mislocalization of aPKC and Crb (Fig. 2 c). Deletion of the N-terminal CR1 domain or the three PDZ domains did not affect the dominant-negative phenotype of GFP-BazS980A overexpression (Fig. 1 a and not depicted). In contrast, overexpression of a GFP-BazS980A version lacking the region from aa 1097–1464, which is required for membrane targeting of Baz (Krahn et al., 2010), did not cause dominant-negative effects (Fig. 1 a and not depicted). Thus, we conclude that GFP-BazS980A has to be localized to the plasma membrane to induce a dominant-negative phenotype.

In embryonic neuroblasts, GFP-BazS980A localized to the apical cortex like wild-type Baz (Kuchinke et al., 1998), without affecting the localization of cell fate determinants, spindle orientation, asymmetric cell division, or viability of the flies (Fig. S2 a and not depicted). Oocyte polarity was not affected upon GFP-BazS980A overexpression, and the mutant protein localized correctly to the anterior cortex of the oocyte (Fig. S2 b).

Genetic interactions of nonphosphorylatable Baz with cell polarity regulators

Proper cell polarity is the prerequisite for the secretion of a contiguous cuticle by the epidermis at the end of embryogenesis.
Figure 1. GFP-BazS980A does not localize properly and causes the formation of protein aggregates when overexpressed. (a) GFP-tagged versions of Baz used in this study. + or − indicate whether overexpression of these variants of Baz causes the dominant-negative phenotype described in Overexpression of nonphosphorylatable Baz phenocopies mutations in crb and sdt. (b) STED imaging reveals localization of GFP-Baz basal to Crb in the embryonic ectoderm. (c) STED imaging reveals colocalization of GFP-BazS980A with Crb (arrow) and localization of GFP-BazS980A apical to Crb (arrowheads) in the embryonic ectoderm. (d) GFP-Baz localizes in dots at the apex of the lateral plasma membrane. (e) Overexpressed GFP-BazS980A localizes to aggregates containing additional proteins, including Crb and DE-cadherin, that are mislocalized to the cytosol. (f and g) Subcellular localization of Baz phosphorylated at S980. Embryos at stage 9 were stained with an antibody that recognizes Baz irrespective of its phosphorylation status (Baz) and with an antibody that recognizes Baz only when it is phosphorylated at S980 (pS980Baz). Note that in the epithelium, both signals overlap only partially, with pS980Baz staining (arrowheads) being enriched at the apical border of larger spots stained with the conventional Baz antibody (arrows). (g) pS980Baz staining is also detectable in the apical cortex of mitotic neuroblasts (asterisks). (d–g) Insets show overviews of the embryos from which the high magnification images were taken. Baz transgenes were overexpressed with da::GAL4. See also Fig. S1. Bars: (b and c) 1 µm; (d–g) 10 µm.
Baz is required for recruitment of Sdt to the plasma membrane

To investigate the functional interactions between Baz and the Crb–Sdt complex, we analyzed the subcellular localization of Baz, Crb, and Sdt in wild-type embryos and in embryos mutant for crb, sdt, and baz. In wild-type embryos at stage 6, Crb staining just started to become detectable, whereas Sdt was already robustly expressed and colocalized with Baz in the apical region of the lateral plasma membrane of the blastoderm epithelium (Fig. 4 a). From stage 7 onward, Sdt colocalized with Crb and partially also with Baz in all ectodermal epithelia (Fig. 4, b and c; Bachmann et al., 2001; Hong et al., 2001). In crb8F105 and crb11A22 mutant embryos at stage 8, a significant amount of Sdt remained colocalized with Baz in the apical region of the lateral plasma membrane (Fig. 4, d and e). In baz815-8 mutant embryos lacking both maternal and zygotic Baz at stage 7, neither Crb nor Sdt were detectable at the plasma membrane (Fig. 4 f and Fig. S3). In sdtK85 mutant embryos at stage 8, Crb was completely mislocalized, whereas Baz was still detectable in apical-lateral spots at the membrane (Fig. 4 g). Together, our data show that Baz is necessary for membrane localization of Sdt in the complete absence of Crb, whereas both Crb and Sdt are dispensable for the apical membrane localization of Baz at early stages of embryonic development.

Baz binds directly to the PDZ domain of Sdt

These data pointed to a function of Baz in the recruitment of Sdt to the membrane, independent of Crb. To test whether Baz directly binds to Sdt, we performed pull-down experiments with recombinant proteins expressed in Escherichia coli (Fig. 5 a). A His-tagged...
Phosphorylation of S980 of Baz weakens the binding between Baz and Sdt

To test whether the phosphorylation status of Baz might affect the interaction between Baz and Sdt, we treated embryo lysates with the phosphatase inhibitor cantharidin, which prevents dephosphorylation of several sites in the Baz protein, including S980 (Krahn et al., 2009). Compared with the DMSO-treated control, the coimmunoprecipitation of Sdt with Baz was strongly reduced upon cantharidin treatment (Fig. 5 c), which suggested that phosphorylation of Baz diminishes the binding affinity between Baz and Sdt.

To investigate whether phosphorylation of S980 of Baz specifically affects the binding between Baz and Sdt, we cotransfected GFP-Baz and GFP-BazS980A together with myc-tagged full-length Sdt and a fragment of Sdt consisting only of the myc-tagged PDZ domain also coimmunoprecipitated with GFP-Baz in S2R+ cells (Fig. 5, d and e). These findings exclude the possibility that in vivo Baz and Sdt interact only indirectly via PAR-6, which can bind to both Baz (Petronczki and Knoblich, 2001) and to a region at the N terminus of Sdt (Wang et al., 2004). We used the same assay to narrow down the region of Baz that is required for binding to Sdt in vivo. Deletion of the three PDZ domains of Baz did not affect binding of Baz to Sdt-PDZ-myc, whereas deletion of the C terminus of Baz (aa 969–1464) or internal deletion of the so-called aPKC-binding region (aa 968–996; Nagai-Tamai et al., 2002) completely abolished the binding of Sdt-PDZ-myc to Baz (Fig. 5 e). Together, these data show that Baz and Sdt bind directly to each other, both in vitro and in vivo. The interaction is mediated by the PDZ domain of Sdt and depends on the presence of the region between aa 968 and 996 of Baz, which contains the aPKC phosphorylation site S980.
**Conclusions**

Based on our results, we propose the following model for the interaction between the PAR-3 (Baz)–PAR-6–aPKC complex and the Crb–Sdt complex during the establishment of apical–basal cell polarity in early embryogenesis. During cellularization, shortly before Crb expression starts, Baz and Sdt form a...
complex that localizes to the apical region of the lateral plasma membrane. In this complex, the PDZ domain of Sdt binds to the region surrounding S980 of Baz, which is a phosphorylation target of aPKC. As long as S980 is not phosphorylated by aPKC, this complex is stable, and the PDZ domain of Sdt is not available for binding to the C terminus of Crb. Upon phosphorylation of S980 of Baz by aPKC, the binding between Baz and Sdt becomes weaker, causing the dissociation of the Baz–Sdt complex at the ZA and releasing Sdt for binding to Crb. This mechanism provides an explanation for the enrichment of the Crb–Sdt complex
in the immediate vicinity of the ZA because Baz initially recruits Sdt to the ZA and then releases it locally for binding to Crb, which localizes to the apical plasma membrane domain. Whether there is direct competition between Baz and Crb for binding to Sdt remains to be further investigated. The separation of the ZA from the adjacent apical membrane domain may then be achieved by the recruitment of aPKC and PAR-6 to the Crb–Sdt complex via binding of PAR-6 to Sdt or directly to Crb (Wang et al., 2004; Kempkens et al., 2006). When the dissociation of the Baz–Sdt complex is blocked, for instance by overexpression of GFP-BazS980A, the Crb–Sdt complex cannot form, which results in phenotypes very similar to those of crb or sdt loss of function mutations (Tepass et al., 1990; Bachmann et al., 2001; Hong et al., 2001; Kim et al., 2009). This hypothesis is supported by the observation that the cuticle phenotype of GFP-BazS980A overexpression was strongly suppressed by concomitant reduction of Lgl or Scrib activity, as was reported for crb and sdt mutant phenotypes (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Also consistent with this model is our observation that aPKC mutant embryos derived from germline clones, in which phosphorylation of S980 of Baz cannot occur, exhibit a very similar epithelial phenotype as embryos mutant for crb or sdt (Kim et al., 2009). We do not think that the dominant-negative phenotype of GFP-BazS980A overexpression is primarily caused by altered binding of aPKC to Baz or misregulation of aPKC kinase activity because, in this case, we would also expect dominant-negative effects upon overexpression of GFP-BazS980A in neuroblasts, in which the regulation of aPKC kinase activity is crucial for asymmetric cell division (Betschinger et al., 2003; Wirtz-Peitz et al., 2008).

Two recently published manuscripts also reported dominant-negative phenotypes in epithelial development similar to the ones we describe in this study upon overexpression of GFP-BazS980A (Morais-de-Sá et al., 2010; Walther and Pichaud, 2010). However, these phenotypes were attributed predominantly to the effect of phosphorylation of BazS980 by aPKC on the binding affinity between aPKC and Baz. According to their model, phosphorylation of Baz by aPKC leads to the dissociation of the Baz–aPKC complex, which triggers the segregation of Baz to the ZA and of aPKC and PAR-6 to the apical membrane domain. Although our data also underline the importance of the phosphorylation of Baz at S980 by aPKC, our model goes beyond the one proposed by Morais-de-Sá et al. (2010) and Walther and Pichaud (2010) by showing that all of the phenotypes observed upon overexpression of GFP-BazS980A can be explained by the phosphorylation-dependent binding of Sdt to Baz.

It has recently been proposed that phosphorylation of mammalian PAR-3 by aPKC-λ1ζ is required for separation of PAR-3 from aPKC and PAR-6, which is the prerequisite for apical domain formation in mammalian epithelia (Horikoshi et al., 2009; McCaffrey and Macara, 2009). One of these studies furthermore proposed that binding of aPKC and PAR-6 to PAR-3 may be an important intermediate step to recruit aPKC and PAR-6 to the membrane before they dissociate from PAR-3 and bind to other apical membrane–anchoring factors such as Cdc42 or the Crb–PALS-1 complex (Horikoshi et al., 2009). Our results are conceptually similar but further extend this model by demonstrating for the first time the direct interaction between Baz and Sdt. These findings represent an important advancement in our understanding of the molecular mechanisms that control the establishment of apical–basal cell polarity in the Drosophila ectoderm. To fully understand this process, it will be important to know how aPKC is activated in early embryogenesis and how the phosphorylation of Baz changes the binding interface between Baz, Sdt, and aPKC. Because all of the components of the molecular mechanism that we describe in this study are conserved in evolution, we are eager to see whether their interactions are regulated in a similar way during the polarization of mammalian epithelia.

Materials and methods

Fly stocks and genetics

The following mutant alleles were used in this study: crb$^{1422}$ (Jürgens et al., 1984), crb$^{505}$ (Wodarz et al., 1993), sdt$^{K85}$ (provided by K. Kolmakov and V. Belov, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany), sheep anti-β1 integrin (provided by D. Bilder, University of California, Berkeley, Berkeley, CA; Mechler et al., 1985; Bilder et al., 2003; Tanentzapf and Tepass, 2003), and scrib$^{2}$ (Bilder et al., 2003; Tanentzapf and Tepass, 2003). baz$^{54}$ (McKim et al., 1996; Krahm et al., 2010) germline clone embryos were obtained using the Flipase recombination target–dominant female sterile method (Chou and Perrimon, 1992). UAS::GFP-Baz transgenes were generated using standard germline transformation. da::GAL4, en::GAL4, arm::GAL4, nos::GAL4, and wp::GAL4 driver lines were obtained from the Bloomington Drosophila Stock Center.

Immunohistochemistry

Embryos were fixed in a 1:1 mixture of 4% formaldehyde, phosphate buffer, pH 7.4, and heptane for 20 min. After removal of the vitelline envelope by vigorous shaking in a 1:1 mixture of methanol and heptane, embryos were rehydrated in PBS and 0.1% Tween 20 (PBT) for 20 min and then incubated with primary antibodies in PBT and 5% normal horse serum. The primary antibodies used were rabbit anti-PKCζ C20 (1:1,000; Santa Cruz Biotechnology, Inc.), rabbit anti-Baz (1:1,000; Wodarz et al., 1999), rabbit anti–Baz phospho-S980 (1:100; Krahm et al., 2009), mouse anti-Crb C4d (1:50; Developmental Studies Hybridoma Bank; Tepass and Knust, 1993), mouse anti-sdt (1:20; provided by E. Knust; Berger et al., 2007), rabbit anti–Lin-7 (1:500; provided by E. Knust; Bachmann et al., 2004), rabbit anti-PAT (1:1,000; provided by E. Knust; Richard et al., 2006), guinea pig anti-Mira (1:1,000; Kim et al., 2009), rat anti–DE-cadherin DCAD2 (1:20; Developmental Studies Hybridoma Bank; Oda et al., 1994), mouse anti-Dlg 4F3 (1:50; Developmental Studies Hybridoma Bank), rabbit anti–Staufen (1:1,000; provided by D. Johnston, Gurdan Institute, Cambridge, England; UK; St Johnston et al., 1991), mouse anti-Gurken 1D12 (1:10; Developmental Studies Hybridoma Bank), and mouse anti-GFP 3E6 (1:1,000; Invitrogen). DNA was stained with DAPI (Invitrogen). Secondary antibodies conjugated to Cy2 and Cy3 were obtained from Jackson ImmunolResearch Laboratories, Inc. Secondary antibodies conjugated to Alexa Fluor 647 were obtained from Invitrogen. After repeated washing in PBT, embryos were mounted in Mowiol 4-88 (Polysciences Europe) supplemented with 1,4-diazabicyclo (2.2.2) octane (DABCO). TUNEL assays, for detection of cell death in situ, were performed with an in situ cell death detection kit (Roche) according to the manufacturer’s instructions (Wang et al., 1999). Images were taken on a confocal microscope (LSM 510 Meta; Carl Zeiss, Inc.) using 25× NA 0.8 Plan-Neofluar and 63× NA 1.4 Plan-Apochromat objectives and processed using Photoshop (Adobe).

STED microscopy

Embryos were fixed and incubated with primary antibodies as described in the previous section before being incubated with the following secondary antibodies: ATO 594 (Ato-TEC GmbH) goat anti–rabbit IgG (dianova GmbH) and KC 114 (provided by K. Kolmakov and V. Belov, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany). sheep anti–IgG (dianova GmbH). Two-color STED images were recorded with a custom-built STED microscope that combined two pairs of excitation and STED laser beams all derived from a single supercontinuum fiber laser source similar to the one described previously (Wildanger et al., 2008). Excitation wavelengths were 570 ± 5 nm (ATTO 594) and 650 ± 3 nm (KK 114), and STED wavelengths were 720 ± 20 nm (ATTO 594) and 755 ± 20 nm (KK 114). The fluorescence was detected in the spectral ranges of 600–640 nm for
from onic neuroblasts and in oocytes. Fig. S3 is related to Fig. 4 and shows that GFP-BazS980A localizes like wild-type Baz in embry-

tion of GFP-BazS980E in the embryonic epidermis. Fig. S2 is related to Fig. S1 is related to Fig. 1 and shows the mislocalization of PATJ, Sdt, and

Online supplemental material

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overexpressing GFP-BazS980A. Online supplemental material is available

Fig. 2 and shows the dramatically abnormal development of an embryo overexpressing GFP-Baz. Video 2 is related to

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