Isoforms of the Polarity Protein Par6 Have Distinct Functions*

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PAR-6 is essential for asymmetric division of the Caenorhabditis elegans zygote. It is also critical for cell polarization in many other contexts throughout the Metazoa. The Par6 protein contains a PDZ domain and a partial CRIB (Cdc42/Rac interactive binding) domain, which mediate interactions with other polarity proteins such as Par3, Cdc42, Pals1, and Lgl. A family of mammalian Par6 isoforms (Par6A–D) has been described, but the significance of this diversification has been unclear. Here we demonstrate that Par6 family members localize differently when expressed in Madin-Darby canine kidney epithelial cells and have distinct effects on tight junction (TJ) assembly. Par6A localizes to the cytosol and inhibits TJ formation, but Par6A co-localizes predominantly with the TJ marker ZO-1 at cell-cell contacts and does not affect junctions. These functional differences correlate with differences in Pals1 binding; Par6B interacts strongly with Pals1, whereas Par6A binds weakly to Pals1 even in the presence of active Cdc42. Pals1 has a low affinity for the isolated CRIB-PDZ domain of Par6A, but analysis of chimeras showed that in addition Pals1 binds with affinity for the isolated CRIB-PDZ domain of Par6A, and even in the presence of active Cdc42. Par6A contains a PDZ domain and a partial CRIB domain, both of which are required for binding to Par6 and Cdc42-GTP. The N terminus of Par6 interacts directly with the regulatory domain of PKCα and PKCζ (3, 4, 10).

Recently, two additional binding partners for Par6 have been identified. One is Pals1, which is a component of a conserved polarity complex containing the transmembrane protein Crumbs (Crb), and Patj, a multi-PDZ domain protein (11, 12). This complex is also localized to TJs, as is the Par complex, and the overexpression of Crb (or of fragments of Pals1 or Patj) interferes with TJ assembly. Par6 interacts directly with the N terminus of Pals1 through the CRIB-PDZ region, and importantly, this interaction is regulated by Cdc42 (12). Another binding partner is Lgl, which is also involved in cell polarization. Lgl interacts directly with Par6 and can be phosphorylated by aPKC (13, 14). Thus, Par3, Pals1, and Lgl are physically linked through Par6 to signaling pathways that converge on the Cdc42 GTPase.

Whereas only one par-6 gene exists in Drosophila and C. elegans, a family of four is present in mammals, designated as Par6A–D (3) (although no full-length clone of Par6D has yet been identified). However, the significance of this diversification has been unclear. We now demonstrate that Par6 isoforms A–C localize differently when expressed in MDCK epithelial cells and have distinct effects on TJ formation. These functional differences correlate with differences in Pals1 binding, suggesting that Par6 family members have distinct functions in mammalian cells, which are mediated by differential targeting and effector interactions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Processing**—MDCK II and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) plus 10% Hyclone fetal bovine serum (5% fetal bovine serum, 5% calf serum for COS-7) plus 1 mM sodium pyruvate and penicillin/streptomycin. MDCK cells were transiently transfected with different constructs using Effectene (Qiagen) or electroporation (Amamax). COS-7 cell transfections, immunoprecipitations, immunoblotting, and immunofluorescence assays were performed as described previously (3, 16). The MDCK cell lines stably expressing Myc-Par6B(102–371) have been described before (5). Images were collected using a Nikon TE200 wide field microscope or a Zeiss Pascal laser-scanning confocal microscope. Primary antibodies were monoclonal anti-Myc 9E10 (1/500), monoclonal anti-IA 12CA5 (1/500), polyclonal anti-ZO-1 (1/500), monoclonal anti-occludin (1/500), polyclonal anti-claudin-1 (1/500) (all from Zymed Laboratories Inc.), polyclonal anti-Par6 (1/100), and polyclonal anti-Pals1 (1/50). The Par6 and Pals1 antibodies were produced in rabbits against recombinant proteins (Cocalico Biologicals). Secondary antibodies were coupled to Alexa Fluor 594 or 488 (Molecular Probes). Calcium switch and transepithelial resistance measurements were performed as previously described (5, 15).

**DNA Constructs**—Par3ΔA, -B, and -C constructs were described previously (3). Deletion mutants and chimeras of Par6 were generated by PCR. Single amino acid mutations were made using the QuickChange site-directed mutagenesis kit (Stratagene).
Distinct Functions of Par6 Isoforms

In Vitro Binding Assays—Purified GST-Pals1-(1–181) or GST alone (2 μg each) was bound to glutathione-Sepharose beads and incubated for 30 min at 4 °C with in vitro translated 35S-labeled Par6 proteins (TnT-coupled wheat germ system, Promega). Wheat germ lysates were used because plants do not possess Par gene homologues, so the in vitro translated proteins are unlikely to bind to bridging proteins or to other factors in the incubation mix. After washing, bound proteins were eluted in SDS sample buffer, separated by SDS-PAGE, and subjected to autoradiography.

RESULTS

The reasons for the diversification of the Par6 family in vertebrates have not been understood. Therefore, to address this issue, we compared the subcellular distribution and biochemical properties of the mammalian Par6 isoforms. Unexpectedly, HA-tagged Par6A, -B, and -C did not give identical staining patterns when expressed in MDCK epithelial cells. (Par6D was not examined because no full-length cDNA has yet been identified.) Par6A co-localized predominantly with the tight junction marker ZO-1 at cell-cell contacts, whereas Par6B was distributed throughout the cytoplasm and disrupted ZO-1 localization (Fig. 1, A and B), as previously described (3, 5). Par6C partially localized to cell-cell contacts and, like Par6A, had no effect on ZO-1 localization (Fig. 1A). Replacement of the HA tag with Myc or other tags did not alter localization patterns (data not shown). Expression of Par6B has been found to inhibit the assembly of many TJ components, including the transmembrane proteins occludin and claudin-1 (5). Therefore, to further determine the effect of Par6A on TJ structure, we examined occludin, claudin-1, and two peripheral components, Par3 and Pals1. As shown in Fig. 1C, the junctional associations of these proteins were all unaffected by Par6A expression.

We surmised that these isoform differences might reflect differences in the interactions with known Par6 binding partners. However, all three isoforms bound with similar efficiencies to Par3, nLgl1/2, and PKCζ (Fig. 1D), but although both Par6B and Par6C showed strong interactions with Pals1 in the presence of a constitutively active Cdc42 (Cdc42L61 mutant), Par6A bound weakly to Pals1 (Fig. 1E). A more subtle difference was also apparent between the B and C isoforms, because although Cdc42-GTP enhanced the binding of both Par6B and -C to Pals1, Par6B exhibited stronger basal binding in the presence of an inactive Cdc42 mutant (Cdc42N17).

Pals1 localizes at TJs and is important for TJ assembly (12, 17). This raised the possibility that Par6B inhibits TJ formation by sequestering Pals1 away from cell-cell contacts. To test this hypothesis, we transfected HA-Par6A into control MDCK cells or MDCK cells stably expressing Myc-tagged Par6B-(102–371) (ΔNPar6B). Cells were subjected to a calcium switch and TJ re-assembly was monitored. We have shown previously that ΔNPar6B interacts with Pals1 and that it delays the reassembly of TJs after calcium depletion (5, 12). Overexpression of Pals1 increased the peak of transepithelial resistance in control cells (Fig. 2A), which was consistent both with the idea that Pals1 plays an important role in TJ formation and with the observation that loss of Pals1 in MDCK cells reduces the transepithelial resistance (17). When expressed in ΔNPar6B cells, Pals1 partially rescued the delay (caused by ΔNPar6B) in assembly of TJs after calcium re-addition. This was shown by improved transepithelial resistance development (Fig. 2A) and by a more uniform association of ZO-1 with cell-cell junctions (Fig. 2, B and C). Similar results were obtained using transient co-transfection of Par6B and Pals1 (not shown). These data indicate that the inhibition of TJ assembly by Par6B is mediated at least partially through sequestration of Pals1.

Because Par6A and B exhibit distinct properties in cellular localization and Pals1 binding, we focused on these two isoforms. The Par6 family contains a highly conserved CRIB and PDZ domain, but the N and C termini differ considerably (3). To determine which region of Par6 is responsible for the differential binding to Pals1, we made several Par6B/A chimeras (Fig. 3A) and tested their ability to interact with Pals1 in an in vitro binding assay. GST alone or GST-Pals1-(1–181), the region necessary and sufficient to bind Par6B, was attached to glutathione-Sepharose beads and incubated with in vitro translated, 35S-labeled Par6A/B chimera proteins. Interestingly, those proteins that contain the N terminus of Par6A (wild type Par6A, Par6A/B/A, Par6A/B/A, Par6A/B/B, and Par6A/A/B) were all unable to bind Pals1-(1–181). However, those chimeras that either contain the N terminus of Par6B (wild type Par6B, Par6B/A/B, and Par6B/A/A) or lack the N terminus of Par6A, such as Par6A/B/A, were competent to bind Pals1-(1–181) (Fig. 3B). Therefore, we conclude that the N terminus of Par6A blocks association with Pals1. Nonetheless, Par6B/A/A showed a much weaker interaction with Pals1 than the Par6B/A/A chimera. These two chimeras have the same N and C termini but different middle regions that contain CRIB and PDZ domains from Par6A and Par6B, respectively. This difference suggested that the middle region also contributes to the differential recognition of Pals1.

To address this point, we first further characterized the interaction between Par6B and Pals1. This interaction is mediated by the N terminus of Pals1 and requires the PDZ domain of Par6B, because a mutation in the Par6B PDZ domain significantly reduces the binding to Pals1 (12). Here, we show that the isolated PDZ domain of Par6B is both necessary and sufficient for interaction with Pals1. A partial deletion or mutation of the PDZ domain led to loss of binding to Pals1 (Fig. 3C). However, by in vivo immunoprecipitation, where we co-expressed a dominant active Cdc42 with different Par6B fragments, we found that the adjacent CRIB motif was essential for enhanced binding to Pals1 induced by Cdc42-GTP. Thus, binding of the Par6B-(126–253) fragment, which contains both the CRIB and PDZ domains, is enhanced by Cdc42-GTP (Fig. 3D), but the Par6B-(140–253) fragment, which contains only the PDZ domain and exhibited similar basal binding to Pals1, is not up-regulated by Cdc42. These results are consistent with the idea that Cdc42 binding induces a conformational change in Par6 that regulates effector binding (18, 19).

Subsequently, we compared the binding of Pals1 to the CRIB-PDZ region of Par6A and Par6B. Pals1 had a very low affinity for the isolated CRIB-PDZ region of Par6A compared with that of Par6B (Fig. 3E). Thus, in addition to the inhibitory property of the N terminus of Par6A, the intrinsic low affinity of the Par6A CRIB-PDZ region for Pals1 further reduces the ability of Par6A to interact with Pals1.

The CRIB-PDZ domains of Par6 are highly conserved. Only a small number of non-conserved residues are present in this region (Fig. 3F). Mutation of Cys-161 in Par6B to the corresponding residue Tyr in Par6A greatly reduced its interaction with Pals1 (Fig. 3G). However, the reciprocal Tyr to Cys mutation in Par6A failed to increase the binding to Pals1, suggesting that this Cys residue alone is not sufficient to confer the high affinity binding. We also tried to mutate other residues not conserved in Par6A but were unable to find any single amino acid mutation that could increase the binding of Par6A to Pals1. These data suggest that a limited number of residues within the CRIB-PDZ regions of the Par6 family regulates the specificity of interactions with its effectors. However, the rules that determine these interactions are complex and are not governed by single amino acid differences.

Finally, we generated a Par6A mutant, Par6AΔPro, which is analogous to Par6BΔPro and does not bind to Cdc42-GTP (Fig. 4A). This mutant still localized efficiently to cell-cell contacts,
Fig. 1. Differential localization and binding properties of Par6 isoforms. A, Par6 isoforms show distinct distributions when expressed in MDCK cells. MDCK cells were transiently transfected with different HA3-tagged Par6 isoforms as indicated. Cells were fixed and stained for the HA epitope and ZO-1. Insets show close-ups of borders between transfected cells and neighboring cells. B, relative fluorescence intensity of Par6A and Par6B at the cortex and cytosol in MDCK cells. A 29 x 29 pixel area across the cell border was selected, and the mean fluorescence intensity along the x axis was measured using ImageJ software. Left panel shows representative profiles of Par6A and Par6B fluorescence intensity across a cell border. Fluorescence intensities at the cortex (pixel 14) and 5 pixels away at the cytosol (pixel 9) were referred to as $F_{\text{cortex}}$ and $F_{\text{cytosol}}$, respectively. Right panel shows the ratio of $F_{\text{cortex}}/F_{\text{cytosol}}$ normalized to $F_{\text{cytosol}}$. Error bars show standard deviation ($n=10$ cells). C, Par6A overexpression does not affect TJ structure. MDCK cells were transiently transfected with Par6A and fixed and stained for different tight junction components. D, Par6A interacts with Par3, mLgl, and PKCζ. COS-7 cells were co-transfected with plasmids encoding different HA3-tagged Par6 isoforms and Myc-Par3. HA3-Par6 was immunoprecipitated (IP) and blotted for the Myc epitope as well as endogenous mLgl and PKCζ. E, Par6A interacts very weakly with Pals1. COS-7 cells were co-transfected with plasmids encoding different Myc-tagged Par6 isoforms and HA3-Pals1 in the presence of Cdc42L61 or Cdc42N17 as indicated. Myc-Par6 was immunoprecipitated and blotted for the HA3 epitope.
indicating that the localization of Par6A is independent of Cdc42 (Fig. 4B).

**DISCUSSION**

There are several notable aspects to the data presented above. First, although Pals1 is associated with tight junctions in epithelial cells and can bind to Par6B, it does not effectively recruit this Par6 isoform to the junctions. Second, even though Par6A binds more weakly to Pals1, it does co-localize with TJ proteins. Third, this localization of Par6A is independent of Cdc42, and fourth, the N-terminal domain of Par6A inhibits binding of Pals1 to the PDZ domain but does not affect the interactions with other binding partners such as mLgl, Par3, and aPKC. We note that there are some discrepancies in this study with data from other laboratories, because Yamanaka et al. (14) observed a weak interaction of Par6C (also called Par6/H9251) with mLgl. However, Plant et al. (13) reported that, consistent with our data, Par6A–C family members could all interact efficiently with this downstream target. There is general agreement that Par3 binds to all three forms of Par6. Par3

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**Fig. 2.** Pals1 partially rescues the tight junction formation defects caused by Par6B overexpression in MDCK cells. Control (con) MDCK cells or MDCK cells stably expressing Myc-ΔNPar6B were transiently transfected with vector alone or Myc-Pals1 by electroporation. Cells were plated on Transwell® filters (A) or Lab-Tek chamber slides (B) and subjected to calcium switch one day after transfection. A, kinetics of transepithelial resistance (TER) development was monitored for 24 h following calcium re-addition. B, cells were fixed and stained for ZO-1 6 h or 12 h after calcium re-addition. C, quantitative analysis of TJ assembly showing percentage of cells that have complete peripheral ZO-1 staining 6 h or 12 h after calcium addition (mean ± S.D., n = 100). Two independent Myc-ΔNPar6B cell lines were examined.
In this figure, the molecular basis for the differential interactions of Par6A and B to Pals1 is explored. 

**A** Schematic representations of Par6A/B chimeras used in the binding assay. The sequence of Par6A is shown in gray and the sequence of Par6B is shown in black. N terminus of Par6A inhibits the interaction with Pals1. Purified GST-Pals1-(1-181) or GST alone was bound to glutathione-Sepharose beads and incubated with in vitro translated, 35S-labeled Par6A/B chimera proteins. After washing, the bead-associated proteins were separated by SDS-PAGE and subjected to autoradiography.

**B** PDZ domain of Par6B is necessary and sufficient for binding to Pals1. In vitro binding assay was performed as described (B). The Par6B-(102–271) PDZ mutant (mut.PDZ) harbors K167A/P168A/L169A/G170A mutations. The schematic domain structure of Par6B is presented below.

**C** The CRIB motif of Par6B is required for enhanced interaction with Pals1 in the presence of active Cdc42. COS-7 cells were co-transfected with plasmids encoding different HA 3-tagged Par6B constructs and Myc-Pals1 in the absence or presence of Cdc42L61 as indicated. HA 3-Par6 was immunoprecipitated (IP) and blotted for the Myc epitope.

**D** The isolated CRIB-PDZ domain of Par6A has very low affinity for Pals1. The in vitro binding assay was performed as described for B. wt, wild type.

**E** Sequence alignment of Par6A, -B, and -C isoforms. Identical residues are shown in black, and closely related residues are shown in gray. The CRIB and PDZ domains are indicated with solid lines. The residue contributing to the binding affinity of Par6B to Pals1 is marked with an asterisk.
might reflect the fact that activated Cdc42 is required to stabilize this interaction. Although Cdc42-GTP appears transiently during junction assembly, there is little evidence that it is essential, and the expression of a dominant-negative mutant of Cdc42 does not block TJ formation (5). These data are consistent with our observation that the association of Par6A with the junctions is also independent of Cdc42 binding. Taken with the fact that all three Par6 isoforms can bind with similar efficiency to Par3, Lgl, and aPKCs, the results indicate that Par6A targeting to junctions involves another, currently unknown binding partner.

Pals1 binding to Par6A is blocked by an inhibitory property of the N terminus. Presumably, this inhibition is independent of the ability of the N terminus to bind aPKCs, which is common to all forms of Par6. An attractive model is that the N terminus folds back and interacts with the CRIB-PDZ domain in all Par6 isoforms, but the Cdc42-GTP binding releases it from Par6B and -C, thereby exposing the Pals1 binding site, whereas in Par6A this self-interaction is too stable to be released. The isolated CRIB-PDZ domain of Par6A also has a reduced affinity for Pals1 as compared with Par6B despite the high sequence similarity between these two domains. Site-directed mutagenesis revealed that Cys-161 in Par6B contributes to Pals1 binding. However, we were unable to find any single residue that could confer high affinity Pals1 binding to the PDZ domain of Par6A.

These data demonstrate that the Par6 family has evolved to interact differentially with the Pals1/Crb/Patj polarity complex and that at least one unknown, isoform-specific binding partner exists that targets Par6A to cell junctions.

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