Phosphoinositide Binding by Par-3 Involved in Par-3 Localization

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ABSTRACT. Electrostatic interactions between lipids and proteins control many cellular events. We found that phospholipids, including phosphatidylinositol 3-phosphate, phosphatidylinositol 4,5-bisphosphate, and phosphatidylinositol 3,4,5-triphosphate, bound to the C-terminal coiled-coil region of par-3 at conserved, basic residues. We identified K1013 and K1014 as the phosphoinositide binding site, because the K1013E/K1014E mutation of rat par-3 abolished its lipid binding. Importantly, the K1013E/K1014E par-3 mutant exhibited significantly weaker localization at the cell-cell junctions than the wild-type par-3. Fluorescence recovery after photo-bleaching analyses confirmed the faster turnover of mutant par-3 at cell-cell junctions. The treatment of cells with an inhibitor of phosphatidylinositol 3-kinases partially increased the turnover of par-3. These data suggested that the putative phospholipid binding by par-3 is important for its localization at cell-cell junctions.

Key words: membrane binding protein/phosphoinositides/cell polarity/par-3

Introduction

The asymmetric distribution of phosphoinositides contributes to the development of three-dimensional cysts of epithelial cells. Phosphatidylinositol 3,4,5-triphosphate (PI(3,4,5)P₃) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) are reportedly localized at the baso-lateral and apical surfaces of MDCK cells, respectively (Martin-Belmonte et al., 2008). The asymmetric distribution of phosphoinositides is thus suggested to be necessary for the establishment of cell polarity in cyst formation (Gassama-Diagne et al., 2006). Interestingly, the border of the localization of PI(3,4,5)P₃ and PI(4,5)P₂ is the tight junction that segregates the apical and baso-lateral membranes.

Partition defective proteins, such as par-3, play an essential role in the establishment of cellular polarity (Macara, 2004; Suzuki and Ohno, 2006). par-3 is localized at the border between PI(3,4,5)P₃ and PI(4,5)P₂, i.e., the tight junction of the cyst, where par-3 functions with atypical protein kinase C (aPKC) and par-6 to establish the apical specification of the cyst (Horikoshi et al., 2009; Macara, 2004; Suzuki and Ohno, 2006).

There are three PDZ domains in par-3. Among them, the second PDZ domain reportedly binds to charged lipids through its surface potentials (Wu et al., 2007). Interestingly, the third PDZ domain binds to PTEN, providing the molecular link between par-3 and phosphoinositides (von Stein et al., 2005).

Recently, the C-terminal coiled-coil region of par-3 from Drosophila was reported to bind to various phospholipids, including PI(3,4,5)P₃ and PI(4,5)P₂ (Krahn et al., 2010; Simoes Sde et al., 2010). In this work, we examined the lipid binding by the rat par-3 coiled-coil region, and found that it binds to various phosphoinositides and phosphatidylserine.

Materials and Methods

Recombinant proteins

The maltose binding protein (MBP)-par-3 PDZ domain (aa 237–708) and C-terminal coiled-coil region (aa 949–1337) (rat) fusions were expressed in Escherichia coli, according to the manufacturer’s
(Stratagene) instructions. The MBP fusion proteins were eluted from the affinity column in buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM MgCl\(_2\), 0.5 mM EDTA and 5 mM 2-mercaptoethanol) supplemented with 20 mM maltose. The His-tagged wild type and K1013E/K1014E mutant of the coiled-coil region of par-3 were expressed in Sf9 cells as His-tagged proteins, and were purified by chromatography on nickel-NTA-agarose (Qiagen). The proteins were eluted with buffer containing 10 mM Tris-HCl [pH 8.0], 300 mM NaCl, 30% glycerol, 1 mM PMSF, and 0.001% leupeptin, supplemented with 300 mM imidazole.

**Liposome preparation**

Liposomes were prepared from total bovine brain lipids (Folch fraction 1; Avanti Polar Lipids) or synthetic phosphoinositides (Echelon Biosciences) with egg PC and PE (Avanti Polar lipids). Lipids solubilized in chloroform or chloroform-methanol were dried under nitrogen gas in glass test tubes, and were reuspended in buffer containing 25 mM Heps, pH 7.5, 100 mM NaCl, and 0.5 mM EDTA by mixing with a vortexer, and then hydrated at 37°C for 1 hr.

**Liposome co-sedimentation assay**

Liposome co-sedimentation assays were performed as follows. To remove the aggregated proteins, the purified proteins were subjected to centrifugation at 100,000×g for 30 min at 25°C in a TL100 rotor (Beckman). Proteins, at the indicated concentrations, were incubated with liposomes in 50 µl XB for 20 min at room temperature, and then centrifuged at 100,000×g for 30 min at 25°C in a TL100 rotor. Supernatants and pellets were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gels were stained with Coomassie Brilliant Blue. Kd values were estimated using the Solver of Excel program (Microsoft) with the equation Bound protein \( (x) = (\text{Bound protein}_{\max} - \text{Bound protein}_{\min}) \cdot (1-e^{-kt}) + \text{Bound protein}_{\min} \) where \( x \) is the concentration of phosphoinositides. The constant (k) was calculated from the fitted curves, and was used to calculate the Kd, with the equation Kd=ln2/k. The Bound protein\(_{\max}\) was assumed to be 100%, and the Bound protein\(_{\min}\) was assumed to be 15%, the value with PE/PC liposomes. Data in the graphs are presented as means and standard errors of the mean [SEM]

**Cell culture and transfection**

Madin-Darby canine kidney (MDCK) cells were cultured in Dulbecco’s modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, penicillin, and streptomycin. The green fluorescent protein (GFP)-par-3 proteins were prepared by subcloning the rat par-3 cDNA into the pEGFP-N3 vector (Clontech). Transfection was performed with the Lipofectamine LTX and PLUS reagents (Invitrogen), according to the manufacturer’s protocols.

**Antibodies, cell staining, and confocal microscopy**

MDCK cells were fixed with 3.7% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 and 1% BSA in TBS, and blocked in 1% BSA in TBS. MDCK cells were labeled with Alexa633-conjugated phalloidin. Fluorescence images were obtained via confocal microscopy (Olympus Fluoview 1000D) at room temperature. The 100× oil immersion objective (NA=1.45; Olympus) was used.

**FRAP analysis**

All FRAP experiments were performed on a confocal microscopy system (Olympus Fluoview 1000D) at room temperature. The 100× oil immersion objective (NA=1.40; Olympus) was used. Images were recorded with laser power settings of 1% for 473 nm (GFP). Two prebleached images were acquired before the GFP signal was bleached, using 2 iterations of the 473 nm laser at 100% power, followed by the acquisition of images with the laser powers described above. The images were despeckled, and then the background was subtracted. The resulting background-subtracted data were then normalized to the first pre-bleached image. Kinetic modeling was performed using the Solver of Excel program (Microsoft) with the equation Fluorescence (t)=Fluorescence\(_{\max}\)·(1–e\(^{-kt}\)) \cdot (1–e\(^{-kt}\))\cdot Fluorescence\(_{\min}\). The rate constant of recovery (k) and the maximum recovery compared to the first pre-bleached image (% recovery) were calculated from the fitted curves. The rate constant of recovery was used to calculate the half time, t\(_{1/2}\)=ln2/k. Data in the graphs are presented as means and standard errors of the mean [SEM] (Weisswange et al., 2009).

**Results**

**Phospholipid binding domain in par-3**

The C-terminus of par-3 contains a coiled-coil region including several conserved, basic residues, which may compose a lipid binding motif. Therefore, we prepared the C-terminal portion of rat par-3, as a recombinant MBP fusion protein expressed in E. coli (Fig. 1A). We also prepared the PDZ fragment of par-3. We prepared protein-free liposomes from the brain Folch fraction, and the binding of the PDZ domain and C-terminal domains was examined. The binding of the PDZ domain to the Folch liposomes was very weak, whereas that of the C-terminal region was easily detectable (Fig. 1B).

We then examined the amino-acid sequence of the C-terminal coiled-coil region of par-3. It contains many basic amino-acid residues; however, most of them had acidic amino-acid residues nearby. Therefore, the charges of these amino-acid residues were considered to be neutralized. However, Lys1013 and Lys1014 are conserved, basic
amino-acid residues without adjacent acidic amino-acid residues (Fig. 1C). These two lysine residues are conserved among species (Fig. 1C).

To examine the contribution of these two lysines to lipid binding, we prepared the mutant with glutamic acid substitutions. The wild type (WT) and K1013E/K1014E mutant of the C-terminal coiled-coil region of par-3 were expressed in insect cells and then purified. These proteins were subjected to a co-sedimentation assay with Folch liposomes (Fig. 1D). The K1013E/K1014E mutant of the par-3 coiled-coil region exhibited significantly decreased binding to the Folch liposomes, as compared to the WT par-3 coiled-coil region.

**Phosphoinositide binding by the C-terminal domain of par-3**

We then examined the phospholipid species bound by par-3. As expected from the binding of the coiled-coil region to the liposomes made from the Folch fraction, which is rich in phosphatidylinerine, the coiled-coil region binds more strongly to phosphatidylinerine (PS) containing liposomes than to those made of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fig. 2). The C-terminal coiled-coil region also binds to various phospholipids, such as phosphatidic acids (PA) and phosphatidylinositol (PI), but more strongly to PI(3)P, PI(5)P, PI(3,4)P₂, PI(4,5)P₂, and PI(3,4,5)P₃ (Fig. 2A).

We then measured the concentration-dependent binding of the coiled-coil region with PI(3)P, PI(5)P, and PI(3,4,5)P₃, which exhibited stronger binding, as well as with PS and PI(4,5)P₂, which are abundant and important lipid species. As shown in Fig. 2B, PI(3)P, PI(5)P, and PI(3,4,5)P₃ exhibited similar affinities to the coiled-coil region of par-3. We estimated the Kd values for PI(3)P, PI(5)P, and PI(3,4,5)P₃ as 16, 10, and 10 µM, respectively, from curve fitting. These values are comparable to the Kd value of 3.5 µM between Drosophila Bazooka and PI(3,4,5)P₃ (Krahm et al., 2010).

**par-3 localization at cell-cell junctions dependent on the lipid binding site**

To examine the contribution of the lipid-binding site on the C-terminal coiled-coil region to the function of par-3, we used Madin-Darby canine kidney (MDCK) cells. The par-3 localization at cell-cell junctions was also analyzed with GFP-tagged par-3. The wild-type (WT) par-3 was localized at cell-cell junctions. In contrast, some of the K1013E/K1014E mutant of par-3 was still localized at cell-cell junctions, but most of the par-3 mutant was cytosolic (Fig. 3).
par-3 turnover at cell-cell junctions is dependent on PI3K and the phospholipid binding site

To quantify the effects of the mutation at the lipid binding site on par-3 localization at the cell-cell junctions, we analyzed the stability of par-3 at cell-cell junctions by the fluorescence recovery after photo-bleaching (FRAP) technique, using MDCK cells expressing par-3 tagged with GFP (Fig. 4A). Consistent with the cytosolic localization of the mutant par-3, the K1013E/K1014E mutant of par-3 had a shorter recovery halftime than WT PAR-3, suggesting that the lipid-binding site contributes to the stabilization of par-3 at the cell-cell junction (Fig. 4B–D).

Phosphatidylinositol 3-kinases (PI3Ks) catalyze the production of PI(3)P and PI(3,4,5)P\(_3\). Therefore, we analyzed the effect of a PI3K inhibitor, wortmannin, on par-3 localization. The recovery halftime of WT par-3 after photobleaching became faster upon wortmannin treatment (Fig. 4B–D). However, the recovery of WT par-3 in the presence of wortmannin was slower than that of the K1013E/K1014E par-3 mutant, suggesting that the localization of par-3 at the cell-cell junction is partially dependent on PI3K products.
In this study, we found that the C-terminal coiled-coil region of mammalian par-3 bound to phospholipids. The C-terminal coiled-coil region of Drosophila Bazooka/par-3 was recently reported to bind to phosphoinositides with broad specificity. The phosphoinositide binding by mammalian par-3 was not specific (Fig. 1). PI(3)P, PI(3,4,5)P$_3$, and PI(4,5)P$_2$ were

**Discussion**

In this study, we found that the C-terminal coiled-coil region of mammalian par-3 bound to phospholipids. The C-terminal coiled-coil region of Drosophila Bazooka/par-3 was recently reported to bind to phosphoinositides with broad specificity. The phosphoinositide binding by mammalian par-3 was not specific (Fig. 1). PI(3)P, PI(3,4,5)P$_3$, and PI(4,5)P$_2$ were
found to bind to par-3. \(\text{PI}(4,5)\text{P}_2\) is more abundant than \(\text{PI}(3)\text{P}\) and \(\text{PI}(3,4,5)\text{P}_3\), suggesting that \(\text{PI}(4,5)\text{P}_2\) could contribute to the par-3 localization. However, the broad specificity of phosphoinositide binding by par-3 and the different localizations of \(\text{PI}(4,5)\text{P}_2\) and \(\text{PI}(3,4,5)\text{P}_3\) (Martin-Belmonte et al., 2008) suggest that lipid binding is not the only determinant of par-3 localization.

Importantly, the specificity of Drosophila par-3 was similar to that of mammalian par-3 (Krahn et al., 2010). The coiled-coil region of Bazooka was localized diffusely to the membrane of Drosophila by itself, and a Bazooka mutant with the coiled-coil region substituted by the PH domain rescued the lethality of a Bazooka mutant allele (Krahn et al., 2010). Therefore, the lipid binding ability of the C-terminal coiled-coil region of par-3 seems to be important and conserved among species.

The par-3 mutant lacking aa 937–1024 reportedly lost the specific localization to the apical area of cell-cell junctions, where tight junctions exist. However, the par-3 mutant lacking aa 937–1024 was still localized at the cell-cell junctions and in the cytosol (Mizuno et al., 2003). This region contains the basic amino acids, Lys1013 and Lys1014, that contribute to phospholipid binding, as shown in this study. Although the K1013E/K1014E mutant of par-3 exhibited more cytosolic localization than WT par-3, the K1013E/K1014E mutant of par-3 could still localize to the cell-cell junction (Fig. 3). Thus, it seems that the subcellular localization of the K1013E/K1014E mutant of par-3 is essentially identical to that of par3 lacking aa 937–1034.

Interestingly, part of the C-terminal coiled-coil region (aa 1039–1220) reportedly bound to PI3K and FAK (Itoh et al., 2010). In contrast, the PDZ domain binds to PTEN, which antagonizes PI3K (von Stein et al., 2005). PTEN is known to catalyze \(\text{PI}(3,4,5)\text{P}_3\) dephosphorylation at the apical membrane (Martin-Belmonte et al., 2007). Although it is still unclear how these molecules act together to establish the polarity of cells, both the phosphoinositide and protein interactions of par-3 are considered to be important for its function.

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**References**


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