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Phosphorylation-dependent binding of 14-3-3 to Par3β, a human Par3-related cell polarity protein☆

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Abstract

Mammalian Par3α and Par3β/Par3L participate in cell polarity establishment and localize to tight junctions of epithelial cells; Par3α acts via binding to atypical PKC (aPKC). Here we show that Par3β as well as Par3α interacts with 14-3-3 proteins in a phosphorylation-dependent manner. In the interaction, Ser-746 of Par3β and the corresponding residue of Par3α (Ser-814) likely play a crucial role, since replacement of these residues by unphosphorylatable alanine results in a loss of interacting activity. The mutant Par3 proteins with the replacement are correctly recruited to tight junctions of MDCK cells and to membrane ruffles induced by an active form of the small GTPase Rac in HeLa cells. Thus, the interaction with 14-3-3 appears to be dispensable to Par3 localization. Consistent with this, the Par3α–14-3-3 interaction does not inhibit the Par3α–aPKC association required for the Par3α localization, although the aPKC-binding site lies close to the Ser-814-containing, 14-3-3-interacting region.

Keywords: 14-3-3; Par3; Par3β; Par3L; aPKC; Par6; Cell polarity; Tight junction; Epithelial cells; Membrane ruffles

Cell polarization is fundamental for a variety of processes such as asymmetric cell division, directed migration, and maintenance of fully functional differentiated cells [1–4]. It is known that various evolutionarily conserved proteins participate in cell polarity establishment. Among them are *par* (partitioning defective) gene products that have been originally identified as determinants of asymmetric cell division of the nematode *Caenorhabditis elegans* zygote [1–4]: PAR-3, PAR-6, and atypical protein kinase C (aPKC) form a trimeric complex that localizes to the anterior cortex of the zygote, while PAR-1, a serine/threonine kinase, is present at the posterior cortex [1–4]. PAR-5, being symmetrically distributed throughout the cytoplasm, is considered to function at the top of a regulatory hierarchy of the *par* genes, since PAR-5 is required for the specific localization of the other Par proteins [5]. PAR-5 belongs to the 14-3-3 family of proteins present in high abundance in all eukaryotic cells [5]; 14-3-3 functions by binding to its target proteins primarily in a manner dependent on phosphorylation, albeit being able to interact well with a variety of nonphosphorylated ligands [6–8]. Recent studies imply that the functional hierarchy likely differs between organisms: in the fruitfly *Drosophila melanogaster*, 14-3-3 may act downstream of PAR-1 [9]; in *Xenopus* and mammals, Par1 becomes phosphorylated by aPKC to interact with 14-3-3, thereby controlling cell polarity [10,11].

The Par3/Par6/aPKC complex is also required for establishment of apical-basolateral polarity in mammalian epithelial cells, and targeted to tight junctions, by which the apical surface is segregated from the

☆ *Abbreviations:* aPKC, atypical protein kinase C; CR, conserved region; Baz, Bazooka; PCR, polymerase chain reaction; GST, glutathione *S*-transferase.

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basolateral membranes [2–4]. In the conserved complex, Par6 constitutively associates with aPKC via a PB1-domain-mediated head-to-head interaction [12,13] and Par3 directly binds to the C-terminal catalytic domain of aPKC with a high affinity [14]. Par3 contains three evolutionarily conserved regions (CRs): the N-terminal CR1 mediates self-oligomerization; CR2 comprises three PDZ domains; and CR3 is required for the binding to aPKC (see Fig. 1A). Another human PAR-3 homologue Par3β [15], also known as Par3L [16], harbors regions homologous to CR1 and CR2 but lacks CR3 (Fig. 1A); Par3β is also recruited to tight junctions, albeit in a manner independent of aPKC, and likely plays an essential role in the establishment of epithelial cell polarity [15,16].

To identify a novel Par3-interacting protein, we have performed a yeast two-hybrid screening using human fetal brain cDNA library and found that several 14-3-3 isoforms interact with the human conventional Par3 (Par3α). During the course of this study, Benton and Johnston [17] have reported that *Drosophila* 14-3-3 proteins bind to the fruitfly PAR-3 homologue Bazooka (Baz) on two conserved serines, Ser-151 in the N-terminus and Ser-1085 close to CR3 in the C-terminus, both of which are phosphorylated by PAR-1. The PAR-1-induced binding of Baz to 14-3-3 likely inhibits formation of the Baz/PAR-6/aPKC complex by blocking the interaction between Baz and aPKC, hence regulating localization of Baz [17]. On the other hand, Hurd et al. [18] have shown that 14-3-3 proteins can interact with an N-terminal fragment of human Par3α via binding to Ser-144 as a phosphoepitope, equivalent to Ser-151 of Baz; however, the role for the C-terminal region of Par3α in the interaction has not been clarified. It has also remained to be elucidated whether Par3β binds to 14-3-3 proteins.

In the present report, we show that Par3β as well as Par3α interacts with 14-3-3 proteins in a phosphorylation-dependent manner. For the interaction, the C-terminally conserved serines, Ser-746 in Par3β and Ser-814 in Par3α, are crucial, whereas Ser-141 of Par3β and its equivalent residue of Par3α (Ser-144) do not appear to play a major role. In contrast to *Drosophila*, the Par3–14-3-3 interaction in mammals is likely dispensable for the localization of the Par3 proteins: their correct recruitment to tight junctions of MDCK cells and to membrane ruffles induced by an active form of the small GTPase Rac in HeLa cells. This agrees with the finding that the interaction between Par3α and 14-3-3 does not prevent the formation of the Par3α/Par6/aPKC complex in mammalian cells, which complex is required for the localization of Par3α.

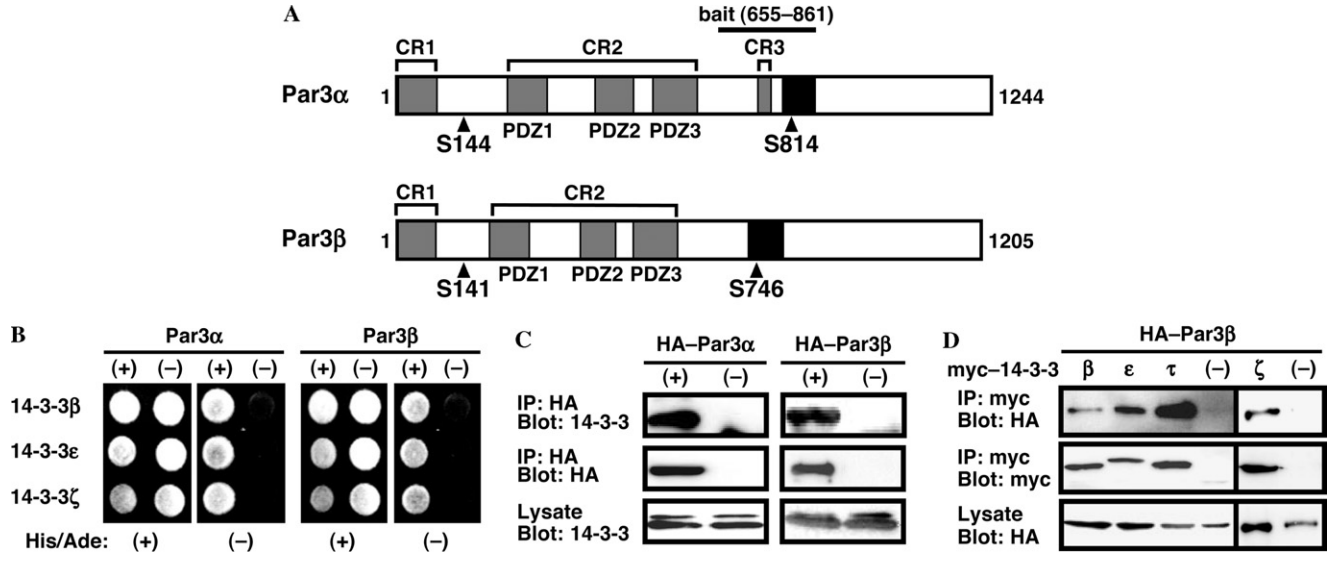

Fig. 1. (A) Schematic structures of human Par3α and Par3β. The conserved regions (CRs) are indicated by gray boxes: the amino acids of CR1 and CR2 comprising three PDZ domains are 60% and 49% identical, respectively, between Par3α and Par3β. Although Par3β lacks CR3, the region C-terminal to CR3 (boxed in black) shares a high amino acid identity (79%) between the human Par3 proteins. The bar denotes the Par3α fragment of amino acids 655–861, used as a bait in the present two-hybrid screening. Arrowheads indicate the positions of the serine residues investigated in this study. (B) Two-hybrid interaction of human Par3β and Par3α with 14-3-3. The yeast AH109 cells were co-transformed with a pair of pGBK encoding the full-length Par3α or Par3β and pGADGH encoding 14-3-3β, ε, or ζ. Following the selection for Trp^+ and Leu^+ phenotype, its histidine- and adenine-dependent growth was tested. (C) Interaction of the Par3 proteins with endogenous 14-3-3 in COS-7 cells. COS-7 cells were transfected with pEF-BOS-HA–Par3α or pEF-BOS-HA–Par3β. Proteins of the cell lysates (lysate) were immunoprecipitated (IP) with the anti-HA antibody, followed by immunoblot (blot) with the anti-14-3-3 or anti-HA antibodies. (D) Interaction of Par3β with various 14-3-3 isoforms. COS-7 cells were transfected with a pair of pEF-BOS-HA–Par3β and pEF-BOS-myc encoding 14-3-3β, ε, ζ or τ. Proteins of the cell lysates (lysate) were analyzed by immunoblot (blot) following immunoprecipitation (IP), using the indicated antibodies.

Materials and methods

Plasmids and antibodies. Complementary DNA (cDNA) fragments encoding Par3α, Par3β, Par6α, Rac1 (G12V), and Rac1 (T17N) were prepared as described previously [12,15,19]. The cDNA for human 14-3-3τ was obtained by polymerase chain reaction (PCR) using a human fetal brain cDNA library (BD Bioscience) as a template. Mutations leading to the indicated amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. The cDNA fragments were cloned into the indicated vectors. All of the constructs were sequenced to confirm their identities.

Anti-aPKC polyclonal antibodies and an anti-14-3-3ζ monoclonal antibody were purchased from Santa Cruz Biotechnology; the mouse anti-myc monoclonal antibody 9E10 and the rat anti-HA monoclonal antibody 3F10 were from Roche Applied Science; the mouse anti-HA monoclonal antibody 16B12 was from Covance Research Products; the mouse anti-FLAG monoclonal antibody M2 was from Sigma; horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Biosciences; Alexa Fluor 488-labeled goat anti-mouse IgG antibodies were from Molecular Probes; and Cy3-conjugated goat anti-rat IgG antibodies from Jackson Immuno Research Laboratories. An anti-ZO–1 monoclonal antibody was kindly provided by Dr. K. Takeuchi (Nagoya University) [20].

Two-hybrid experiments. Two-hybrid screening was performed using a Matchmaker Two-Hybrid System (BD Biosciences) according to the manufacturer's instructions. Briefly, a cDNA fragment encoding a CR3-spanning region of human Par3α (amino acid residues 655–861), which can directly interact with aPKC, was prepared by PCR [15]. The cDNA was ligated to the pGBK vector, containing the DNA-binding domain [21], which was used as a bait in the following two-hybrid screening. The yeast reporter strain AH109, containing the *HIS3*, *ADE*, and *LacZ* reporter genes, was transformed simultaneously with a pGBK, which contains the Par3α CR3-spanning region, and a plasmid of the human fetal brain cDNA library (BD Biosciences) using a lithium acetate-based method. The transformants were selected for the Trp^+, Leu^+, His^+, and Ade^+ phenotype. Twenty-five independent positive clones were obtained from about 7.6×10^5 clones screened. Sequencing analysis revealed that 23 of the positive clones encode isoforms of 14-3-3 proteins: 13 clones of 14-3-3β, four of 14-3-3ε, and six of 14-3-3ζ. All the clones contained their respective entire coding regions with both 5′- and 3′-untranslated regions. The coding regions of these 14-3-3 proteins were PCR-amplified and ligated to the pGADGH vector containing the GAL4 *trans* activation domain. Various combinations between a pGBK encoding a Par3 protein and a pGADGH encoding a 14-3-3 protein were co-transformed into AH109 cells or HF7c cells, the latter of which contained *HIS3* and *LacZ* reporter genes. Following selection for the Trp^+ and Leu^+ phenotypes, the transformed AH109 cells were tested for their ability to grow on plates lacking both histidine and adenine; the transformed HF7 cells were tested for their ability to grow on plates lacking histidine supplemented with 2 mM 3-aminotriazole for the suppression of background growth, according to manufacturers' recommendation (BD Biosciences).

An in vivo binding assay by immunoprecipitation. For expression of proteins in COS-7 cells, cDNAs were ligated to the mammalian expression vector pEF-BOS as previously described [15,19]. COS-7 cells were transfected using LipofectAMINE (Invitrogen) with the indicated cDNAs and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The cells were lysed at 4 °C with a lysis buffer (138 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton X-100, and 20 mM Tris, pH 8.0) containing protease inhibitors (80 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, 20 μg/ml chymostatin, and 10 μg/ml pepstatin) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_5, and 10 mM $Na_4P_2O_7$). The lysates were centrifuged for 30 min at 20,630*g*, and the supernatant was precipitated with the anti-myc, anti-FLAG, or anti-HA monoclonal antibody in the presence of protein G–Sepharose (Amersham Biosciences). The precipitates were washed three times with the lysis buffer and applied to SDS–PAGE, followed by immunoblot analysis with the indicated antibodies. The blots were developed using ECL-plus (Amersham Biosciences) to visualize the antibodies.

Treatment of Par3α and Par3β with the calf intestine alkaline phosphatase. For expression of a recombinant 14-3-3ζ protein fused to glutathione *S*-transferase (GST), the cDNA for human 14-3-3ζ was ligated to pGEX-6P (Amersham Biosciences). GST–14-3-3ζ was expressed in the *Escherichia coli* strain BL21 and purified by glutathione–Sepharose 4B (Amersham Biosciences), as previously described [12,15,19]. FLAG–tagged Par3α or Par3β was expressed in COS-7 cells and precipitated with the anti-FLAG monoclonal antibody in the presence of protein G–Sepharose beads (Amersham Biosciences). The Par3 proteins precipitated were washed three times with the lysis buffer and once with a solution containing 1 mM $MgCl_2$ and 50 mM Tris–HCl (pH 9.0). The Par3 proteins bound to the beads were then treated for 30 min at 37 °C with or without the calf intestine alkaline phosphatase (Takara), and washed with the lysis buffer and subsequently with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). The proteins were then incubated for 30 min at 4 °C with 20 μg GST-14-3-3ζ in 100 μl PBS. After the beads were washed five times with PBS containing 0.1% Triton X-100, the bound proteins were eluted from the beads with Laemmli's sample buffer (2.5% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptoethanol, and 62.5 mM Tris–HCl, pH 6.8). The eluates were analyzed by immunoblot with the anti-14-3-3ζ or anti-FLAG antibody.

Localization of the wild-type and mutant proteins of Par3α and Par3β in MDCK cells and Rac-treated HeLa cells. Localization of HA-tagged Par3 proteins was tested using the MDCK epithelial cells and HeLa cells as described previously [12,15,19] with minor modifications. MDCK cells were transfected with pEF-BOS-HA encoding Par3α (wt), Par3α (S144A), Par3α (S814A), Par3β (wt), Par3β (S141A), or Par3β (S746A), using LipofectAMINE 2000 (Invitrogen). HeLa cells were transfected with a pair of pEF-BOS-myc encoding Rac1 (G12V) or Rac1 (T17N), and pEF-BOS-HA encoding Par3α (wt), Par3α (S144A), Par3α (S814A), Par3β (wt), Par3β (S141A), or Par3β (S746A), using LipofectAMINE. After cultured for 18–24 h in DMEM supplemented with 10% fetal calf serum, the cells were fixed for 10 min in 3.7% formaldehyde, and permeabilized for 20 min in PBS containing 0.5% Triton X-100 and 3% bovine serum albumin. The samples were incubated with the rat anti-HA monoclonal antibody 3F10 and with the mouse anti ZO-1 or anti-myc monoclonal antibody for 60 min at room temperature, and subsequently probed with the Cy3-conjugated goat anti-rat IgG antibodies and the Alexa Fluor 488-labeled goat anti-mouse IgG antibodies as secondary antibodies for 30 min at room temperature. Images were visualized with an Axiovert 200M confocal laser-scanning microscope (Carl Zeiss).

Results and discussion

Not only Par3α but also Par3β interacts with 14-3-3 proteins

To identify a novel Par3-interacting protein, we screened a human fetal cDNA library in the yeast two-hybrid system using, as a bait, a CR3-spanning region of human Par3α (amino acids 655–861; see Fig. 1A), which region is known to be sufficient for the interaction with aPKC [15]; CR3 is required but not enough for the binding to aPKC [15]. In the screening, we obtained 25 independent positive clones, 23 of which encoded

isoforms of 14-3-3 proteins: thirteen 14-3-3β, four 14-3-3ε, and six 14-3-3ζ. As shown in Fig. 1B, the full-length Par3α of 1244 amino acids, as well as the bait region alone, interacted with the three human 14-3-3 isoforms.

Human Par3β lacks CR3, but contains a region highly homologous to the one C-terminal to CR3 of Par3α, which is involved in the bait used in the present two-hybrid screening (Fig. 1A). This raises the possibility that Par3β also interacts with 14-3-3 proteins. Indeed, the full-length Par3β of 1205 amino acids exhibited a two-hybrid interaction with 14-3-3β, 14-3-3ε, and 14-3-3ζ (Fig. 1B). To verify that the interaction of the two Par3 proteins with 14-3-3s occurs in mammalian cells, we expressed Par3α and Par3β as HA-tagged proteins in COS-7 cells and precipitated them with an anti-HA antibody. As shown in Fig. 1C, endogenous 14-3-3 proteins were co-precipitated with Par3β as well as with Par3α, indicating the association of these Par3 proteins with 14-3-3 proteins. When various 14-3-3 isoforms such as β, ε, ζ, and τ were ectopically expressed in COS-7 cells, they associated with Par3β (Fig. 1D) and Par3α (data not shown). Thus, not only Par3α but also Par3β interacts with 14-3-3 proteins.

Par3β as well as Par3α binds to 14-3-3ζ in a phosphorylation-dependent manner

It is well documented that 14-3-3 proteins bind to their target proteins via a conserved amphipathic groove [6–8]. In the groove, Lys-49, a completely conserved residue in all 14-3-3 proteins, forms a basic cluster coordinating the phosphate group in phosphoserine-containing ligands, while the invariant Val (Val-176 in 14-3-3ζ) locates on the hydrophobic surface of this groove [22,23]. Both Lys-49 and Val-176 are known to play crucial roles; the K49E or V176D substitution each results in a complete loss of phosphorylation-dependent interaction of 14-3-3ζ with various proteins such as the protein kinase Raf [22,23] and human Par3α [18]. As shown in Fig. 2A, Par3β as well as Par3α was incapable of interacting with 14-3-3ζ (K49E) or 14-3-3ζ (V176D) in the yeast two-hybrid system. When expressed in COS-7 cells, the interactions were also abrogated by either the K49E or the V176D substitution (Fig. 2B). In addition, the same substitutions in human 14-3-3τ, also termed θ, resulted in a loss of its activity to bind to Par3β (Fig. 2C). Taking together, 14-3-3 proteins appear to bind to not only Par3α but also Par3β via the conserved amphipathic groove, which engages phosphoserine-containing proteins.

To verify the phosphorylation dependence of the binding of Par3α and Par3β to 14-3-3, we dephosphorylated the Par3 proteins expressed in COS-7 cells by treating with the calf intestine alkaline phosphatase and tested their ability to bind to a recombinant 14-3-3ζ fused to GST. As shown in Fig. 2D, the phosphatase-treated Par3β failed to interact with 14-3-3ζ. Similarly, the treatment of Par3α led to a drastic decrease in its

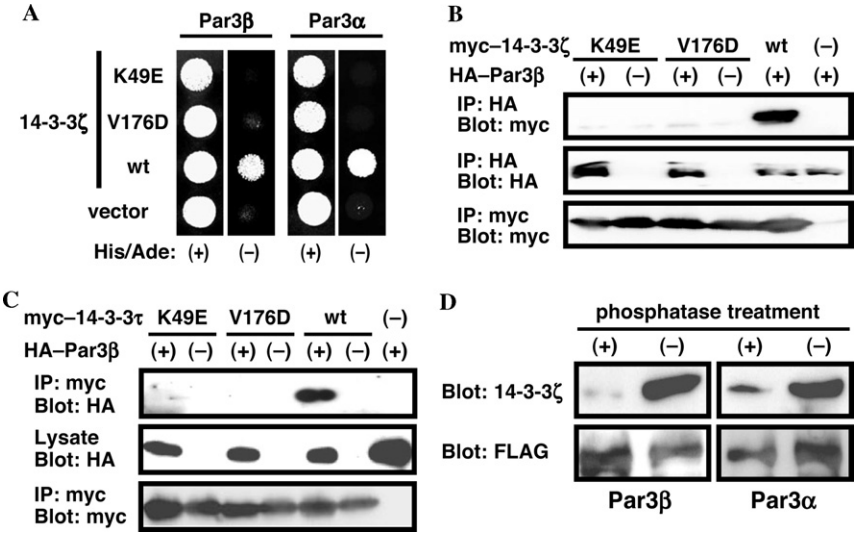

Fig. 2. Phosphorylation-dependent interaction of Par3β with 14-3-3ζ. (A) The yeast AH109 cells were cotransformed with a pair of pGBK encoding Par3β or Par3α and pGADGH encoding the wild-type (wt) 14-3-3ζ or a mutant 14-3-3ζ carrying the K49E or V176D substitution. Following the selection for Trp^+ and Leu^+ phenotype, its histidine- and adenine-dependent and -independent growth was tested. (B) COS-7 cells were transfected with a pair of pEF-BOS-HA–Par3β and pEF-BOS-myc encoding 14-3-3ζ (wt), 14-3-3ζ (K49E), or 14-3-3ζ (V176D). Proteins of the cell lysates were immunoprecipitated (IP) and then analyzed by immunoblot (blot), using the indicated antibodies. (C) COS-7 cells were transfected with a pair of pEF-BOS-HA–Par3β and pEF-BOS-myc encoding 14-3-3τ (wt), 14-3-3τ (K49E), or 14-3-3τ (V176D), and analyzed as in B. (D) FLAG-tagged Par3 proteins expressed in COS-7 cells were precipitated with the anti-FLAG antibody, followed by treatment with (+) or without (−) the calf intestine alkaline phosphatase. After the Par3 proteins were incubated with recombinant GST–14-3-3ζ, bound 14-3-3 was estimated by immunoblot analysis. For details, see Materials and methods.

binding to the 14-3-3 protein (Fig. 2D). Thus, Par3β as well as Par3α binds to 14-3-3 proteins in a phosphorylation-dependent manner.

Ser-814 of Par3α and Ser-746 of Par3β play a major role in the interaction with 14-3-3ζ

We next attempted to determine the residues of Par3α and Par3β that participate in the phosphorylation-dependent interaction with 14-3-3 proteins. Benton and Johnston [17] have recently shown that the *Drosophila* PAR-3, Baz, can bind to two independent regions of 14-3-3 proteins: N- and C-terminal regions containing Ser-151 and Ser-1085 as a phosphoepitope, respectively (see Fig. 3A). The N-terminal binding region closely matches one of a canonical 14-3-3 recognition motif, RSXpSXP (X is any residue and pS denotes phosphoserine) [6–8]; the C-terminal region does not resemble any of the well-defined consensus motifs, but is similar to the 14-3-3-binding site in the protein kinase PKCμ, RTSpSAELS [24]. A Baz fragment containing Ser-151 or Ser-1085 is each capable of binding to 14-3-3 in a phosphorylation-dependent manner [17], although it is not clear which serine residue is crucial for the interaction in the context of the full-length Baz. The involvement of the serine of the Par3 C-terminus appears to be conserved between *Drosophila* and *C. elegans*. PAR-5, a 14-3-3 homologue of *C. elegans*, interacts with the PAR-3 C-terminus via binding to Ser-950, corresponding to Ser-1085 of Baz (see Fig. 3A), as the phosphoepitope [18], whereas an N-terminal fragment of *C. elegans* PAR-3 does not associate with PAR-5 [17]. In mammals, the substitution of unphosphorylatable alanine for Ser-144 in human Par3α, equivalent to Ser-151 in Baz (see Fig. 3A), has been shown to abrogate the binding of a Par3α N-terminal fragment to 14-3-3, but does not affect the interaction between the full-length Par3α and 14-3-3 [18]; these findings suggest the presence of multiple 14-3-3-binding sites in human Par3α, although other sites involved have remained unknown.

The above observations led us to focus on the two serine residues of human Par3 proteins, equivalent to

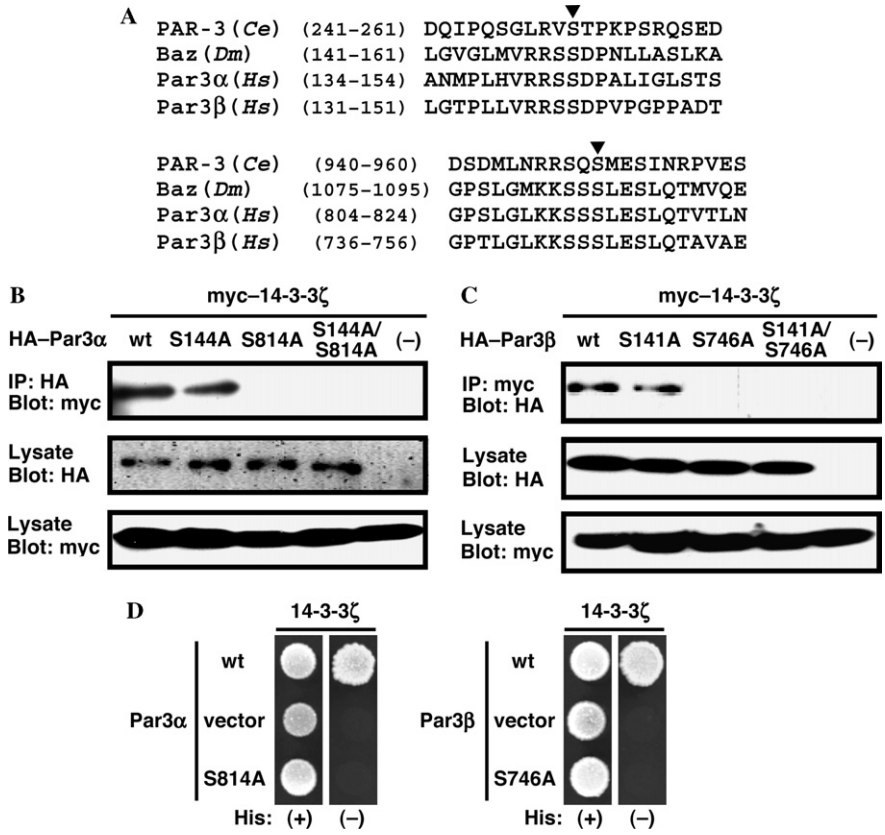

Fig. 3. Role for conserved serine residues of Par3β and Par3α in the interaction with 14-3-3. (A) Alignment of amino acid sequences surrounding the two conserved serine residues in the N- (upper) and C-terminal regions (lower) of Par3 proteins in *Caenorhabditis elegans* (*Ce*), *Drosophila malanogaster* (*Dm*), and *Homo sapiens* (*Hs*). Arrowheads indicate the positions of the conserved serine residues. (B) COS-7 cells were transfected with a pair of pEF-BOS-myc–14-3-3ζ and pEF-BOS-HA encoding Par3α (wt), Par3α (S144A), or Par3α (S814A). Proteins of the cell lysates (lysate) were immunoprecipitated (IP) and then analyzed by immunoblot (blot), using the indicated antibodies. (C) COS-7 cells were transfected with a pair of pEF-BOS-myc–14-3-3ζ and pEF-BOS-HA encoding Par3β (wt), Par3β (S141A), or Par3β (S746A), and analyzed as in (B). (D) The yeast HF7c cells were cotransformed with a pair of pGADGH–14-3-3ζ and pGBK encoding a fragment of Par3α (amino acids 655–861) or Par3β (amino acids 621–792). Following the selection for Trp^+ and Leu^+ phenotype, its histidine-dependent growth was tested.

Ser-151 and Ser-1085 of Baz: Ser-144 and Ser-814 of Par3α ; Ser-141 and Ser-746 of Par3β (Fig. 3A). As shown in Fig. 3B, a mutant full-length Par3α carrying the replacement of Ser-144 by alanine was co-precipitated with 14-3-3ζ to a similar extent as the wild-type protein in COS-7 cells, which agrees with the previous observation by Hurd et al. [18]. Thus, phosphorylation of Ser-144 does not seem to play a crucial role. In contrast, the S814A substitution led to an impaired binding to 14-3-3ζ (Fig. 3B), indicative of a major role of Ser-814. It should be noted that Ser-814 is present in the region used as the bait in the present two-hybrid screening for identification of Par3α-interacting proteins (Fig. 1A). Similarly, the substitution of alanine for Ser-746 in Par3β also resulted in a complete loss of interaction with 14-3-3ζ, whereas Par3β (S141A) fully bound to 14-3-3ζ (Fig. 3C). The crucial roles for Ser-814 of Par3α and Ser-746 of Par3β were verified in the yeast two-hybrid system (Fig. 3D). Thus, the conserved serine residue in the Par3 C-terminus—Ser-814 in Par3α; Ser-746 in Par3β—likely plays a major role in the interaction with 14-3-3.

Interactions of Par3α and Par3β with 14-3-3 are not involved in their localization to tight junctions of epithelial cells or to membrane ruffles in HeLa cells

In the *C. elegans* one-cell embryo, the 14-3-3 homologue PAR-5 is required for the anterior localization of PAR-3 [5]; and, in *Drosophila*, mutant PAR-3/Baz proteins, defective in binding to 14-3-3, fail to correctly localize to the apical membrane domain of epithelial cells, indicating involvement of the Par3–14-3-3 interaction [17]. To study the role of the interaction in mammalian cells, we expressed mutant Par3 proteins in MDCK epithelial cells and tested their localization. As shown in Fig. 4A, the wild-type Par3β colocalized with the tight junction protein ZO-1, as previously described [15]. Neither the S141A nor the S746A substitution affected the localization of Par3β to tight junctions (Fig. 4A), indicating that the interaction with 14-3-3 does not participate in the Par3β localization. Similarly, the recruitment of Par3α to tight junctions was not influenced by the S144A or the S814A substitution (Fig. 4A). Hence, the interaction of the Par3 proteins with 14-3-3 is likely dispensable to their localization to tight junctions of mammalian epithelial cells.

We have previously shown that Par6 and aPKC are targeted to Rac1-induced membrane ruffles in HeLa cells [19]. As shown in Fig. 4B, the wild-type Par3α also colocalized with the active Rac (G12V) at membrane ruffles; similar co-localizations were observed when Par3α (S814A) and Par3α (S144A) were expressed. When Par3β was expressed in HeLa cells, it was recruited to membrane ruffles induced by Rac (G12V) as well (Fig. 4B). Neither the S141A nor the S746A substitution exerted an effect on the recruitment (Fig. 4B). The Par3–14-3-3 interaction thus does not appear to be responsible for the targeting to membrane ruffles in HeLa cells. Taken together, the subcellular localization of Par3 is likely independent of the binding to 14-3-3.

Interactions of Par3α with 14-3-3 and with aPKC are not mutually exclusive

It is well established that the targeting of Par3α to tight junctions of mammalian epithelial cells depends on its interaction with aPKC that is stably complexed

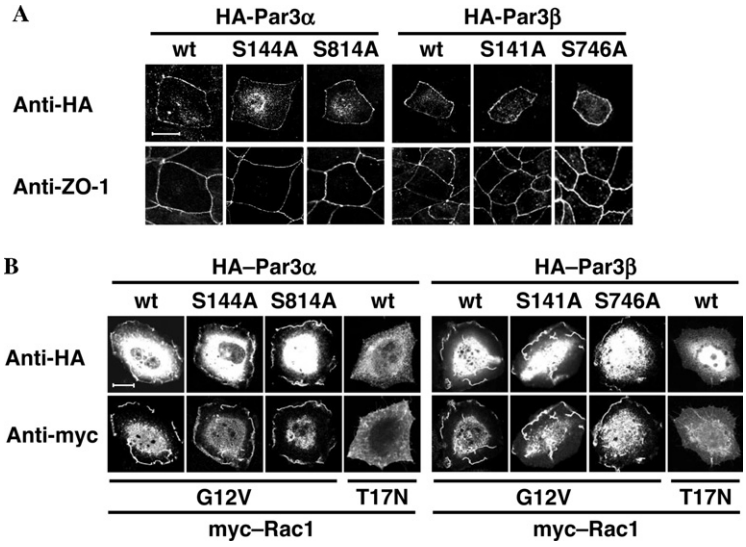

Fig. 4. Localization of the wild-type and mutant Par3β and Par3α in MDCK and HeLa cells. MDCK (A) and HeLa (B) cells were transfected with the expression plasmids as indicated. The cells were fixed and stained with the anti-HA and anti-ZO-1 antibodies (A), or with the anti-HA and anti-myc antibodies (B). Bar = 20 μm.

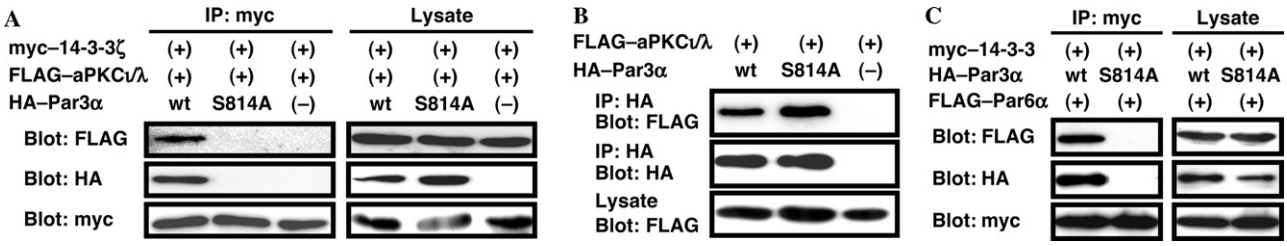

Fig. 5. Par3α-mediated interaction of 14-3-3ζ with aPKCι/λ and Par6α. (A) COS-7 cells were transfected with pEF-BOS-myc–14-3-3ζ, pEF-BOS-FLAG–aPKCι/λ, and pEF-BOS-HA encoding Par3α (wt) or Par3α (S814A). Proteins of the cell lysates (lysate) were immunoprecipitated (IP) and then analyzed by immunoblot (blot), using the indicated antibodies. (B) COS-7 cells were transfected with pEF-BOS-FLAG–aPKCι/λ and pEF-BOS-HA encoding Par3α (wt) or Par3α (S814A), and analyzed as in (A). (C) COS-7 cells were transfected with pEF-BOS-myc–14-3-3ζ, pEF-BOS-FLAG–Par6α, and pEF-BOS-HA encoding Par3α (wt) or Par3α (S814A), and analyzed as in (A).

with Par6; disruption of the interaction leads to mislocalization of Par3α [2–4]. It was thus considered that the interaction between Par3α and Par6/aPKC should not be prevented by the binding of Par3α to 14-3-3, because the localization of Par3α is independent of its binding to 14-3-3 (Fig. 4). On the other hand, since the Ser-814-containing 14-3-3-binding region is adjacent to CR3, a region essential for the binding to aPKC (Fig. 1A), it seemed also possible that the interactions of Par3 with 14-3-3 and with aPKC are mutually exclusive. To clarify this, we tested whether Par3α simultaneously binds to 14-3-3 and aPKC. As shown in Fig. 5A, aPKCι/λ was co-precipitated with 14-3-3ζ when expressed in COS-7 cells. The interaction between 14-3-3ζ and aPKCι/λ was completely dependent on the presence of Par3α, i.e., Par3α binds to 14-3-3ζ and aPKCι/λ at the same time. Although Par3α (S814A), defective in interaction with 14-3-3, was capable of fully binding to aPKCι/λ (Fig. 5B), the mutant Par3α failed to support the association of aPKCι/λ with 14-3-3ζ (Fig. 5A), which agrees with the idea that 14-3-3ζ interacts with aPKCι/λ via the binding to Par3α. This is further supported by the observation that a mutant 14-3-3ζ (K49E), defective in binding to Par3α, did not form a complex with aPKC (data not shown). In addition, the interaction of Par6α with 14-3-3ζ also required the binding of Par3α to 14-3-3ζ (Fig. 5C). Thus, 14-3-3 likely enters into the Par3α/Par6/aPKC complex via binding to Par3α but without disrupting the Par3α–aPKC interaction.

Concluding remarks

In the present study, we show that human Par3β as well as Par3α interacts with 14-3-3 proteins in a phosphorylation-dependent manner (Figs. 1 and 2). Although the two conserved serine residues, one existing in the N-terminus and the other in the C-terminal region (Fig. 1A), are considered to be possibly involved in the interaction as the phosphoepitope, the present findings indicate that the C-terminal serine—Ser-814 in Par3α; Ser-746 in Par3β—plays a major role (Fig. 3). This 14-3-3-binding site is adjacent to CR3 of Par3α, a region required for the interaction with aPKC (Fig. 1); however, the binding does not block the interaction of Par3α with aPKC (Fig. 5), which interaction plays an essential role in the correct targeting of Par3α to tight junctions of mammalian epithelial cells. Consistent with this, Par3α is recruited to tight junctions independently of its binding to 14-3-3 (Fig. 4). Similarly, the subcellular localization of Par3β does not appear to be regulated by 14-3-3 (Fig. 4). In contrast, the binding of *Drosophila* 14-3-3 to its PAR-3 homologue Baz has been shown to prevent the interaction between Baz and aPKC, and to control the localization of Baz in insect epithelial cells [17]. Thus, whereas the 14-3-3-binding sites seem to be evolutionarily well conserved, roles for the interactions are likely diverged. Further studies are required for understanding of roles for Par3–14-3-3 interactions in mammalian cells.

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