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Slp2-a inactivates ezrin by recruiting protein phosphatase 1 to the plasma membrane

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ABSTRACT

Synaptotagmin-like protein 2-a (Slp2-a) was originally described as a membrane trafficking protein that consists of a Slp homology domain (SHD), a linker domain, and tandem C2 domains (named the C2A domain and C2B domain). Slp2-a mediates docking of Rab27-bearing vesicles to the plasma membrane through simultaneous interaction with Rab27 and phospholipids in the plasma membrane. We have recently reported that Slp2-a regulates renal epithelial cell size through interaction with Rap1GAP2 via the C2B domain independently of Rab27 and demonstrated the presence of excess activation of ezrin, a membrane-cytoskeleton linker and signal transducer, in Slp2-a-knockdown Madin–Darby canine kidney II (MDCK II) cells. However, the precise mechanism of ezrin inactivation by Slp2-a in cell size control has remained largely unknown. In this study, we investigated the functional relationship between Slp2-a and ezrin in MDCK II cells. The results showed that activation of ezrin in control MDCK II cells either pharmacologically or by overexpression of a constitutively active ezrin mutant caused an increase in cell size, whereas inactivation of ezrin in Slp2-a-knockdown cells by a specific ezrin inhibitor restored them to their normal cell size. We also found that Slp2-a interacts via its previously uncharacterized linker domain with protein phosphatase 1β (PP1β), which inactivates ezrin, and that the interaction is required for the plasma membrane localization of PP1β. These results indicate that Slp2-a inactivates ezrin by recruiting PP1 to the plasma membrane.

1. Introduction

The synaptotagmin-like protein (Slp) family was originally identified as a family of synaptotagmin-related molecules lacking an N-terminal transmembrane domain [1–3]. The Slp family consists of five members (Slp1–5) in mice and humans, and all members share an N-terminal Slp homology domain (SHD), a linker domain in the middle of the molecule, and C-terminal tandem C2 domains (named the C2A domain and C2B domain) [reviewed in ref. 4]. Since the SHD is a well-known Rab27 effector domain that specifically recognizes the GTP-bound active form of Rab27 [5–7], Slp family members are generally thought to function as Rab27 effectors that regulate the trafficking of Rab27-bearing vesicles [4]. The phospholipid (e.g., phosphatidylserine and phosphoinositide) binding activity of the C2A domain of certain Slps has also been suggested to be involved in the docking of Rab27-bearing vesicles to the plasma membrane [8–11].

In contrast to the established role of Slps in Rab27-dependent membrane trafficking, we have recently shown that Slp2-a interacts via its C2B domain with Rap1GAP2 and that it regulates renal epithelial cell size through Rap–ezrin signaling independently of Rab27 [12]. Ezrin is a member of the ezrin–radixin–moesin (ERM) family that acts as a linker between the plasma membrane and the actin cytoskeleton [13], and it is also involved in the regulation of cell size [12,14]. Ezrin exists in two conformational states, an inactive or closed state, in which the N-terminal domain and the C-terminal domain containing the F-actin-binding site interact with each other, and an active or open state, in which phosphorylation of the C-terminal threonine 567

Abbreviations: EGFP, enhanced green fluorescent protein; ERM, ezrin–radixin–moesin; MDCK, Madin–Darby canine kidney; OA, okadaic acid; KD, knockdown; PP, protein phosphatase; PP1Cα, protein phosphatase 1 catalytic subunit α isoform; SHD, Slp homology domain; Slp, synaptotagmin-like protein; WT, wild-type.

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disrupts the intramolecular interaction [13]. Ezrin in the inactive state is mainly cytosolic, whereas ezrin in the active state is localized at the plasma membrane through interaction via its N-terminal domain with phospholipids in the plasma membrane. Active ezrin directly links the actin cytoskeleton to the plasma membrane and promotes cell spreading [14,15]. Excess activation of ezrin and increased size of Madin–Darby canine kidney II (MDCK II) cells have been observed in the absence of Slp2-a [12,16], but the precise molecular mechanism by which Slp2-a suppresses ezrin activation remains to be determined.

Although multiple cellular signalings, e.g., PKC signaling and ROCK signaling, are known to induce ezrin phosphorylation [17], the mechanism of ezrin dephosphorylation is poorly understood. In this study, we used MDCK II cells to investigate the involvement of Slp2-a in controlling the activity of ezrin and showed that Slp2-a interacts via its previously uncharacterized linker domain with protein phosphatase 1β (PP1β), thereby resulting in the recruitment of PP1β to the plasma membrane and inactivation of ezrin there. We discuss the possible mechanism of ezrin inactivation by Slp2-a in cell size control based on our findings.

2. Materials and methods

2.1. Materials

Materials, including antibodies, reagents, and plasmids, used in this study are described in Supplementary information.

2.2. Cell culture, transfection, and drug treatment

Control and Slp2-a-knockdown (KD) stable MDCK II cells [12,16] and COS-7 cells were cultured in DMEM (Wako Pure Chemical Industries, Osaka, Japan) containing 10% FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Stable MDCK II cells were grown in DMEM containing 800 μg/ml G418 (Invitrogen). Plasmids were transfected into stable MDCK II cells or COS-7 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Stable MDCK II cells were treated for 24 h with various concentrations of okadaic acid (OA) (2.5, 5, 10, 20, and 40 nM; LC Laboratories, Woburn, MA) or of an ezrin inhibitor, NSC668394 (1, 5, 10, and 20 μM; EMD Millipore).

2.3. Immunofluorescence and immunoblot analyses

Immunostaining and immunoblotting were performed essentially as described previously [12].

2.4. Co-immunoprecipitation assays

COS-7 cells (3×10^5 cells/6-cm dish, the day before transfection) were transfected with plasmids (pEF-FLAG-PPs, pEF-FLAG-Slp2-a linker (wild-type and mutants), pEF-FLAG-Rap1GAP2, pEF-FLAG-ezrin (wild-type and mutants), pEF-T7-Slp2-a, or pEF-T7-PP1Cβ), cultured for 1 day, and harvested. Co-immunoprecipitation assays with anti-FLAG tag or anti-T7 tag antibody-conjugated agarose were performed as described previously [18].

2.5. Statistical analysis

The data are expressed as means and S.E. Data were tested for statistical significance by using Student's unpaired *t*-test, Dunnett's test, or the Tukey–Kramer test as indicated in the legend of each figure. A probability (p) level < 0.05 was considered statistically significant.

3. Results

3.1. Activation of ezrin regulates MDCK II cell size

We previously showed that cell size was significantly increased in Slp2-a-KD MDCK II cells [16] (Supplementary Fig. S1A), presumably because of excess activation of ezrin (i.e., phosphorylation) (Supplementary Fig. S1B) [12]. To investigate the relationship between ezrin activity and cell size in more detail, we pharmacologically activated endogenous ezrin in control MDCK II cells and pharmacologically inactivated endogenous ezrin in Slp2-a-KD cells and observed their morphology. When the control cells were exposed to okadaic acid (OA), an inhibitor of the serine/threonine protein phosphatases (PPs) PP1 and PP2A, which are known to inactivate ezrin, both endogenous ezrin activity (Fig. 1A) and cell size increased in a dose-dependent manner (Fig. 1B). In contrast, when the Slp2-a-KD cells were exposed to the ezrin inhibitor NSC668394, which is known to directly bind to and inhibit ezrin phosphorylation [19] (Fig. 1C), the size of the Slp2-a-KD cells decreased in a dose-dependent manner (Fig. 1D). To determine whether activation of ezrin alone affects cell size, we overexpressed a phosphomimetic form of ezrin (ezrin-T567D), which is a constitutively active mutant of ezrin [12], in control MDCK II cells and evaluated its effect on cell size. As expected, expression of ezrin-T567D resulted in a dramatic increase in cell size (Fig. 1E). Intriguingly, however, neither expression of wild-type ezrin nor expression of a constitutively inactive mutant of ezrin (a non-phosphorylated ezrin-T567A mutant) [20] in control cells affected their size (Fig. 1E). Taken together, these results indicated that ezrin activity regulates MDCK II cell size.

3.2. Inactive ezrin in the cytoplasm is dramatically decreased in Slp2-a-KD cells

Because ezrin is known to exist in two conformational states, an inactive/dephosphorylated state localized in the cytoplasm and an active/Thr-567-phosphorylated state localized at the plasma membrane [13], we compared the localization of endogenous ezrin in control cells and Slp2-a-KD cells by performing an immunofluorescence analysis. As shown in Fig. 2A, ezrin was mainly localized at the plasma membrane in the control cells, but some punctate ezrin signals were clearly observed in the cytoplasm. In contrast, hardly any cytoplasmic ezrin signals were detected in the Slp2-a-KD cells, and ezrin appeared to exclusively localize at the plasma membrane. We used anti-phospho-ezrin(Thr567) (anti-phospho-ERM)-specific antibody to determine the activity state of cytoplasmic ezrin in the control cells. The results showed no active, phosphorylated ezrin signals in the cytoplasm of the control cells (Fig. 2B, bottom middle panel), indicating that cytoplasmic ezrin must be inactive. To confirm this finding, we expressed the above-described ezrin mutants or wild-type (WT) ezrin with a Venus (a variant of yellow fluorescent protein) tag in control cells and examined their localization. Although all of the ezrin proteins were mainly localized at the plasma membrane in the control cells, the cytoplasmic patterns were markedly different (Fig. 2C). The same as endogenous ezrin signals, recombinant ezrin-WT was also localized at some small punctate signals in the cytoplasm of the control cells. In contrast, larger punctate signals of ezrin-T567A (inactive state) were observed in the cytoplasm, whereas hardly any cytoplasmic ezrin-T567D (active state) signals were detected in the control cells. We therefore concluded that the inactive form of ezrin is accumulated in the cytoplasm and assume that Slp2-a positively regulates inactivation of ezrin in a more direct fashion than we had previously thought [12].

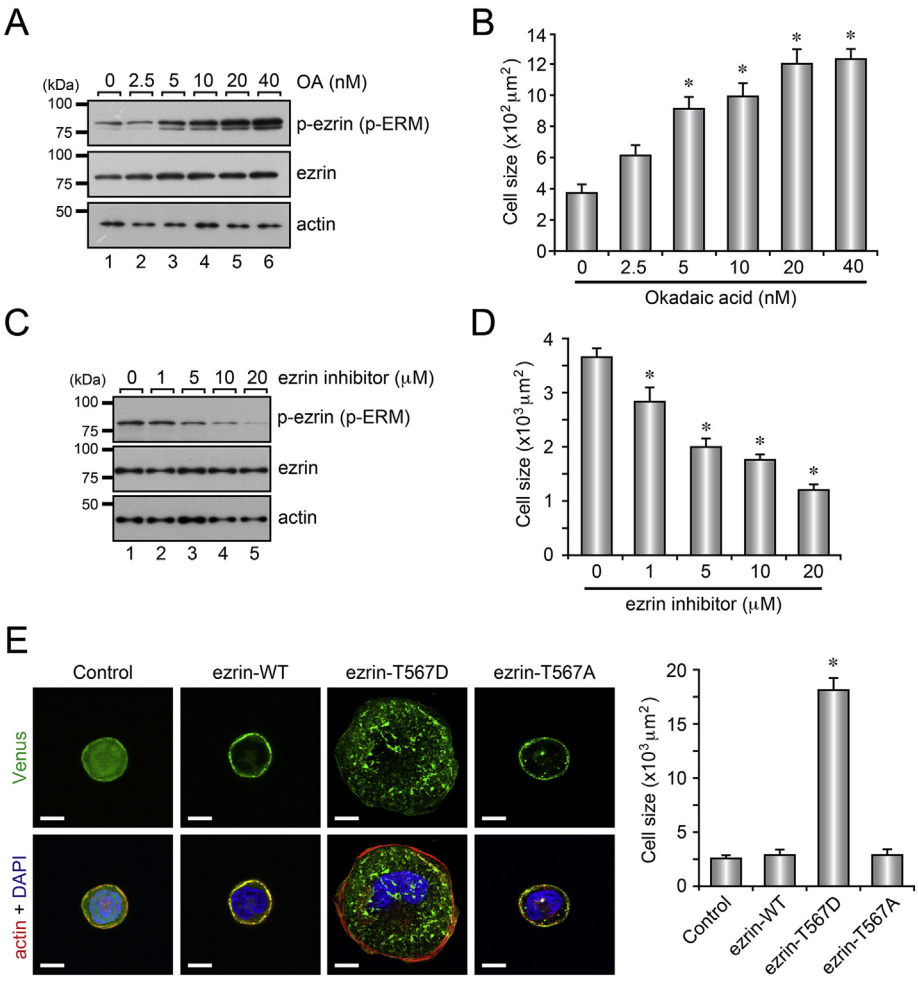

Fig. 1. Activation of ezrin induces increased MDCK II cell size. (A) Activation of ezrin by OA treatment. Control cells were treated with the indicated concentrations of OA for 24 h and harvested. The cell lysates were analyzed by immunoblotting with the antibodies indicated. Actin was used as an internal control. The positions of the molecular mass markers (in kDa) are shown on the left. (B) Increased size of control cells in response to OA treatment. Control cells were treated with the indicated concentrations of OA for 24 h. Their cell sizes were measured as described in Supplementary Fig. 1A. *, $p < 0.001$, Dunnett's test ($n = 35$ from three independent experiments). (C) Inactivation of ezrin in Slp2-a-KD cells by treatment with an ezrin inhibitor. Slp2-a-KD cells were treated with the indicated concentrations of the ezrin inhibitor for 24 h and harvested. (D) Decreased size of Slp2-a-KD cells in response to ezrin inhibitor treatment. Slp2-a-KD cells were treated with the indicated concentrations of the ezrin inhibitor for 24 h. *, $p < 0.001$, Dunnett's test ($n = 40$ from three independent experiments). (E) Expression of ezrin mutants in control cells. (Left) Control cell were transfected with pVenus-N1, pVenus-N1-ezrin-WT, pVenus-N1-ezrin-T567D, or pVenus-N1-ezrin-T567A, and cultured for 24 h. The cells were then re-plated on 35-mm glass-bottomed dishes and cultured for 10 h, and their cell sizes were measured. Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *, $p < 0.001$, Tukey–Kramer test ($n = 30$ from three independent experiments). Values in B, D, and E are means and S.E.

3.3. Slp2-a interacts with protein phosphatase 1

Reports of two recent studies, one demonstrating that both PP1 and PP2 have the ability to dephosphorylate ezrin at threonine 567 [21], and the other showing that PP1 interacts with Slp2-a *in vitro* [22], prompted us to further investigate the possible involvement of PPs in Slp2-a-mediated ezrin inactivation. Consistent with the results of these previous studies, the results of our co-immunoprecipitation assays in COS-7 cells indicated that all three isoforms of the catalytic subunits of PP1, but neither of the catalytic subunits of PP2s, interacted with Slp2-a (Fig. 3A). Because of the availability of anti-PP1β-specific antibody and endogenous expression of PP1β in MDCK II cells (the levels of PP1β expression in the control cells and in the Slp2-a-KD cells were almost the same) (Fig. 3B), we focused our subsequent analysis of the mechanism of Slp2-a-mediated ezrin inactivation on PP1β (and PP2α as a negative control). To investigate the subcellular localization of PP1β or PP2α, we expressed EGFP (enhanced green fluorescent protein)-tagged PP1Cβ or PP2Cα in control cells, and, as shown in Fig. 3C, observed that PP1Cβ was localized both at the plasma membrane and in the nucleus, whereas PP2Cα was localized in the cytoplasm alone. It should be noted that PP1Cβ, but not PP2Cα, co-localized with Slp2-a at the plasma membrane in the control cells (Fig. 3D) and that PP1Cβ was dispersed in the cytoplasm of the Slp2-a-KD cells (Fig. 3E). These results indicated that Slp2-a is required for the plasma membrane-localization of PP1β.

3.4. The Slp2-a linker domain interacts with PP1

Because PP1 has been shown to recognize a classical RVxF motif and a SILK motif, which is an additional common PP1 binding element [22], we searched for these motifs in the protein sequence of mouse Slp2-a *in silico*, and similar motifs, i.e., GILK^{272} and KHVRF^{331}, were found to be present in the linker domain of Slp2-a. To determine whether the interaction between Slp2-a and PP1Cβ was mediated by these motifs in the linker domain, we introduced

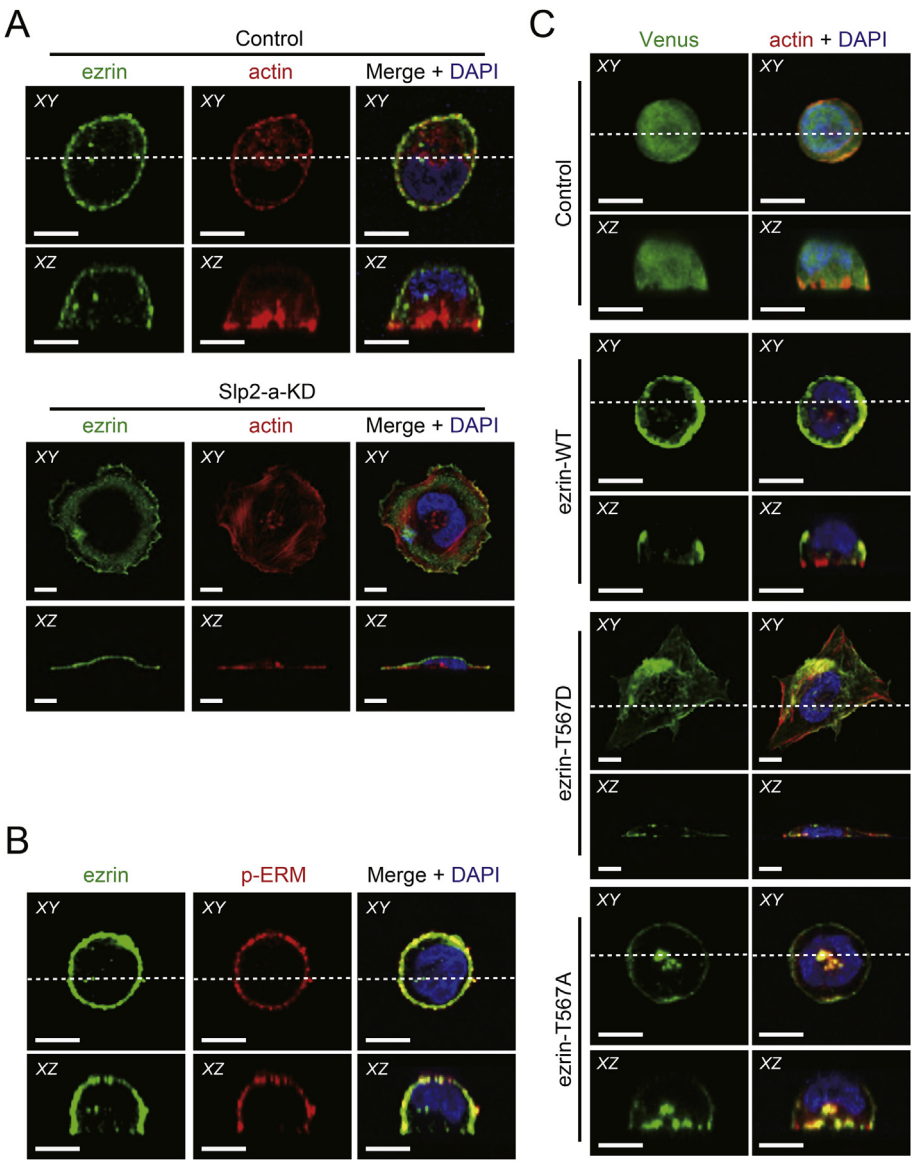

Fig. 2. The inactive form of ezrin was observed in the cytoplasm of control MDCK II cells. (A) Localization of endogenous ezrin in control and Slp2-a-KD cells. The cells were seeded on 35-mm glass-bottomed dishes, cultured for 4 h, and then stained with anti-ezrin antibody (green), Texas-Red-conjugated phalloidin (red), and DAPI (blue). (B) Activity of intracellular ezrin in control cells. The cells were seeded as described above and then stained with anti-ezrin antibody (green), anti-phospho-ezrin(T567) (anti-p-ERM) antibody (red), and DAPI (blue). (C) Localization of ezrin mutants in control cells. The cells were transfected with pVenus-N1, pVenus-N1-ezrin-WT, pVenus-N1-ezrin-T567D, or pVenus-N1-ezrin-T567A (green), and cultured for 24 h. The cells were then re-plated on 35-mm glass-bottomed dishes for 4 h and stained with Texas-Red-conjugated phalloidin (red) and DAPI (blue). Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutations into these motifs, i.e., substitutions for Ala (Fig. 4A; GILK^{272} to AAAA^{272} and KHVRF^{331} to KHARA^{331} mutations), and evaluated the PP1Cβ binding activity of these mutants by co-immunoprecipitation assays. Although the wild-type Slp2-a linker domain interacted with PP1Cβ, neither Slp2-a linker mutant interacted with PP1Cβ at all (Fig. 4B). If the interaction between Slp2-a and PP1β is actually involved in the control of cell size, disruption of the interaction by a dominant-negative construct, i.e., Slp2-a linker domain, should affect cell size. As expected, overexpression of an EGFP-tagged Slp2-a linker domain in the control cells resulted in an increase in cell size, whereas overexpression of the PP1Cβ-binding-deficient mutant (Slp2-a linker AAAA/KHARA) had no effect on cell size (Fig. 4C). These results indicated that Slp2-a regulates MDCK II cell size through interaction with PP1β via the RVxF- and SILK-like motifs.

4. Discussion

Although Slp2-a was originally identified as a Rab27 effector protein that through its SHD and C2A domain mediates trafficking of Rab27-bearing vesicles and organelles to the plasma membrane in the secretory pathway [10,11,16,23–27], we have previously shown that Slp2-a has an additional Rab27-independent function in controlling the size of renal epithelial cells [12,16]. We have also shown that Slp2-a interacts via its C2B domain with Rap1GAP2 and controls renal epithelial cell size through regulation of Rap–ezrin signaling [12], but the precise mechanism of the ezrin regulation by Slp2-a had remained unknown. In the present study, we discovered a novel function of the Slp2-a linker domain in the control of ezrin activity. Slp2-a was found to interact via its previously uncharacterized linker domain with PP1β, which is known

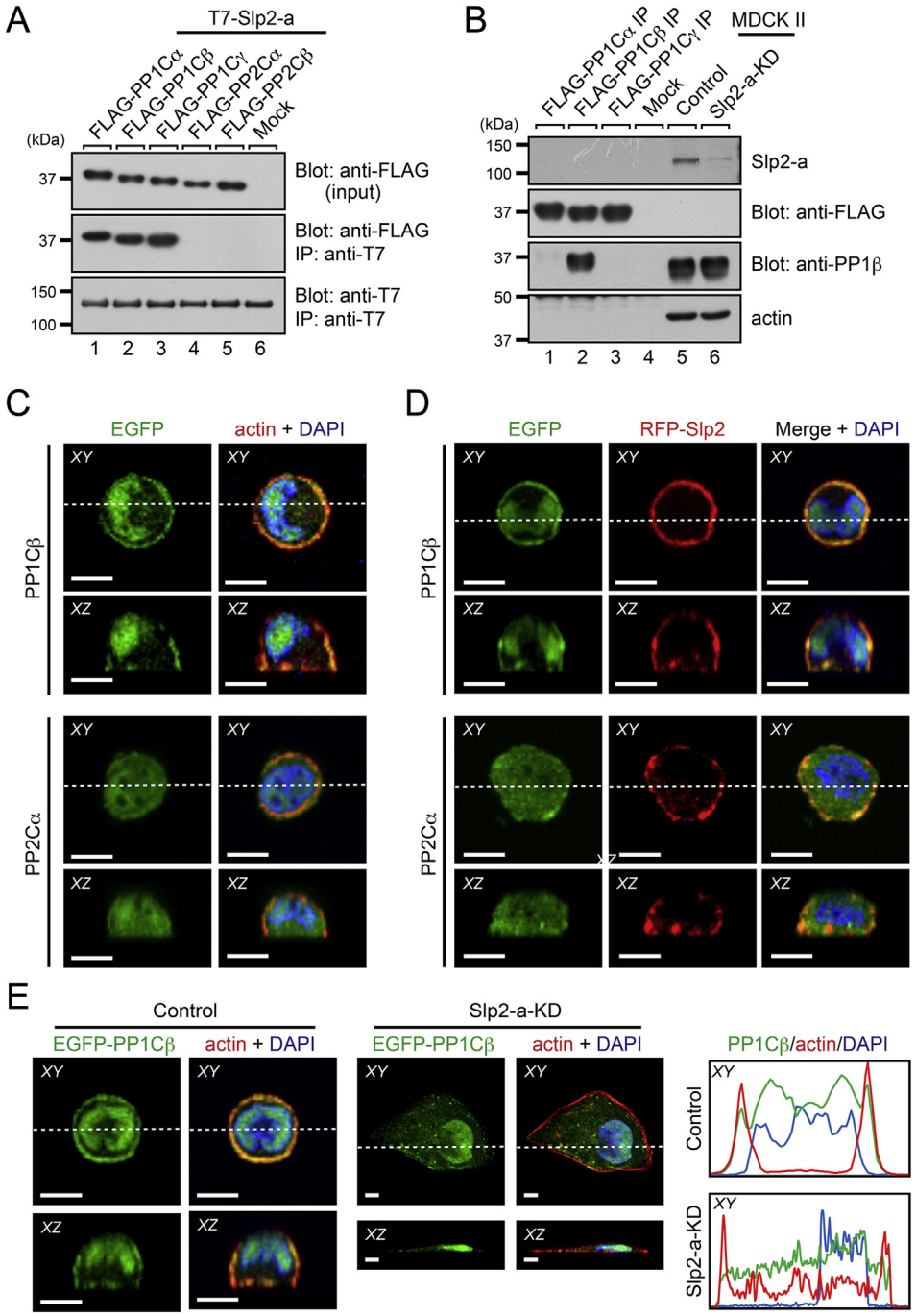

Fig. 3. Slp2-a interacts with PP1 at the plasma membrane. (A) Interaction between Slp2-a and PPs. T7-tagged Slp2-a and FLAG-tagged PPs were co-expressed in COS-7 cells, and their associations were analyzed by co-immunoprecipitation assays with anti-T7 tag antibody-conjugated agarose beads. The positions of the molecular mass markers (in kDa) are shown on the left. (B) Expression of PP1β in MDCK II cells. FLAG-tagged PP1Cα, β or γ was expressed in COS-7 cells and immunoprecipitated (IP) with anti-FLAG-tag antibody-conjugated agarose beads. Immunoisolated FLAG-tagged proteins (lanes 1–3) and total cell lysates of subconfluent control and Slp2-a-KD cells (lanes 5 and 6) were analyzed by immunoblotting with the antibodies indicated. (C) Localization of PPs in control MDCK II cells. The control cells were transfected with pEGFP-C1-PP1Cβ (or -PP2Cα) (green) and cultured for 24 h, then re-plated on 35-mm glass-bottomed dishes for 4 h and stained with Texas-Red-conjugated phalloidin (red). Nuclei were stained with DAPI (blue). Confocal *xy* and *xz* sections are shown. Top, an *xy* image; bottom, an *xz* section at the broken line (*xy*) shown in the image above. (D) Co-localization between Slp2-a and PP1β in control MDCK II cells. The cells were co-transfected with pEGFP-C1-PP1Cβ (or -PP2Cα) (green) and pmRFP-C1-Slp2-a (red) and cultured for 24 h. They were then re-plated on 35-mm glass-bottomed dishes for 4 h and stained with DAPI (blue). (E) Slp2-a-dependent plasma membrane localization of PP1Cβ. Control and Slp2-a-KD cells were transfected with pEGFP-C1-PP1Cβ (green) and stained as described above. Fluorescence intensity along the broken white lines (upper panels) is shown at the right. Scale bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to inactivate ezrin through dephosphorylation at Thr-567 [21] (Fig. 4), and to recruit PP1β to the plasma membrane of MDCK II cells (Fig. 3). In the absence of Slp2-a, PP1β was unable to target to the plasma membrane (Fig. 3E) and excess activation of ezrin was therefore observed in Slp2-a-KD MDCK II cells (Supplementary Fig. S1B). Activated ezrin is likely to be the primary cause of the increase in size of Slp2-a-KD MDCK II cells, because pharmacological inhibition of ezrin phosphorylation resulted in a decrease in the size of Slp2-a-KD MDCK II cells (Fig. 1C and D) and overexpression of the phosphomimetic ezrin-

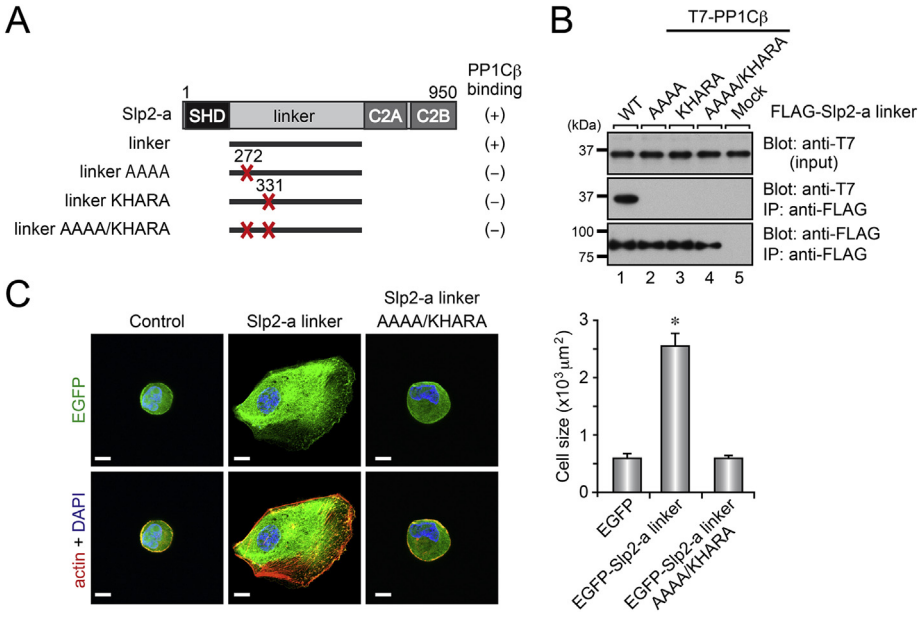

Fig. 4. The Slp2-a linker domain controls cell size through interaction with PP1β. (A) Schematic representation of the functional domains of Slp2-a and its truncated mutants (solid lines) and point mutants (crossed out in red; Ala substitution at the position of the amino acid number indicated) used in this study. Slp2-a contains an N-terminal SHD, a linker domain in the middle of the molecule, and C-terminal tandem C2 domains (C2A domain and C2B domain). The PP1Cβ binding activity of each mutant is shown on the right. (B) Mutations in the PP1Cβ-binding site in the Slp2-a linker domain. FLAG-tagged Slp2-a linker point mutants and T7-tagged PP1Cβ were co-expressed in COS-7 cells, and their associations were analyzed as described above. The positions of the molecular mass markers (in kDa) are shown on the left. (C) Effect of the Slp2-a linker domain on MDCK II cell size. (Left) Control cells were transfected with pEGFP-C1, pEGFP-C1-Slp2-a linker, or pEGFP-C1-Slp2-a linker (AAAA/KHARA) (green) and cultured for 24 h. The cells were then re-plated on 35-mm glass-bottomed dishes and cultured for 24 h, and their sizes were measured. Scale bars, 10 μm. (Right) Quantification of the cell sizes shown in the images on the left. *, $p < 0.001$, Tukey–Kramer test (n = 55 from three independent experiments). Values are means and S.E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

T567D mutant in control MDCK II cells was sufficient to increase their size (Fig. 1E).

In addition to the ezrin inactivating activity of Slp2-a as a result of recruiting PP1β to the plasma membrane as demonstrated in this study, we have previously shown that the C2B domain of Slp2-a indirectly contributes to ezrin inactivation by recruiting Rap1-GAP2 to the plasma membrane [12]. Because the PP1-binding site and Rap1GAP2-binding site in Slp2-a were completely different, Slp2-a is likely to interact with both proteins at the same time. Actually, the results of our biochemical and cellular analyses indicated that Slp2-a forms a complex with both PP1β and Rap1GAP2 at the plasma membrane (Supplementary Fig. S2), suggesting that they co-operatively function in cell size control by efficiently inactivating ezrin (see the proposed model in Supplementary Fig. S3).

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.099.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.099.

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