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Regulated Interaction of Protein Phosphatase 1 and Protein Phosphatase 2A with Phospholipase C-Related but Catalytically Inactive Protein

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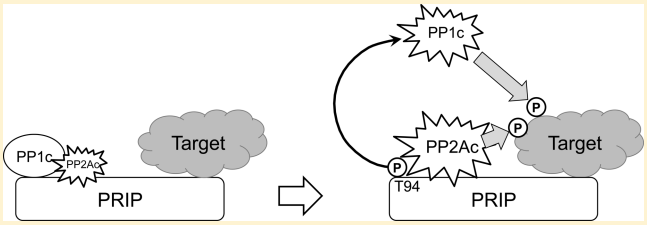
ABSTRACT: Protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are major members of the protein serine/threonine phosphatase families. We have identified PP1 and PP2A as interacting partners of PRIP (phospholipase C-related but catalytically inactive protein), a protein isolated in our laboratory. We first investigated the interaction of PRIP with two phosphatases, using purified recombinant proteins. PRIP immobilized on beads pulled down the catalytic subunits of both PP1 and PP2A, indicating that the interactions were in a direct manner, and the binding of PP1 and the binding of PP2A to PRIP were mutually exclusive. Site-directed mutagenesis experiments revealed that the binding sites for PP1 and PP2A on PRIP were not identical, but similar. Phosphorylation of PRIP by protein kinase A (PKA) resulted in the weakened binding of PP1, but not PP2A. Rather, the dissociation of PP1 from PRIP by phosphorylation accompanied the strengthened binding of PP2A in in vitro experiments. This regulation of binding of PP1 and PP2A to PRIP by PKA-dependent phosphorylation was also observed in living cells treated with forskolin or isoproterenol. These results suggested that PRIP directly interacts with the catalytic subunits of two distinct phosphatases in a mutually exclusive manner and the interactions are regulated by phosphorylation, thus functioning as a scaffold to regulate the activities and subcellular localizations of both PP1 and PP2A in phospho-dependent cellular signaling.



Reversible phosphorylation is one of the most important post-translational modifications regulating protein characteristics, such as function and stability, especially those involved in cellular signaling. The importance of this modification is suggested by the fact that approximately one-third of all eukaryotic proteins are phosphorylated on specific serine, threonine, and/or tyrosine residues based on cellular status.[1] Dynamic changes in the phosphorylation state of a protein result from the balance between localized activities of protein kinases and protein phosphatases, because each has a particular substrate preference.[2] Approximately 500 protein kinases are encoded by the human genome, ~100 of which are tyrosine kinases, countered by comparable numbers of tyrosine phosphatases. On the other hand, huge families of ~400 serine/threonine kinases are countered by smaller numbers (~45) of serine/threonine phosphatases.[3,4] Despite the unbalanced ratio of phosphatases to kinases, the phospho states of a wide variety of substrate proteins need to be regulated in a spatiotemporal manner, which is caused by the great diversity of regulatory proteins that form comparable numbers of holoenzymes; i.e., a wide variety of regulatory proteins allows small numbers of core phosphatases to be responsible for dephosphorylating variable substrates with a diverse substrate specificity.[5]

Protein phosphatase 1 (PP1) and PP2A are the most studied members of the phosphoprotein phosphatase family, both of which acquire versatile substrate specificity by forming complexes with a variety of regulatory proteins. Nearly 200 different regulatory subunits have been reported to interact with the PP1 catalytic subunit (PP1c) in a mutually exclusive manner.[6] The dominant form of PP2A in cells is a heterotrimeric holoenzyme; the core enzyme (AC core) consists of a 36 kDa catalytic subunit (PP2Ac) and a 65 kDa structural subunit A (PP2Aa). Numerous regulatory B subunits confer unique characteristics, for example, substrate specificity to the AC core dimer (PP2Aac).[1,7] The regulatory/targeting proteins for these phosphatases are still being discovered, and most do not share a common motif for interacting with the catalytic subunit, except protein phosphatase 1 (PP1), the regulatory subunits of which often share the relatively conserved sequence RVxF.[6]

PRIP (phospholipase C-related but catalytically inactive protein) was first identified in the brain cytosol fraction as a novel D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] binding protein whose primary structure was similar to that of phospholipase C (PLC) δ1, although it lacked catalytic activity.[8−11] Later, an isoform with a relatively broad tissue distribution was reported,[12,13] indicating that PRIP is composed

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of types 1 and 2. Subsequent studies identified several interacting proteins, including GABARAP [γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein],[14] PP1c,[15−17] PP2Ac,[18] and the β subunit of the $GABA_A$ receptor,[18−20] in addition to Ins(1,4,5)P_3. Analyses of phenotypes shown with PRIP gene-deficient mice (KO mice), genetically deficient in type 1 or type 2 isoforms or both, provided information regarding the physiological functions of PRIP, i.e., modulation of $GABA_A$ receptor signaling,[21−24] dysfunction of reproduction, negative regulation of multiple-hormone secretion,[25] and bone properties.[26] Altered phosphorylation states of several proteins relating to $GABA_A$ receptors,[16,18,23] bone properties (Smad-1/5/8),[26] and exocytosis (SNAP-25)[27] were observed in the mutant mice, indicating that PRIP is involved in the regulation of phospho states of various proteins, probably relating to the phenotypes observed in mutant mice. Indeed, we demonstrated that PRIP modulates the phospho states of $GABA_A$ receptors, thus regulating $GABA_A$ receptor function and trafficking.[16,18] Recently, we also found that PRIP interacts with a protein kinase, Akt, in its phosphorylated form, in insulin-stimulated cells.[23] Considering that PRIP interacts with two different families of protein phosphatases, PP1 and PP2A, and protein kinase, Akt, we assume that PRIP might work as a hub in a phospho-dependent signaling pathway by recruiting multiple enzymes involved in protein phosphorylation and dephosphorylation to target molecules in specific locations required for functions.

Despite the large numbers of regulatory subunits reported for PP1c and PP2Ac, there are only limited examples in which multiple phosphatases interact with a single molecule, for example, integrin $\alpha_{IIb}\beta_3$[28,29] and CG-NAP (centrosome- and Golgi-localized PKN-associated protein).[30] PRIP might be one of a limited number of adaptor proteins. To understand how these adapter proteins serve multiple enzymes to target proteins for fine-tuning their phospho states in a variety of cellular conditions, the relationship between an adaptor and each enzyme has to be investigated.

In this study, PRIP, as a multivalent adaptor protein, was investigated with reference to the regulation of two distinct protein phosphatases. We examined the relationship of the interaction between PRIP and PP1c and between PRIP and PP2Ac and obtained results indicating that the interaction of PP1c and PP2Ac with PRIP was mutually exclusive and the phosphorylation of PRIP by PKA regulated the binding profiles.

MATERIALS AND METHODS

Antibodies and Reagents. The catalytic subunit of PKA was obtained from Promega (Madison, WI). Forskolin was obtained from Calbiochem/Merck (Darmstadt, Germany). The anti-PP2Ac polyclonal antibody was purchased from Cell Signaling Technology (Danvers, MA). Monoclonal antibodies against PP1c, the PP2A structural subunit (PP2Aa), and the anti-GST (glutathione *S*-transferase) polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PP1c polyclonal antibody and isoproterenol were purchased from Sigma-Aldrich (St. Louis, MO). Normal rabbit and mouse globulins were purchased from Jackson Immuno Research Laboratories (West Grove, PA). [γ-^{32}P]ATP (185 MBq/mL, specific activity of 111 TBq/mmol) was purchased from Perkin-Elmer (Boston, MA).

Cell Culture and Transfection. COS-7 cells were supplied by RIKEN CELL BANK. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator. Transfection of the cells with the plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

DNA Constructs. The plasmid for expressing the GST-fused extended pleckstrin homology (PH) domain of PRIP-1 (74−298) (GST-PRIP-1PH), which contains the binding region for PP1c and PP2Ac,[15,18] was prepared as described previously[31] and was used to prepare the mutants using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequences of the mutagenic primers were as follows: K92Q/K93A, 5′-GTGGTGGCAGACAGGCAACAGTGTCTTTCAGC-3′ and 5′-GCTGAAAGACATTGTTGCCTGTCTGCCACCAC-3′; K104A/K105Q, 5′-GCATGCCATCGGCGCAGAAGATTAGCAGTG-3′ and 5′-CACTGCTAATCTTCTGCGCCGATGGCATGC-3′; K151Q/K152A, 5′-CTGGGAACCTTCCCAGGCAGACCTCGAAAAAGC-3′ and 5′-GCTTTTTCGAGGTCTGCCTGGGAAGGTTCCCAG-3′; K277D/K280D, 5′-CTCAACCCTACTCTGGACGAATCTGACATCAGGTTAAAG-3′ and 5′-CTTTAACCTGATGTCAGATTCGTCCAGAGTAGGGTTGAG-3′. The cDNA for PP2Ac was amplified by polymerase chain reaction from reverse transcripts of rat brain total RNAs using the following primers and subcloned into *Bam*HI/*Sal*I site of each vector [pET-His30[14] or pCold-TF (TaKaRa Bio)]: 5′-TAGGATCCATGGACGAGAAGTTGTTCACCAAG-3′ and 5′-GACGTCGACTACAGGAAGTAGTCTGGGG-3′. The plasmid for expressing subunit A of PP2Ac in the bacterial expression system was generated in a manner similar to that used for PP2Ac using the following primers: 5′-TAGGATCCATGGCAGCTGCCGACGG-3′ and 5′-ACGTCGACTCAGGCAAGAGAGAGAACAGTCAGAG-3′. The construction of the plasmid for expressing PP1cα in the bacterial expression system has been previously described.[15]

Preparation of Recombinant Proteins. For purification of the His-tagged recombinant proteins, *Escherichia coli* BL-21(DE3) cells transformed with appropriate plasmids were grown to an absorbance at 600 nm of 0.4 at 37 °C and then with 250 μM isopropyl β-D-1-thiogalactopyranoside at 18 °C for an additional 12−14 h. Bacterial lysate was prepared by sonication in lysis buffer [50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 25 μg/mL *p*-amidinophenylmethanesulfonyl fluoride, 3 μg/mL aprotinin, 5 μg/mL leupeptin, and 2.5 μg/mL pepstatin], followed by rotation after the addition of 1% Triton X-100 for 30 min. Purification was achieved using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen). After being extensively washed with lysis buffer without protease inhibitors, the proteins were eluted with 300 mM imidazole in lysis buffer without protease inhibitors. Purity was checked by staining with Coomassie Brilliant Blue after sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The recombinant molecules of interest were dialyzed against the solution for an overnight assay, followed by centrifugation at 100000*g* for 30 min before use. His-tagged PP2Ac was also prepared as a chimera with TF (trigger factor; His-TF-PP2Ac) to increase the solubility during preparation by following the manufacturer's protocol. GST-tagged proteins were prepared using the standard procedure with the same schedule to grow bacteria and induce the expression of the recombinant proteins described above.

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Phosphatase Assay. The phosphatase assay using [^{32}P]-phosphorylase *a* as a substrate was performed as described previously[16,17] in the presence or absence of His-tagged full-length PRIP-1. The reaction buffers for PP1c and PP2Ac were buffer 1 [50 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, and 0.03% Triton X-100] and buffer 2 [50 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, and 1 mM dithiothreitol], respectively.

GST Pull-Down Assays. The purified protein of wild-type or mutant GST-PRIP-1 (74−298) was first incubated with glutathione Sepharose 4B (GE Healthcare) in reaction buffer containing 20 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol for 30 min at 4 °C. After being washed with the same buffer, the beads were incubated with His-tagged PP1c and/or PP2Ac at 4 °C for 30 min. At the end of the incubation, the beads were washed with the reaction buffer three times, boiled in 50 μL of sample buffer for 5 min, and subjected to SDS−PAGE followed by Western blotting. Bound PP1c and PP2Ac were detected with the antibodies against each phosphatase. In some experiments, His-TF-PP2Ac was used instead of His-PP2Ac, giving results identical to those for His-PP2Ac.

To examine the effect of the phosphorylation of PRIP-1 on the binding to PP1c or PP2Ac, GST-PRIP-1 (74−298) was first phosphorylated by incubation in 50 μL of 40 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 200 μM ATP, and 0.1 μg of the catalytic subunit of PKA for 30 min at 30 °C, followed by further incubation with glutathione Sepharose 4B beads in binding buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol at 4 °C for 30 min. The beads were washed with binding buffer and subjected to a pull-down assay as described above.

Immunoprecipitation. COS-7 cells were transiently transfected to express full-length PRIP-1 in a 6 cm culture dish as described above and divided into a six-well plate the next day. The cells were serum-starved overnight, followed by a 50 μM forskolin treatment at 37 °C for 15 min and an immunoprecipitation assay. Cells were washed with phosphate-buffered saline and collected in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol, 25 μg/mL *p*-amidinophenylmethanesulfonyl fluoride, 3 μg/mL aprotinin, 5 μg/mL leupeptin, and 2.5 μg/mL pepstatin A, followed by rotation at 4 °C for 1 h, and the lysate was cleared by centrifugation at 15000*g* for 20 min. The lysate was incubated with 2 μg of the anti-PRIP-1 rabbit polyclonal antibody at 4 °C overnight and then mixed with a 10 mg bed volume of protein G-Sepharose followed by additional rotation at 4 °C for 1 h. After rotation, the beads were washed with lysis buffer three times, boiled in SDS−PAGE sample buffer for 5 min, and then subjected to Western blotting. Male mice [C57BL/6J (Charles River Laboratories Japan, Inc.)] at 12 weeks of age were treated intraperitoneally with isoproterenol (10 mg/kg), followed by isolation of whole brains to prepare the lystaes for immunoprecipitation, SDS−PAGE, and Western blotting. The handling of mice and all procedures were approved by the Animal Care Committee of Kyushu University, which follows the guidelines of the Japanese Council on Animal Care.

Statistics. All statistical analyses were performed using GraphPad Prism (GraphPad Software).

RESULTS AND DISCUSSION

Binding of the Catalytic Subunit of PP1 and PP2A with PRIP. We previously investigated the direct interaction of PRIP with PP1c using recombinant proteins,[15] but with regard to PP2A, the binding to PRIP was shown only by the data as described below. The catalytic subunit of PP2A (PP2Ac) was contained in the immunocomplex by the anti-PRIP-1 antibody from lysates of mouse brain and COS-7 cells that exogenously express the wild type or deleted mutants of PRIP-1.[18] The β3 subunit of $GABA_A$ receptors immobilized on the beads pulled down PP2Ac along with PRIP-1 from brain lystaes.[18] Thus, it is unclear whether the binding of PRIP to PP2Ac occurs in a direct or indirect manner. Here we first confirmed the direct binding of PP2Ac with PRIP-1 using recombinant proteins. PP2Ac (His-tagged form) was applied to GST alone or GST-PRIP-1 immobilized on glutathione beads, followed by immunoblotting with the anti-PP2Ac antibody. The pull-down assay in reverse mode was also performed; PRIP-1 (His-tagged form) was assayed using immobilized GST alone, GST-PP2Ac, or GST-PP2Aa. As shown in Figure 1A, the direct binding of PP2Ac to PRIP-1 is shown. Subunit A of PP2A (PP2Aa) alone did not show binding with PRIP-1, but when the mixture of the catalytic and A subunits of PP2A was applied to immobilized PRIP-1, subunit A along with the catalytic subunit showed binding (results not shown).

For quantitative analysis of the interaction, brain lysates prepared from wild-type (WT) and KO mice were subjected to immunoprecipitation by the anti-PRIP-1 antibody, followed by immunoblotting with each antibody, as designated in Figure 1B. Brain lysates contained PP1c and PP2Ac, and PP2Aa, as well as PRIP-1 for WT. The result clearly showed that the catalytic subunits of PP1 and PP2A were coprecipitated with PRIP-1 only from the brain lysate of WT. From the band density in the precipitates compared to that in the lysates, as assessed by the anti-PRIP-1 antibody, we estimated that ∼9.0% PRIP-1 present in the lysates was immunoprecipitated by the anti-PRIP-1 antibody. When this value is taken into account, a similar calculation based on band densities by each antibody led to the result that ∼0.55 ± 0.05 and ∼0.31 ± 0.03% of PP1c and PP2Ac were coprecipitated with PRIP-1, respectively, whereas the structural A subunit of PP2A appeared to be coprecipitated maximally up to 0.19 ± 0.05% with PRIP-1, probably through the interaction with the catalytic subunit. This rate difference between subunits C and A of PP2A indicated preferable binding of PRIP-1 to the monomeric catalytic subunit rather than the preformed AC core dimer of PP2A (PP2Aac). Given that there is as much PRIP-2 as PRIP-1, and type 2 also interacted with the catalytic subunits of PP1 and PP2A with an affinity similar to that of PRIP-1,[17] the percentage mentioned above would be twice as high in the brain. Therefore, ∼3% cellular PP1c and PP2Ac appear to form a complex with PRIP; this percentage is relatively high for a single molecule among many PP1c and PP2Ac binding proteins, indicating the importance of PRIP in the regulation of phosphatases relating to cellular functions.

We further examined the effect of formation of the complex with PRIP-1 on the catalytic activities of PP2A, together with PP1, using [^{32}P]phosphorylase *a* as a protein substrate. Purified PRIP-1 dose-dependently inhibited PP1c phosphatase activity in a manner similar to that reported in the previous paper using [^{32}P]myosin light chain,[15] whereas it did not affect PP2A phosphatase activity at concentrations of a 100-fold molar excess of PRIP over the phosphatase (Figure 1C).

Mode of Binding of PP1c and PP2Ac with PRIP. We previously showed that PP1c interacts with PRIP-1 through the consensus binding motif for PP1c, ^{93}KTVSF^{97}, existing just at

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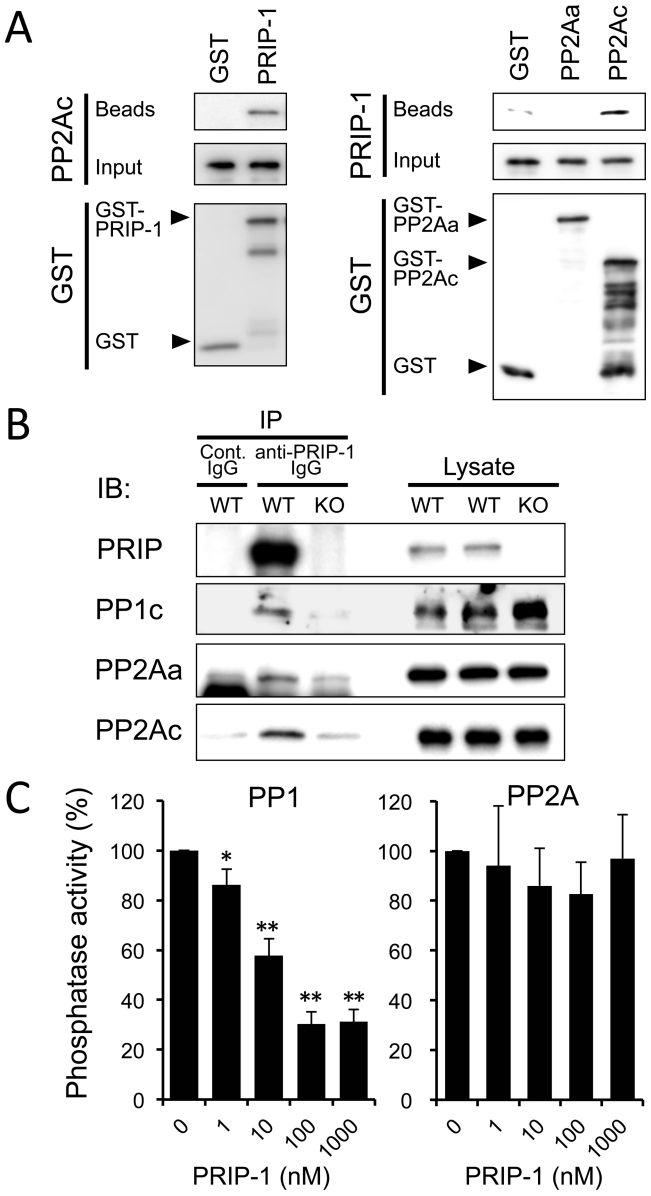

Figure 1. Interaction of PRIP with the catalytic subunits of PP1 and PP2A. (A) Binding of PP2Ac to PRIP-1. (left) Recombinant PP2Ac was applied to GST-PRIP-1 or GST alone immobilized on glutathione beads, followed by immunoblotting with the anti-PP2Ac antibody. In the right panel, His-tagged PRIP-1 was applied to GST-PP2Ac, GST-PP2Aa, or GST alone immobilized on glutathione beads, followed by immunoblotting with the anti-PRIP-1 antibody. Bands observed with the anti-GST antibody were identified by arrowheads, as judged by the molecular sizes. Other unidentified bands were degradative products. Typical blots are shown, and many other blots provided similar results. (B) Formation of the complex of PRIP with PP1c and PP2Ac in the mouse brain. PRIP-1 was immunoprecipitated using the anti-PRIP-1 antibody from brain lysates of WT and KO mice. The lysates and immunoprecipitates were subjected to Western blotting by the indicated antibodies; 0.1 or 20% of the total amount of brain lysates or immunoprecipitates, respectively, was subjected to SDS–PAGE, the values of which were taken into account for the calculation (see Results and Discussion). The blots shown are typical of six independent experiments. The other experiments gave similar results. The dense band seen slightly below PP2Aa (65 kDa) is unknown but is probably the heavy chain of IgG because 10 times more IgG was used for the control. PP2Aa and PP2Ac seemed to be precipitated slightly with normal IgG (Cont. IgG) because faint bands were seen even when the brain lystaes of PRIP-KO mice were used. (C) Effect of

Figure 1. continued

PRIP on phosphatase activity. Phosphatase activity was assayed with each phosphatase at 10 nM using [^{32}P]phosphorylase *a* as a substrate in the presence of various concentrations of PRIP-1. The ^{32}P radioactivity released during the 10 min incubation was counted and expressed as a percentage of that in the absence of PRIP-1. Radioactivities of approximately 5800 and 2300 dpm were counted in the absence of PRIP-1 for PP1c and PP2Ac, respectively. Results are expressed as means ± SE of five separate experiments. *p < 0.05; **p < 0.01.

the amino terminus to the PH domain of PRIP-1,[15] and that PP2Ac also interacts with PRIP-1 through the region containing the PH domain (amino acids 83–297 of PRIP-1),[18] indicating that the sites responsible for binding are identical or very close. We here examined the relationship of the binding of two molecules. As shown in Figure 2A, the GST-fused PH domain of PRIP-1 (PRIP-1), but not GST alone, immobilized on glutathione beads interacted with PP1c (3 nM), which was displaced by recombinant PP2Ac in a dose-dependent manner. In reverse, the level of binding of PP2Ac to the beads increased dose-dependently (Figure 2A). Figure 2B

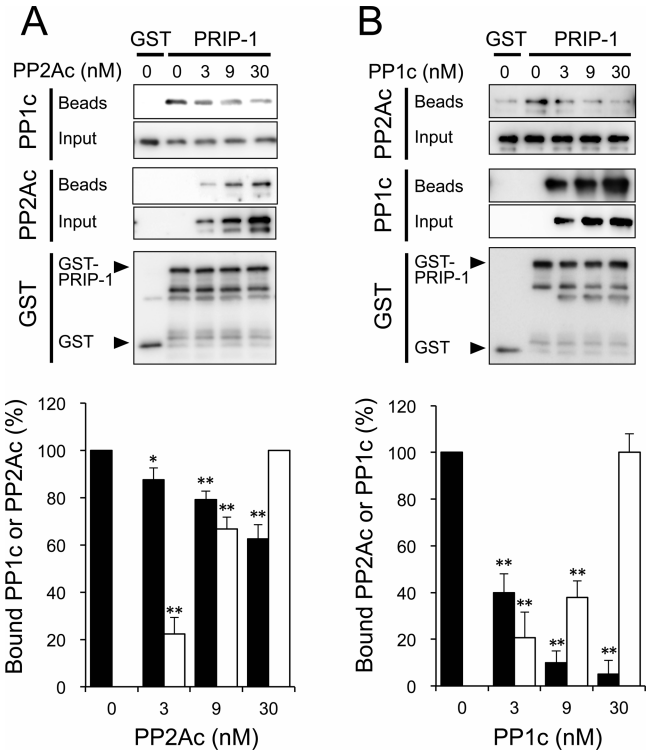

Figure 2. Modes of binding of PP1c and PP2Ac to PRIP. GST or GST-PRIP-1PH (74–298) (0.6 pmol) immobilized on glutathione beads was incubated with 3 nM His-tagged PP1c or PP2Ac in the presence of 3, 9, or 30 nM His-tagged PP2Ac (A) or PP1c (B), respectively. Materials bound to the beads were separated by SDS–PAGE, followed by immunoblotting with the indicated antibodies. Bands observed with the anti-GST antibody were identified by arrowheads, as judged by the molecular sizes. Other unidentified bands were degradative products. The blots shown are representative of five independent experiments. The binding of phosphatase in the absence of its partner phosphatase was taken to be 100% for black bars, while that at 30 nM partner phosphatase was taken to be 100% for white bars. *p < 0.05; **p < 0.01.

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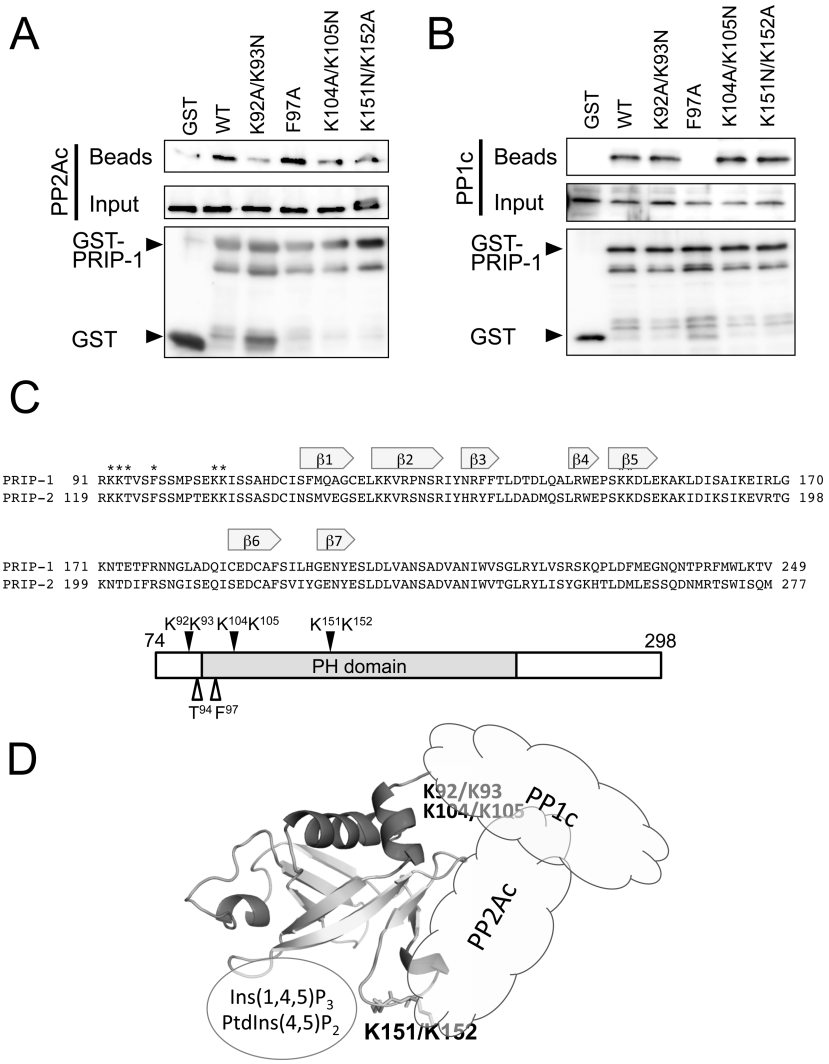

Figure 3. Mutational analysis of the binding site for PP2Ac on PRIP. GST alone or WT or mutant GST-PRIP-1PH (0.6 pmol) immobilized on glutathione beads was incubated with His-tagged PP2Ac (A) or PP1c (B). Materials bound to the beads were separated by SDS−PAGE, followed by Western blotting with the anti-GST (A and B, bottom panels), anti-PP2Ac (A, top panel), or anti-PP1c (B, top panel) antibody. Bands observed by anti-GST were identified by arrowheads, as judged by the molecular sizes. Other unidentified bands were degradative products. The blots shown are representative of five independent experiments. (C) Sequence alignment of PRIP-1 and -2 and schematic representation of PRIP-1 (amino acids 74−298). The sequences including the PH domains of rat PRIP-1 (GenBank entry NP_445908) and PRIP-2 (GenBank entry NP_001100350) were aligned using ClustalW2 and a Gonnet-weight matrix with a gap-opening penalty of 5 and an extension penalty of 0.05. The β-strands conserved among PH domains are aligned over the sequences. Amino acid residues mutated in this study are denoted with asterisks; their positions are shown with black arrowheads, and the mutations denoted with white arrowheads were generated in previous studies.[15,16] (D) Structure of the region of PRIP-1 shown in panel C and possible binding sites for Ins(1,4,5)P_3, PP1c, and PP2Ac. The model was drawn using PyMol (http://www.pymol.org/). The three-dimensional coordinates of the PH domain of PRIP-1 were generated with the SWISS-MODEL server using the X-ray crystallographic structure of the PH domain of PLC-δ1 (Protein Data Bank entry 1MAI) as the template and visualized with PyMol.

shows the results of experiments performed in reverse, which indicate that the binding of the catalytic subunits of both phosphatases to PRIP-1 is mutually exclusive in an either competitive or sterically exclusive manner.

To discriminate the two possible reasons for the mutual exclusion, we attempted to identify the binding site of PP2Ac. For this purpose, we generated several mutants based on the reasons described below. Mutant F97A, which was already generated for PP1c in previous studies,[15,16] was also used. No common sequences have been identified for binding to PP2Ac in the binding proteins reported. For instance, Yang et al. reported that PP2Ac interacts with Tap42/α4 via a region of this protein conserved among various species.[32] They replaced positively charged residues Arg-163 and Lys-166 present in the conserved region (RxxKI, where x indicates any residue) by reversing the charged residues, Glu and Asp, respectively (R163E/K166D), which resulted in weakened binding to PP2Ac.[32] We found a similar sequence, ^{277}KESKI^{281}, in the region following the PH domain of PRIP-1 and tested PP2Ac binding of mutated PRIP-1, in which Lys-277 and Lys-280 were replaced with aspartate; however, there was no effect on PP2Ac binding (data not shown).

The integrin α_{IIb} subunit was also shown to interact with the catalytic subunits of both PP1 and PP2A through a consensus motif, ^{989}KVxF^{992}, and a region, ^{989}KVGFFKR^{995}, respectively,[28] indicating that the binding sites of PP1c and PP2Ac overlapped. We also found the presence of double basic amino acids (KK at positions 92 and 93) close to the consensus motif for PP1c

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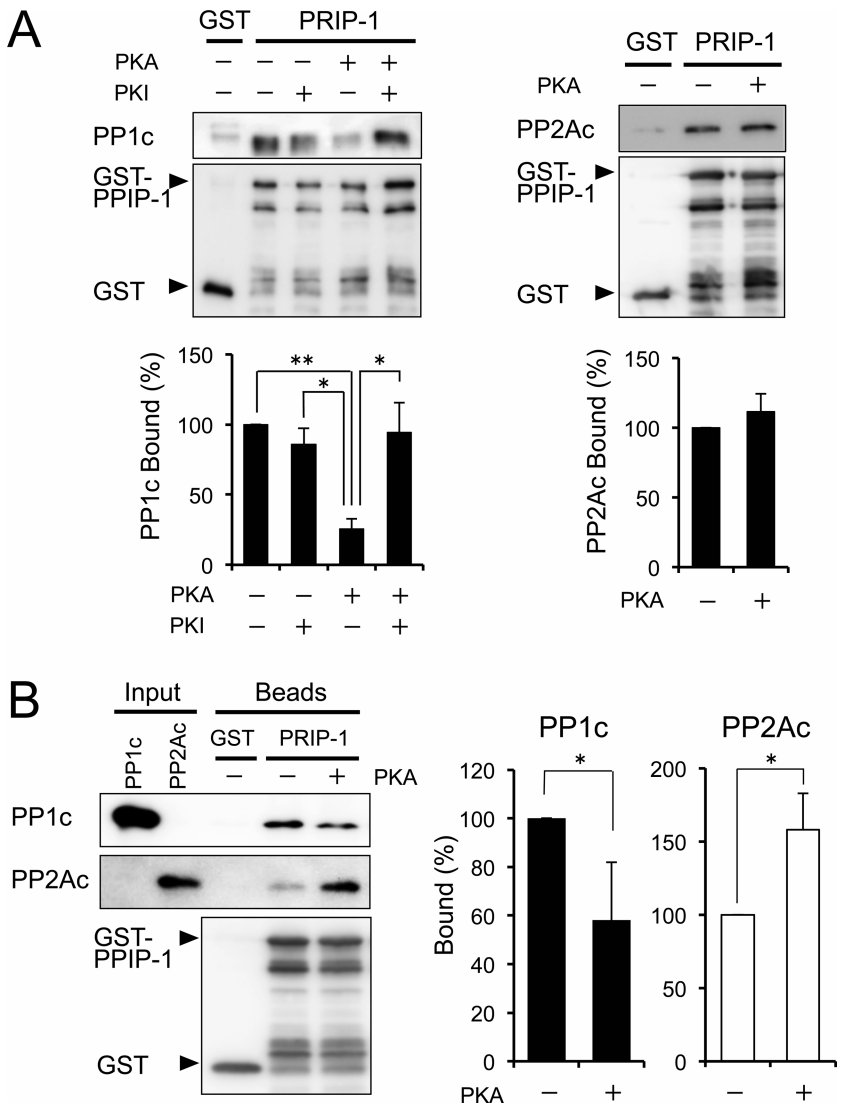

Figure 4. Effect of PRIP phosphorylation by PKA on PP1c and PP2Ac binding. (A) GST-PRIP-1PH (36 ng) was incubated with Mg-ATP in the presence or absence of the catalytic subunit of PKA (2 pmol) for 30 min at 30 °C, and the mixture was applied to glutathione beads, followed by incubation separately with His-tagged PP1c (25 ng) or PP2Ac (25 ng). (B) His-tagged PP1c and PP2Ac were simultaneously incubated with phosphorylated PRIP-1 immobilized on beads. Materials bound to the beads were separated by SDS–PAGE, followed by Western blotting with the antibodies against GST, PP1c, or PP2Ac. Bands observed with the anti-GST antibody are identified by arrowheads, as judged by the molecular sizes. Other unidentified bands were degradative products. The blots shown are representative of five independent experiments. The binding of PP1c or PP2Ac to nonphosphorylated PRIP-1 is taken to be 100%. Each bar represents the mean of five independent experiments ± SE. *$p < 0.05$; **$p < 0.01$.

(Figure 3C); thus, we generated a mutant in which lysines at positions 92 and 93 were mutated to alanine and asparagine (K92A/K93N). Either GST-fused PRIP-1 WT or mutants F97A and K92A/K93N immobilized on glutathione beads were assayed for binding to PP1c and PP2Ac. As shown in Figure 3A, mutant K92A/K93N robustly weakened the binding of PP2Ac, whereas F97A yielded binding similar to that of the WT. In reverse, the level of binding of PP1c was not decreased with the mutants at K92 and K93 but apparently decreased with mutant F97A, as described previously (Figure 3B),[15] indicating that the amino acid residues responsible for the binding to PP1c and PP2Ac were different but close in space.

PP2Ac binding was also identified in another protein, Rb2/p130, by Purev et al.[33] Two nuclear localization signals ([1080]SPSKRLRE and [1097]TPTKKRGI) in the C-terminus of Rb2/p130 were both responsible for binding to PP2Ac, and therefore, replacement of the basic residues within the motifs with alanine abrogated PP2Ac binding.[34] Thus, we also examined whether the similar sequences present in PRIP-1 [[101]PSEKKISS and [149]PSKKDLE (see Figure 3C)] were involved in PP2Ac binding. Introduction of the mutations by replacing lysine residues with alanine or asparagine (K104A/K105N and K151N/K152A) resulted in a reduction in the level of binding of PP2Ac to PRIP-1, as shown in Figure 3A, whereas the binding of PP1c was not affected (Figure 3B).

The basic residues mutated here (K151 and K152) correspond to those for recognizing Ins(1,4,5)P_3/phosphatidylinositol 4,5-diphosphate by forming hydrogen bonds with the phosphate group of these inositol compounds.[35] We therefore examined whether inositol phosphates and PP2Ac both affect the binding to PRIP-1. Neither a 100-fold excess of Ins(1,4,5)P_3 over PP2Ac nor a 10-fold excess of PP2Ac over

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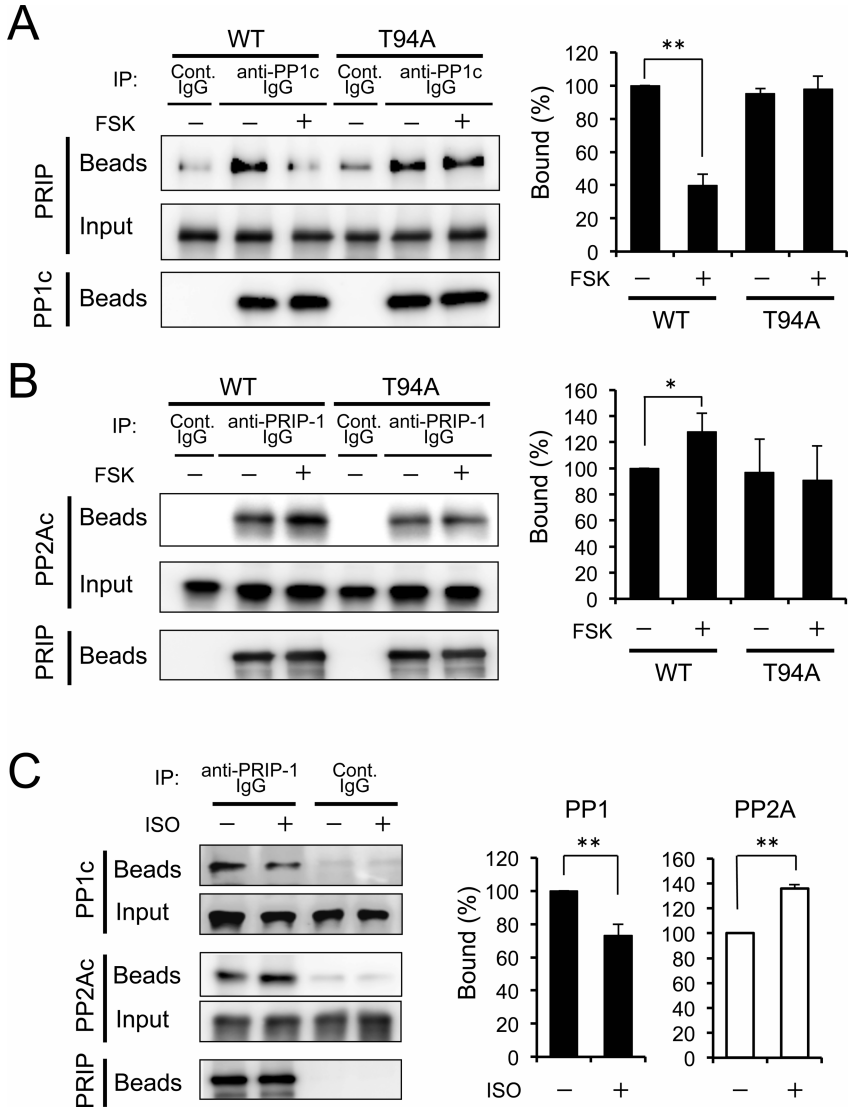

Figure 5. Treatment of cells with forskolin upon binding of PP1c and PP2Ac to PRIP. COS-7 cells were transiently transfected to express WT or mutant (T94A) PRIP-1. After stimulation with 50 μM FSK for 15 min, the cell lysate was subjected to immunoprecipitation using the anti-PP1c antibody (A) or anti-PRIP-1 antibody (B). The lysate and immunoprecipitate were separated by SDS−PAGE, followed by Western blotting using the indicated antibodies. The blots shown are representative of five independent experiments. Band density (immobilized/total) assayed using WT cells without forskolin stimulation was taken to be 100%. The binding of PRIP with phosphatase is indicated as a percent of the binding of WT PRIP-1 in control cells. Each bar represents the mean of five independent experiments ± SE. *$p < 0.05$; **$p < 0.01$. (C) Mice at 12 weeks of age were treated intraperitoneally with isoproterenol (10 mg/kg). Thirty minutes after the injection, the whole brain was isolated, followed by the preparation of lysates and immunoprecipitation using the anti-PRIP-1 antibody. The immunoprecipitates were subjected to SDS−PAGE and Western blotting using antibodies of interest. The binding of PRIP with PP1c or PP2Ac is represented as described for panels A and B.

$Ins(1,4,5)P_3$ showed an effect on the respective binding (data not shown), indicating that PRIP-1 accommodates both $Ins(1,4,5)P_3$ and PP2Ac simultaneously. In the structural model of the PH domain of PRIP-1 formed on the basis of that of PLC-δ1, $Ins(1,4,5)P_3$ appears to bind to the bottom surface that is formed by variable loops 1 and 3 in an analogy to the PH domain of PLC-δ1,[35] while the binding to PP2Ac appears to be another surface of the PH domain composed of variable loop 3 containing Lys-151 and Lys-152 and the amino-terminal extension to the PH domain containing Lys-92, Lys-93, Lys-104, and Lys-105 (see Figure 3C,D). This might partially explain the lack of competition between PP2Ac and $Ins(1,4,5)P_3$ for PRIP binding. It is also possible that the mode of binding of PRIP to $Ins(1,4,5)P_3$ is different from that of PLC-δ1, although the binding affinity assayed in a solution using $[^3H]Ins(1,4,5)P_3$ was similar.[36]

Effect of Phosphorylation of PRIP by PKA on PP1c and PP2Ac Binding. We then examined the effect of phosphorylation of PRIP-1 on the binding of PP1c and PP2Ac, because we reported previously that Thr-94 adjacent to the PP1c binding motif in PRIP-1 was phosphorylated by PKA both in vitro and in vivo, and the phosphorylation at Thr-94 weakened PP1c binding.[16,27] We first confirmed the previous results in vitro. When PRIP-1 immobilized on the beads was incubated with PKA along with Mg-ATP, the level of binding of PP1c was apparently decreased, as shown in Figure 4A (left panel). Weakened PP1c binding was not seen when either PKA or Mg-ATP was omitted, or PKA was added together with PKI (inhibitor of PKA), indicating that the phosphorylation of PRIP

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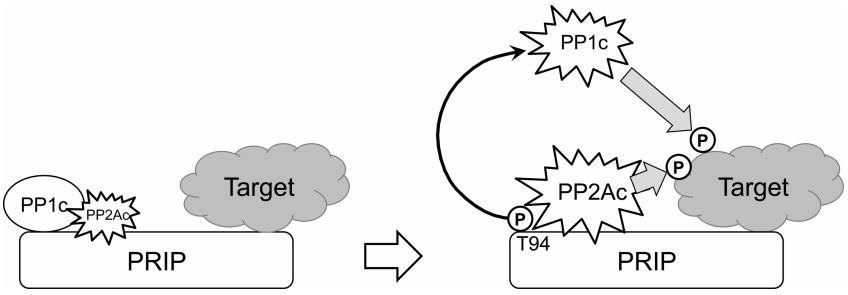

Figure 6. Schematic representation of modes of regulation of PP1c and PP2Ac by PRIP. The left and right panels represent unstimulated PRIP and PRIP stimulated for phosphorylation, respectively. A phosphatase with a round or serrated margin indicates the inactive or active form, respectively. For details, see Results and Discussion.

caused the decrease in the level of PP1c binding. On the other hand, the binding of PP2Ac was not changed by the phosphorylation of PRIP (right panel of Figure 4A).

On the basis of the mutually exclusive binding of PP1c and PP2Ac to PRIP-1 shown in Figure 2, we assumed that the reduction in the level of binding of PP1c to PRIP-1 by phosphorylation results in an increased level of binding of PP2Ac. We therefore tested this possibility by comparing the amount of PP1c and PP2Ac bound to PRIP-1 on beads analyzed by Western blotting. As shown in Figure 4B, PKA treatment of PRIP-1 weakened PP1c binding, while that of PP2Ac was strengthened, indicating that not only PP1c but also PP2Ac binding to PRIP is regulated by the phosphorylation of PRIP in cells where the phosphatases coexist.

To examine whether PKA-dependent phosphorylation of PRIP also changes the binding profile between PP1c and PP2Ac in living cells, we first examined COS-7 cells, which contain intrinsic PP1c and PP2Ac, but not PRIP-1. COS-7 cells transiently transfected with genes for WT PRIP-1 or the T94A mutant, which fails in phosphorylation at the site relating to PP1c binding,[16] were stimulated with forskolin to increase the level of cellular cAMP causing PKA activation, followed by the preparation of cell lysates and immunoprecipitation by anti-PP1c (Figure 5A) or anti-PRIP-1 antibodies (Figure 5B). The immunoprecipitates were blotted for PRIP-1 or PP1c and PP2Ac. The results show that the binding of PRIP-1 to PP1c was weakened by stimulation with forskolin, while that of PP2Ac to PRIP-1 was reversely strengthened when COS-7 cells transfected with WT-PRIP-1 were used. On the other hand, when COS-7 cells transfected with T94A PRIP-1 were used, such changes were not observed. We also used brain lysates from mice treated with isoproterenol as a physiologically relevant example and found similar binding profiles (Figure 5C).

Although many proteins have been shown to regulate the function of a single protein phosphatase by direct binding,[30] a few proteins are known to interact with multiple families of phosphatases.[30] Such proteins often bind not only multiple phosphatases but also protein kinases to function as a scaffold to modulate the phospho-dependent signaling pathway.[37] We here assumed that PRIP is a novel protein belonging to such a protein family, because we have shown that PRIP interacts with protein phosphatases, PP1c and PP2Ac, and a protein kinase, Akt.[15,18,23] Other examples of such multivalent adaptor proteins are integrin $\alpha_{IIb}\beta_3$[29] and CG-NAP.[30] Of these proteins, Vijayan et al. showed that integrin $\alpha_{IIb}\beta_3$ interacts with catalytic subunits of both PP1 and PP2A through the α_{IIb} subunit, and only PP1, but not PP2A, dissociates by thrombin-induced aggregation or the engagement with fibrinogen in platelets.[28] Through the binding of PP1c and PP2Ac, in addition to the binding of other enzymes, including c-src, protein tyrosine phosphatase 1B, and protein kinase Cβ, integrin $\alpha_{IIb}\beta_3$ regulates the phospho states of downstream target proteins, thus controlling cellular functions.[28] The properties of binding of PRIP-1 to protein phosphatases shown in this study are similar to those of the integrin α_{IIb} subunit; i.e., both PRIP-1 and integrin α_{IIb} directly interact with PP1c through consensus PP1c binding motifs and inhibit catalytic phosphatase activity. The proteins no longer bind PP1c as an active form by cellular activation to induce their own phosphorylation but retain an increased amount of PP2Ac, resulting in accelerated dephosphorylation of their downstream target proteins by PP1c and PP2Ac. The proximity of the binding sites for PP1c and PP2Ac is also similar to those in both integrin α_{IIb} and PRIP. The binding sites for PP1c and PP2Ac were not identical, but PP2Ac binding was inhibited by introducing the mutation into the amino acids adjacent to the PP1c consensus motif (K92A/K93N) of PRIP-1, which is also similar to that of integrin α_{IIb}.[29]

Summarizing the results obtained in this study, we depict regulatory modes of protein phosphatases, PP1c and PP2Ac, by PRIP (see Figure 6). PRIP associates with the catalytic subunits of PP1 and PP2A in a mutually exclusive manner, whose catalysis is inactive and active, respectively, and recruits the phosphatases to the sites required inside cells by binding via other regions present in PRIP, including the PH domain, the EF hand motif, the X-Y domain, the C2 domain, etc. When cells are stimulated for the phosphorylation of target proteins to advance the cellular signaling pathway, PRIP itself is also phosphorylated, liberating PP1c to an active form and then associating more active PP2Ac, leading to the promotion of the dephosphorylation of target proteins to terminate the signaling pathway promptly. Thus, the presence of PRIP would generate the transient sharp phospho regulation of target proteins; otherwise, cellular signaling proceeds improperly because of persistent phosphorylation of target proteins. Such transient phospho regulation by PRIP has already been found in PKA-dependent phospho regulation of the β subunit of $GABA_A$ receptors[16,18] and SNAP-25,[27] because these substrates phosphorylated by PKA are preferentially catalyzed by PP1c. In addition, association of PRIP with more active PP2Ac, in place of PP1c liberated from PRIP by the phoshorylation (phosphatase swapping), might also contribute to the regulation of events requiring protein substrates specific to PP2Ac. For instance, it was recently reported that phosho-Akt in the insulin signaling pathway is dephosphorylated by PP2Ac through complex formation along with Clk2 and B56β,[38] so it is

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possible that PRIP is implicated in insulin signaling via phosphatase swapping, because PRIP binds to the phosphorylated form of Akt as reported previously.[23] Furthermore, we recently found that PP2Ac was more actively implicated in SNAP-25 phosphorylation by PKC than by PKA,[27] so phosphatase swapping on PRIP might be actively implicated in PKC regulaton of SNAP-25. Further work on the physiological relevance of these findings is apparently needed.

In addition, further studies considering an individual cell signaling pathway would explain the phenotypes observed in PRIP-KO mice, such as hypersecretion of dense-core vesicles,[39] bone properties,[26] $GABA_A$ receptor signaling,[21,23,24] and malfunction of the reproduction system.[25]

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Notes

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ABBREVIATIONS

FSK, forskolin; GABA, γ-aminobutyric acid; GST, glutathione *S*-transferase; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; KO, knockout; PKA, cAMP-dependent protein kinase; PP, protein phosphatase; PRIP, phospholipase C-related but catalytically inactive protein; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SE, standard error; SNAP-25, synaptosome-associated protein of 25 kDa; TF, trigger factor; WT, wild type.

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