Background: PRIP inhibits exocytosis, but the underlying mechanism is unknown.
Results: PRIP interacts with syntaxin-1 and SNAP-25 through its C2 domain and inhibits SNARE complex formation.
Conclusion: Inhibition of exocytosis by PRIP is attributed to the direct binding to SNAREs and the inhibition of SNARE complex formation.
Significance: PRIP is a new member of SNARE-binding proteins bearing C2 domain that are involved in regulating exocytosis.

Membrane fusion for exocytosis is mediated by SNAREs, forming trans-ternary complexes to bridge vesicle and target membranes. There is an array of accessory proteins that directly interact with and regulate SNARE proteins. PRIP (phospholipase C-related but catalytically inactive protein) is likely one of these proteins; PRIP, consisting of multiple functional modules including pleckstrin homology and C2 domains, inhibited exocytosis, probably via the binding to membrane phosphoinositides through the pleckstrin homology domain. However, the roles of the C2 domain have not yet been investigated. In this study, we found that the C2 domain of PRIP directly interacts with syntaxin 1 and SNAP-25 but not with VAMP2. The C2 domain promoted PRIP to co-localize with syntaxin 1 and SNAP-25 in PC12 cells. The binding profile of the C2 domain to SNAP-25 was comparable with that of synaptotagmin I, and PRIP inhibited synaptotagmin I in binding to SNAP-25 and syntaxin 1. It was also shown that the C2 domain was required for PRIP to suppress SDS-resistant ternary SNARE complex formation and inhibit high K⁺-induced noradrenalin release from PC12 cells. These results suggest that PRIP inhibits regulated exocytosis through the interaction of its C2 domain with syntaxin 1 and SNAP-25, potentially competing with other SNARE-binding, C2 domain-containing accessory proteins such as synaptotagmin I and by directly inhibiting trans-SNARE complex formation.

Exocytosis is one of the fundamental cellular events by which cells secrete neurotransmitters, neuropeptides, and peptide hormones and also distribute membrane proteins such as receptors, channels, and transporters to the cell surface. The final step of exocytosis, membrane fusion, is mediated by heterotrimERIC complexes of SNARE proteins (1–3) consisting of members of the vesicle-associated membrane protein (VAMP, also called synaptobrevin) family on the vesicular membrane (v-SNARE) and syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) families on the target plasma membrane (t-SNARE). α-Helical SNARE motifs from VAMP and syntaxin and two from SNAP-25 forming parallel coiled-coil bundles are believed to promote fusion of vesicular and target membranes (4, 5). A number of accessory proteins regulating SNARE-mediated membrane fusion have been shown to interact directly with individual SNARE proteins and/or with assembled SNARE protein complexes (6).

PRIP (phospholipase C-related but catalytically inactive protein), consisting of type 1 and type 2, was originally isolated as a novel d-myoinositol 1,4,5-trisphosphate-binding protein in our laboratory. It was named for its lack of catalytic activity despite structural similarity to phospholipase C (PLC)-δ1 (7, 8), comprising a pleckstrin homology (PH) domain, EF-hand motifs, X and Y motifs, and a C2 domain. In addition to d-myoinositol 1,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) binding to the PH domain (9, 10), a...
number of interacting partners have been identified for PRIP including GABARAP (GABA_\alpha_ receptor-associated protein) (11, 12), \( \beta \) subunit of GABA_\alpha_ receptor (13, 14), the catalytic subunit of protein phosphatase 1\( \alpha \) and 2A (13, 15, 16), and the phosphorylated (active) form of Akt (17). To explore the biological functions of PRIP in relation to these interacting proteins, we generated PRIP-1 and/or PRIP-2 KO mice whose phenotypes were partly reported (11, 13, 14, 17) and also found that the mice exhibited increased exocytosis of various peptide hormones such as gonadotropins and insulin (18, 19), suggesting that PRIP exerted inhibitory effects on exocytosis. Therefore, female KO mice exhibited impaired reproduction, probably attributed to the hypersecretion of gonadotropins.\(^7\) We subsequently investigated the molecular mechanism by which PRIP inhibited dense core vesicle exocytosis. PtdIns(4,5)\( \_P_2 \) is required for vesicle exocytosis (20–22), and we found that PtdIns(4,5)\( \_P_2 \) binding to its PH domain was required for PRIP to suppress exocytosis. By binding to PtdIns(4,5)\( \_P_2 \), PRIP localizes to sites of exocytosis and competes with other molecules such as CAPS (Ca\(^{2\,+}\)-activated protein for secretion) for PtdIns(4,5)\( \_P_2 \) binding required for exocytosis.\(^5\) In the course of these experiments, however, we noticed that other mechanisms besides PtdIns(4,5)\( \_P_2 \) binding of the PH domain are also needed for PRIP to exert full inhibition.

In this study, we investigate the role of the PRIP-C2 domain in the inhibition of exocytosis in light of the many reports that a variety of proteins with C2 domains participate in exocytosis (23, 24). We found that the PRIP-C2 domain showed little interaction with phospholipids but interacted with t-SNARE proteins in a Ca\(^{2\,+}\)-dependent manner. The C2 domain was required for the co-localization of PRIP with t-SNAREs in cells. Moreover, the C2 domain also had direct inhibitory effects on ternary SNARE complex formation and on the participation of synaptotagmin. Thus, we propose that PRIP is a new member of C2 domain-containing proteins that regulate membrane traffic by its negative regulation of exocytosis through a combination of PtdIns(4,5)\( \_P_2 \) binding by its PH domain\(^7\) and t-SNARE binding by its C2 domain.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—**\([\text{\textsuperscript{3}}H]\text{Noradrenalin (NA)}\) was obtained from GE Healthcare. The Duolink \textit{in situ} kit for proximity ligation assay (PLA) was from Olink Bioscience (Uppsala, Sweden). The antibodies used were as follows: SNAP-25 (Sigma-Aldrich), VAMP2 (Synaptic Systems, Göttingen, Germany), Munc18 (BD Transduction Laboratories), and syntaxin 1, synaptotagmin I, GFP and GST (Santa Cruz Biotechnology, Santa Cruz, CA). Alexa Fluor 488 anti-rabbit antibody was from Invitrogen. Cy3 anti-mouse antibody, normal rabbit and mouse antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Anti-PRIP-1 mouse monoclonal antibody (2F9) and antigen-purified rabbit polyclonal antibody were prepared in this laboratory as described previously (11, 25).

\(^5\)H. Takeuchi, J. Gao, Z. Zhang, D. James, T. Martin, and M. Hirata, manuscript in preparation.

\(^7\)M. Matsuda, M. Kotani, and M. Hirata, manuscript in preparation.

**DNA Constructs—**The plasmid to express EGFP-PRIP-1 in mammalian cells and the full-length (PRIP-WT, amino acid residues 1–1096) and deletion mutant (PHXY, amino acid residues 82–704) of His-tagged PRIP-1 for baculovirus expression system were prepared as described previously (10). EGFP-PRIP-1 lacking the C2 domain (PRIP\( \Delta C_2 \)) was prepared as follows. Both the 5' and 3' end regions corresponding to outside the C2 domain of PRIP-1 were amplified by PCR, and the HindIII/Sall fragment of the 5' end region was first subcloned into HindIII/Sall-digested vector, pEGFP-C3 (Clontech), followed by subcloning the Xhol/Sall fragment of 3'-end region into Sall site of the plasmid prepared as above. The resulting construct was used to express PRIP-1 lacking the residues 714–850 (PRIP\( \Delta C_2 \), PRIP, whose Arg at position 134 was replaced with Gln to produce the mutants of R134Q and R134Q/\( \Delta C_2 \) for diminishing PtdIns(4,5)\( \_P_2 \) binding, was prepared from the templates, EGFP-PRIP-WT and EGFP-PRIP\( \Delta C_2 \), respectively, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (26). Domain organization of PRIP-1 and the related proteins used in this study are depicted in Fig. 1.

Plasmids to express recombinant C2 domain proteins in the bacterial expression system were prepared by subcloning cDNA amplified by PCR from reverse transscripts of rat brain total RNAs into BamHI/Sall site of pGEX (GE Healthcare) or pET-His30 (11) vectors. The primers to amplify each cDNA were as follows: the C2 domains of PRIP-1 (PRIP-C2, amino acid residues 709–849), PRIP\( \Delta C_2 \), PRIP-C2, respectively, for PRIP-C2, and PRIP-C2/R134Q were expressed in PC12 cells, whereas His-tagged PRIP-WT, PHXY, PRIP-C2, and GST-tagged PRIP-C2 were expressed in baculovirus or bacterial expression systems and purified for \textit{in vitro} assays.

**FIGURE 1. Schematic representation of PRIP-1 used in the study.** Domain organization of PRIP-1 and the related proteins used in this study is depicted. EGFP-tagged PRIP-WT, PRIP\( \Delta C_2 \), PRIP R134Q, and PRIP\( \Delta C_2 /R134Q \) were expressed in PC12 cells, whereas His-tagged PRIP-WT, PHXY, PRIP-C2, and GST-tagged PRIP-C2 were expressed in baculovirus or bacterial expression systems and purified for \textit{in vitro} assays.
and 5′-GGTCTGGGGATCCGCACCTGACCCAC-3′ (Syt-C2B, amino acid residues 272–408), 5′-GGGATCCCTGGGTTGACATCCTGGTCCTCTC-3′ and 5′-GGTCTGGGGATCCCCACTGACAGTGTTGCCACTG-3′, (Syt-C2AB, amino acid residues 142–408), 5′-GGGATCCCTGGGTTGACATCCTGGTCCTCTC-3′ and 5′-GGTCTGGGGATCCCCACTGACAGTGTTGCCACTG-3′. The GenBank accession numbers of parental proteins are as follows: rat PRIP-1, NP459908; rat PLC-β1, NP058731; rat synaptotagmin I, NP001028852; and rat rabphilin 3A, NP598202.

The plasmid to express SNARE proteins including the synaptotagmin 1 (Stx) lacking the C-terminal transmembrane region (StxΔC) has been described elsewhere (27). All of the constructs were fully sequenced to confirm their integrity at the Research Support Center of the Graduate School of Medical Sciences at Kyushu University.

Expression and Purification of Recombinant Proteins—Recombinant full-length SNARE proteins with transmembrane region were prepared by bacterial expression system as described previously in the presence of β-octyl glucoside (28). Other recombinant proteins were prepared as described elsewhere by bacterial (16, 29) or baculovirus expression system (10).

Lipid-Protein Overlay Assay—1 nmol of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine, 1,2-dipalmitoyl-sn-glycero-3-phosphoserine, and phosphatidylglycerol 4,5-bisphosphate were blotted on nitrocellulose membranes. The membranes were air-dried overnight at 4 °C and then were immersed in blocking buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% Tween 20, and 3% bovine serum albumin) to reduce background, followed by an incubation with purified GST or GST-fused PRIP-C2, PLCδ-γ-PH, or Syt (synaptotagmin)-C2A at 10 μg/ml in blocking buffer containing free Ca²⁺ of 10 μM. After extensive washing with the buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% Tween 20, and 10 μM CaCl₂), the membranes were immunoblotted for bound GST-C2 or PH proteins using anti-GST antibody.

t-SNARE Liposome Binding Assay—Preparation of t-SNARE-incorporated liposomes and the flotation assays were performed as described previously (28), except that GST alone or GST-fused C2 domain was used instead of CAPS. All of the phospholipids used in this study were purchased from Avanti Polar Lipids (Alabaster, AL).

Immunoprecipitation and Western Blotting—The cerebrum of wild type or PRIP-KO mouse was homogenized in the lysis buffer (20 μM Tris-HCl, pH 7.5, 150 μM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol) containing protease inhibitors (5 μg/ml pepstatin A, 10 μM leupeptin, 1.7 μg/ml aprotinin, and 50 μM 4-amidinophenylmethanesulfonyl fluoride hydrochloride). The lysates were cleared by centrifugation and incubated with antibody of interest or control immunoglobulin at 4 °C overnight, followed by incubation with protein G beads at 4 °C for 1 h. Then the beads were washed with the lysis buffer four times, and the proteins bound to the beads were separated by SDS-PAGE, followed by transfer to polyvinylidene fluoride membranes (Merck-Millipore, Billerica, MA). After blocking, the membrane was blotted with the appropriate antibody, and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare), and detected for chemiluminescent signals using an LAS-3000 mini gel documentation system (Fujiﬁlm, Tokyo, Japan). Digital images were analyzed with Image Gauge software (Fujiﬁlm) or National Institutes of Health Image J software to measure the density of each band. The handling of mice and all of the procedures were approved by the Animal Care Committee of Kyushu University, which follows the guidelines of the Japanese Council on Animal Care.

Cell Culture, Transfection, and Stable Cell Lines Expressing PRIP—Rat pheochromocytoma (PC12) cells were maintained and used to establish stable cell lines expressing wild type or several mutants of PRIP as described previously (29). GH3 cells were also maintained routinely.

Immunofluorescence and PLA—The cells were plated onto poly-d-lysine-coated glass coverslips at a density of 4 × 10⁵ cells/well in a 12-well plate and subjected to immunofluorescent observation as described elsewhere (30). In some experiments, the cells were transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) and OPTI-MEM (Invitrogen) according to the manufacturer’s protocol, and 24 h after transfection, the cells were plated onto glass coverslips as described above.

In situ PLA was performed using a Duolink in situ kit following to the manufacturer’s protocol, but the cells were prepared, permeabilized, and blocked in the same manner as for immunofluorescent studies. Briefly, two molecules present in permeabilized cells were recognized by respective primary antibody raised in mouse or rabbit, respectively, and secondary antibody to mouse or rabbit Ig conjugated with an unique short DNA strand was then added, followed by ligation of these secondary antibodies and amplification of the ligates. When two molecules are close within 40 nm, successful ligation and amplification are performed for further analysis. The confocal images were obtained using LSM510 META (Carl Zeiss) and analyzed using Duolink Image Tool (Olink Bioscience) to obtain objective quantification of PLA signals.

GST Pulldown Assay—PC12 cell lysate was prepared by homogenizing in the lysis buffer (50 mM Heps-KOH, pH 7.3, 150 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 1% Triton X-100) containing protease inhibitors. Cell lysate or recombinant SNARE proteins whose GST tags had been removed by thrombin digestion were mixed with GST alone or GST-fused C2 domain at an equal molar ratio in the binding buffer (10 mM Heps-KOH, pH 7.3, 100 mM KCl, 3.5 mM MgCl₂, 1 mM EGTA, 0.1% Nonidet P-40) and incubated at 4 °C for 2 h. Then glutathione-Sepharose 4B (GE Healthcare) equilibrated with appropriate buffer was added to the mixture and incubated at 4 °C for another 1 h. At the end of the incubation, the beads were washed with the same buffer four times, boiled in a sample buffer for 5 min, and then subjected to SDS-PAGE followed by Western blotting. Similar experiments in a reverse mode using GST-fused SNARE protein and His-tagged PRIP protein were performed in the same condition as described above. The internal standard sample with known protein concentration was included in each blot and used to calculate the amount of protein of interest by fitting to the standard curve. To examine the effect of Ca²⁺ on the binding of the C2 domains to syntaxin 1 (Stx) or SNAP-25, the pulldown assay was performed in the same binding buffer as above for that in the presence of the Ca²⁺-free condition using the standard curve.
**SNARE Binding of PRIP through the C2 Domain**

**FIGURE 2. Phospholipid binding of PRIP-C2.** Lipid overlay assay was performed using a nitrocellulose membrane on which 1 nmol of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol 4,5-bisphosphate (PIP2) were spotted. Each membrane was probed with GST-fused protein indicated on the left followed by immunodetection with anti-GST antibody. GST-fused PRIP-C2, PLCβ-C2, or Syt-C2A were used. Ca2+ (−) indicates incubation with 1 mM EGTA alone.

calculated amount of Ca2+ to give the free Ca2+ concentration of 10 μM or the buffer containing 1 mM EGTA.

In Vitro SDS-resistant SNARE Complex Formation—The mixture of 10 pmol (equivalent to 0.2 μM) each of the purified GST-SNAP-25, StxΔC-His, and His-VAMP2ΔC were incubated overnight in 48 μl of the binding buffer. At the end of incubation, the mixture was either boiled or incubated in 37 °C for 5 min in a SDS sample buffer and immediately subjected to SDS-PAGE followed by immunoblotting with antibodies indicated in the figures. Additional proteins were included in the mixture of SNAREs during the incubation period. To test the effect of Ca2+ on the SNARE complex formation, the mixture was incubated in the binding buffer containing calculated amounts of CaCl2 to give free Ca2+ concentration of 10 μM with 1 mM EGTA.

Measurement of [3H]Noradrenalin Release—[3H]NA secretion from the stable PC12 cell lines were measured as previously described (29, 30).

Statistical Analysis—All of the statistical analyses were performed by Student’s t test, with a two-tailed value of p < 0.05 considered significant using GraphPad Prism (GraphPad Software).

**RESULTS**

Interaction of PRIP with t-SNARE Proteins through the C2 Domain—We first examined phospholipid binding of the PRIP-C2 domain in a lipid-protein overlay assay (31). The isolated PRIP-C2 domain fused to GST did not show more binding than GST alone to phospholipids tested in the presence of Ca2+ (Fig. 2). By contrast, strong lipid binding as a positive control was observed as follows: PtdIns(4,5)P2 binding by the PH domain of PLC-δ1 and phosphatidylinositol-binding by the C2A domain of synaptotagmin I (Syt-C2A), which was abolished in the absence of Ca2+ (Fig. 2).

Because there are several C2 domain-containing proteins that promote SNARE-mediated membrane fusion by direct SNARE protein interactions (32–36), we examined whether PRIP binds to SNARE proteins via its C2 domain. Anti-PRIP-1 antibody precipitated PRIP-1 along with syntaxin 1 and SNAP-25 from brain lysate of wild type but not from that of PRIP-KO mouse, but no VAMP2 was precipitated (Fig. 3A), indicating that PRIP-1 interacts with syntaxin 1 and SNAP-25 either in a direct or indirect manner. This result was further confirmed, and the region responsible for the binding was iden-
identified using the recombinant PRIP-C2 domain protein. The lysate from PC12 cells was applied to GST alone and GST-fused C2 domains of PRIP-1 (PRIP-C2) or GST-C2B of rabphilin 3A (Rph-C2B) as a positive control (known to bind to SNAP-25, Ref. 34) immobilized on glutathione beads, followed by immunoblotting with the indicated antibodies (Fig. 3B). The result clearly showed that both syntaxin 1 and SNAP-25 bound to PRIP-C2, but VAMP2 and Munc18 did not, whereas only SNAP-25 bound to Rph-C2B as previously reported (34, 35). Thus, the C2 domain of PRIP interacts with two t-SNARE proteins, i.e. syntaxin 1 and SNAP-25, but not with the v-SNARE protein, VAMP2. To clarify whether the interactions of PRIP-C2 with syntaxin 1 and SNAP-25 are direct or indirect, because the experiments shown in Fig. 3 (A and B) were done using the lysates from brain or PC12 cells, we next performed GST pulldown assays using recombinant purified protein samples. GST alone or GST-C2 domain proteins immobilized on glutathione beads were incubated with soluble syntaxin 1 lacking the C-terminal transmembrane region (StxΔC) or SNAP-25. Both syntaxin 1 and SNAP-25 bound to PRIP-C2 (Fig. 3C). The binding was comparable with that of the C2B domain of synaptotagmin I (Syt-C2B) as a positive control. The Rph-C2B interacted with SNAP-25, but not with syntaxin 1, agreeing with previous reports (34, 35) and Fig. 3B. The C2 domain of PLC-δ1 (PLCδ-C2), albeit with a high homology to PRIP (8), did not bind to syntaxin 1 or SNAP-25 (Fig. 3C). The GST pulldown assay in a reverse mode was also performed. His-tagged forms of full-length PRIP-1 (PRIP-WT), PRIP-C2, or PHXY (Fig. 1) lacking the C2 domain and the N-terminal extension were assayed using immobilized GST alone, GST-SNAP-25 or GST-StxΔC. PRIP-WT and PRIP-C2, but not PHXY, showed binding to both SNAP-25 and syntaxin 1 (Fig. 3D) confirming that the C2 domain of PRIP directly interacts with t-SNARE proteins.

Because the binding of syntaxin 1 and SNAP-25 to PRIP-C2 shown in Fig. 3 was assayed by pulldown method using truncated soluble SNARE proteins, we further confirmed the interaction using full-length membrane-integrated SNARE proteins that might exhibit properties different from truncated soluble SNARE proteins (37, 38). Proteoliposomes incorporating full-length syntaxin 1 and SNAP-25 were prepared and incubated with PRIP-WT, PRIP-C2, or GST alone, followed by a gradient centrifugation to detect protein samples in the liposome fraction. Both PRIP-WT and PRIP-C2, but not GST alone, were detected in the floating liposome fraction in the case of proteoliposomes, but not in the protein-free liposome, indicating that PRIP and PRIP-C2 proteins bound to membrane-associated t-SNARE proteins (Fig. 4).

The C2 domain binding at increasing amounts with t-SNARE proteins were examined by a GST pulldown assay (Fig. 5). The EC_{50} (effective concentration required for 50% effect) value of 1.1 μM for PRIP-C2 binding to syntaxin 1 was comparable with that of Syt-C2B (2.4 μM), whereas Rph-C2B showed no binding to syntaxin 1 (Fig. 5A). On the other hand, the binding of PRIP-C2 to SNAP-25 (EC_{50} = 1.9 μM) was lower than that of Rph-C2B (EC_{50} = 0.4 μM), although it was still comparable with the apparent affinity of Syt-C2B to SNAP-25 (EC_{50} = 2.1 μM) (Fig. 5B). Molar ratio for the binding was smaller compared with the positive control; to SNAP-25, the

**B**_{max} Values were 0.18 and 0.18 mol for PRIP-C2 and Syt-C2B, respectively, whereas B_{max} = 0.35 mol for Rph-C2B as a positive control. To syntaxin 1, the B_{max} value was 0.17 mol for PRIP-C2, whereas it was 0.55 mol for Syt-C2B as a positive control. These results, however, indicate that the binding of PRIP-C2 to both syntaxin 1 and SNAP-25 may be regulatory for SNARE-mediated membrane fusion (27, 34).

Co-localization of PRIP with t-SNARE Proteins in Cells—We next examined the subcellular localization of PRIP with SNAP-25 and syntaxin 1 in rat pheochromocytoma, PC12 cells where we observed PRIP-mediated down-regulation of NA release (29). Because PC12 cells do not express detectable levels of endogenous PRIP (Fig. 6F), we established stable PC12 cell lines expressing wild type or the mutant version of PRIP-1 fused to EGFP (Fig. 6A). The cells were processed for immunofluorescence of EGFP-PRIP-1 and endogenous SNAP-25 or syntaxin 1 with respective antibodies. PRIP-WT appeared abundant because of exogenous expression but localized to both cytosol and plasma membrane, whereas both syntaxin 1 (Fig. 6B and C, upper panels). By contrast, PRIP-1 lacking its C2 domain (PRIPΔC2) showed less localization at the plasma membrane with a diffuse distribution in the cytosol, resulting in less co-localization with either syntaxin 1 or SNAP-25 (Fig. 6, B and C, lower panels). These results indicate that PRIP-C2 partly contributes to the co-localization of PRIP with t-SNAREs in the cells.
**SNARE Binding of PRIP through the C2 Domain**

**A**

- PRIP-C2
- Syt-C2B
- Rph-C2B
- GST-StxΔC

**B**

- PRIP-C2
- Syt-C2B
- Rph-C2B
- GST-SNAP-25

**FIGURE 5. Comparison of binding profile of C2 domains to t-SNARE proteins.** Purified C2 domains were incubated with GST-StxΔC (A) or GST-SNAP-25 (B) immobilized on glutathione beads at the indicated concentrations, and the C2 domains bound to the beads were determined by Western blotting. The proteins bound on the beads were quantified as described under "Experimental Procedures" and expressed as molar ratio of the C2 domain of PRIP-1 (PRIP-C2; circle), synaptotagmin I (Syt-C2B; square), or raphillin 3A (Rph-C2B; triangle) to StxΔC (A) or SNAP-25 (B) bound on the beads. The EC_{50} and B_{max} values described under "Results" were obtained by nonlinear regression curve fits to the data (means ± S.D., n = 3).

We further confirmed the importance of PRIP-C2 in co-localization with t-SNARE proteins by using in situ PLA technology, which visualizes protein-protein interactions quantitatively as fluorescent spots by rolling circle amplification reactions dependent on the close proximity (<40 nm) of the target proteins (39). PC12 cells were probed with a combination of mouse antibody against SNAP-25 or syntaxin 1 and rabbit antibody against PRIP-1 as primary antibodies, followed by further probing with PLA probes for mouse and rabbit primary antibodies, ligation, and amplification reactions. As shown in Fig. 6D, red fluorescent spots indicating co-localization of PRIP and syntaxin 1 were observed in the PC12 cells expressing EGFP-PRIP-WT and, to a smaller extent EGFP-PRIPΔC2, with none in the cells expressing EGFP alone (upper panels). Similar results were obtained for SNAP-25 proximity with PRIP or PRIPΔC2 (Fig. 6E). Consistent with the results in Fig. 6 (B and C), the number of signals per cell were significantly decreased by the lack of the C2 domain of PRIP, suggesting that the C2 domain contributes to the co-localization of PRIP with syntaxin 1 and SNAP-25 (Fig. 6, D and E, bar graphs). Because PC12 cells expressing similar amounts of exogenous PRIP-WT or the related proteins were used for PLA experiments (see the blot in Fig. 6D), the signals were comparable. However, the results using PC12 cells were observed by exogenous expression. Thus, GH3 endocrine cells that express endogenous PRIP were analyzed by PLA (Fig. 6F); positive signals indicating proximal presence of endogenous PRIP with SNAP-25 and syntaxin 1 were observed (Fig. 6G).

We found that PtdIns(4,5)P_2 binding to the PH domain is required for PRIP to inhibit exocytosis. Therefore, further experiments using the mutation (R134Q) in the PH domain, which lacks binding to PtdIns(4,5)P_2, were performed. Double mutation of ΔC2 and R134Q showed an almost complete loss of the co-localization of PRIP with t-SNARE proteins in PC12 cells (Fig. 6, D and E, graphs).

**PRIP Effect on the Binding of Synaptotagmin I to t-SNARE Proteins**—Binding parameters of the C2 domain of PRIP to t-SNAREs were comparable with those of synaptotagmin I, suggesting the possibility that PRIP competes with synaptotagmin for binding to t-SNARE proteins. To test this possibility, brain lysates prepared from WT and PRIP-KO mice were immunoprecipitated by anti-SNAP-25 antibody, followed by immunoblotting with antibodies of interest (Fig. 7A). From the band density in the precipitates compared with that in the lysates, we estimated that ~60% of the SNAP-25 present in the lysates was immunoprecipitated by anti-SNAP-25 antibody, and the value was similar for both WT and PRIP-KO mice. The amount of synaptotagmin I (Syt1) precipitated along with SNAP-25 was increased in PRIP-KO mouse by 2-fold (WT, 4.8 ± 0.8%; KO, 10.2 ± 1.9%), whereas the amount of syntaxin 1 (WT, 24.1 ± 2.5%; KO, 24.6 ± 1.8%) was not affected by the absence of PRIP (Fig. 7B). A similar effect of the absence of PRIP in increasing Munc18 in SNAP-25 immunoprecipitates was observed (Fig. 7A). It should also be noted that PRIP (5.1 ± 1.3%) was found in the immunocomplex among syntaxin 1, SytI, and SNAP-25 in WT mice. The results suggest that PRIP inhibits the binding of synaptotagmin I to the t-SNARE complex of syntaxin 1 and SNAP-25, leading to the inhibition of regulated exocytosis. In vitro pulldown assays were also performed first using the isolated C2 domains from PRIP (PRIP-C2) and synaptotagmin I (Syt-C2B); GST-SNAP-25 or GST-StxΔC was incubated with increasing amounts of PRIP-C2 at a fixed amount of Syt-C2B. The results show that the addition of increasing PRIP-C2 caused increased binding of PRIP-C2 along with decreased binding of Syt-C2B, indicating competition (Fig. 7, C and D). PRIP-C2 appeared to bind to StxΔC well, particularly in the presence of Ca^{2+}. Similar results were observed in the assay with t-SNARE complexes of GST-SNAP-25 plus StxΔC (Fig. 7E). When full-length molecules of PRIP and synaptotagmin 1 (without membrane spanning region) were used in the pulldown assay using GST-SNAP-25 and t-SNARE (GST-SNAP-25 plus StxΔC), similar results indicating competition were
observed (data not shown). The figure also includes the results obtained in the presence or absence of Ca$^{2+}$, which will be described later.

**Effect of PRIP on Ternary SNARE Complex Formation**—The formation of SDS-resistant heterotrimeric SNARE complexes consisting of VAMP2, SNAP-25, and syntaxin 1 was assayed because there is growing evidence to indicate that the amount of the SNARE complex correlates well with the extent of exocytosis (6, 40–42). To examine the effect of PRIP on SNARE complex formation, we used recombinant SNAP-25, soluble syntaxin 1AC, and soluble VAMP2AC prepared by bacterial expression. An equimolar mixture of these three SNARE proteins were incubated and treated with SDS sample buffer with or without boiling, followed by SDS-PAGE analysis and Western blotting. Two major high molecular mass bands (∼110 and 220 kDa) were detected with antibodies against GST-SNAP-25, syntaxin 1, and VAMP2, which were lacking in boiled samples (Fig. 8A). The band densities of individual SNARE proteins were more intense following boiling, indicating that high molecular mass bands prior to boiling represented SNARE complexes as previously reported (4, 40). We then examined the effect of PRIP and the related proteins on complex formation. PRIP-WT inhibited SNARE complex formation in a dose-dependent manner, whereas PHX0 showed no effect (Fig. 8, B and C). Isolated PRIP-C2 at higher concentrations showed inhibitory effects, but PLC-δ1 even at high concentrations had no effect. The results indicate that PRIP inhibits the SDS-resistant SNARE complex formation in a dose-dependent manner, likely by binding to SNARE proteins through its C2 domain. By contrast, Syt-2C2 was ineffective in the inhibition of SNARE complex formation (Fig. 8, B and C). Studies of the time-dependent formation of SDS-resistant SNARE complexes revealed that PRIP delayed complex formation (data not shown).

**Ca$^{2+}$ Dependence of the Binding to t-SNAREs and the Inhibitory Effect on SNARE Complex Formation by PRIP**—Because the binding of the C2 domain of synaptotagmin I to t-SNARE proteins is Ca$^{2+}$-dependent (32), and the aspartate residues required for Ca$^{2+}$ binding are relatively well conserved in PRIP-C2, we tested whether the binding of PRIP-C2 to t-SNARE pro-
imates is Ca\textsuperscript{2+}-dependent. The GST pulldown assay was performed in the presence or absence of 10 \(\mu\)M Ca\textsuperscript{2+}. Both the binding of PRIP-C2 to syntaxin 1 and to SNAP-25 exhibited stimulation by Ca\textsuperscript{2+} with an overall Ca\textsuperscript{2+} dependence less than that observed for the positive control, Syt-C2B (Fig. 9A). The Ca\textsuperscript{2+} effect on the competition of PRIP-C2 with Syt-C2B on t-SNARE complexes was also assayed by pulldown assay, whose results were shown in Fig. 7E. GST-SNAP-25 plus syntaxin 1 (Stx\DeltaC) was incubated with increasing amount of PRIP-C2 at fixed Syt-C2B in the presence or absence of Ca\textsuperscript{2+}. The results showed similar competition independent of Ca\textsuperscript{2+} but more binding of PRIP-C2 in the presence of Ca\textsuperscript{2+}.

The Ca\textsuperscript{2+} dependence of the effect of PRIP and PRIP-C2 on the SDS-resistant SNARE complex formation was assayed. The inhibition of SNARE complex formation by full-length PRIP and PRIP-C2 shown in Fig. 8 (B and C) was stronger in the presence of 10 \(\mu\)M Ca\textsuperscript{2+} (Fig. 9, B and C). These results suggest that PRIP or PRIP-C2 might inhibit exocytosis in both Ca\textsuperscript{2+}-independent and Ca\textsuperscript{2+}-dependent manners but more strongly in the presence of Ca\textsuperscript{2+}. Because PRIP competed with synaptotagmin I for SNAP-25 in brain (Fig. 7A), we also examined the effect of PRIP on SDS-resistant SNARE complex formation in the presence of synaptotagmin I. Syt-C2AB by itself showed little effect on SNARE complex formation, as already shown with Syt-C2B in Fig. 8B, and also did not affect the inhibition by PRIP (Fig. 9D). However, when the isolated PRIP-C2 was used,
the inhibition was rescued by Syt-C2B only in the presence of Ca\textsuperscript{2+} (data not shown).

Effect of PRIP on NA Release from PC12—Lastly, we examined the role of full-length PRIP-1 and PRIP-C2 in \[^{3}H\]NA release using PC12 cell lines stably expressing EGFP, EGFP-PRIP-WT, or EGFP-PRIPΔC2 (Fig. 10, upper panel). \[^{3}H\]NA release without stimulation for 2 min was slightly decreased in cells expressing PRIP-WT or PRIPΔC2 but did not achieve statistical significance. High K\textsuperscript{+}-induced secretion of \[^{3}H\]NA was almost completely inhibited in the cells expressing wild type PRIP-1, whereas the inhibitory effect of PRIPΔC2 was partial but still significant (Fig. 10, graph). The results indicate that full-length PRIP-1 inhibits regulated exocytosis, and the C2 domain is involved in the inhibition. The inhibition may result from the direct binding of the C2 domain to t-SNAREs, leading to the inhibition of SNARE complex formation. The rest of the inhibition observed with PRIPΔC2 may involve other regions of PRIP-1 such as the PH domain.\textsuperscript{7}

**DISCUSSION**

In this study, we identified PRIP as a new C2 domain-containing protein involved in the regulation of exocytosis. The study was initiated by the finding that PRIP-KO mice exhibited up-regulation of dense core vesicle exocytosis (18, 19). To explore the molecular mechanisms by which PRIP negatively regulates exocytosis, studies were first undertaken to show the involvement of the PH domain in binding to PtdIns(4,5)P\textsubscript{2}. However, the role of the PH domain could not fully explain the inhibition of exocytosis by PRIP.\textsuperscript{7} The involvement of the C2 domain of PRIP in the inhibitory process was investigated in the present study, and the results show that PRIP directly interacts with both syntaxin 1 and SNAP-25. Despite structural similarity, the C2 domain of PLC-\textbeta\textsubscript{1} bound to neither syntaxin 1 nor SNAP-25, indicating the specificity of the PRIP C2 domain. The majority of the cellular experiments in this study were performed using PC12 cells with exogenously expressed PRIP, because PC12 cells contain no detectable level of PRIP by the antibody. However, the inhibitory effect of PRIP is not limited to overexpression, because PRIP-KO mice exhibit increased secretion of gonadotropins and insulin, indicating that physiologically relevant levels of PRIP are inhibitory.

There is a group of C2 domain-containing proteins with enzymatic activity regulating membrane traffic through the regulation of small G-proteins or the synthesis of membrane phospholipids (43–45). On the other hand, there is another group of C2 domain-containing proteins with no enzymatic activity that play important roles by physical interaction with...
proteins involved in exocytosis. The latter group, which includes synaptotagmin, Munc13, raphilin, DOC2, and CAPS, binds directly to SNARE proteins (3, 28, 32–35, 46, 47). They play important roles as regulatory proteins specific to distinct vesicular populations and/or specific to different phases of exocytosis, depending on their affinities, specificities, or Ca$^{2+}$ dependence. The C2 domain of PRIP is in this latter group and thus might be involved in inhibiting exocytosis by competing for SNARE complex assembly with proteins that promote membrane fusion. The present work also suggested that PRIP interferes with synaptotagmin function; thus competition at multiple levels may be responsible for the inhibition of exocytosis.

PRIP or its C2 domain showed direct effects on the formation of SDS-resistant SNARE complexes in vitro. Although the precise role of SDS-resistant SNARE complexes in vivo have not yet been revealed, it is generally accepted that ternary SNARE complex formation is required for vesicles to fuse or become ready to fuse with target membranes (42). In this study, we showed that PRIP inhibited the formation of SDS-resistant ternary SNARE complexes in a dose-dependent manner through the direct binding of the PRIP-C2 domain to SNARE proteins. Although the isolated C2 domain was less potent than the full-length PRIP-1, which is often observed (9), it could be concluded that the C2 domain of PRIP participates in the inhibition. In this context, PRIP may be involved in the inhibition of exocytosis not only by competing for SNAREs with other SNARE-binding proteins that promote exocytosis, but also by inhibiting SNARE complex formation directly.

The binding of PRIP to SNARE proteins was enhanced by Ca$^{2+}$, which also correlated with the inhibitory effect of PRIP on SDS-resistant SNARE complex formation. Structural analyses on multiple C2 domains revealed that Ca$^{2+}$-binding sites reside in loops at the top of a β-barrel structure with conserved aspartate residues in loops 1 and 3 and 3 primarily responsible for direct recognition of Ca$^{2+}$ (43, 48). In PRIP-C2, only one aspartate in the loop 1 is conserved, but it is the same with the C2 domain of PLC-δ1, which was shown to bind Ca$^{2+}$ (49). In calcium-binding loop 3 of PRIP-C2, two of three aspartate residues are conserved, and the other is replaced by glutamate. However, the structural analysis of Rph-C2A, in which an aspartate residue is also replaced by glutamate at the same position as PRIP, revealed that the glutamate could be directly involved in the recognition of Ca$^{2+}$ (50). Thus, the results regarding promotion of PRIP binding with SNARE protein by Ca$^{2+}$ is probably attributed to the conserved acidic residues for Ca$^{2+}$ binding in PRIP-C2.

There are several C2 domains that directly interact with SNARE proteins (32–36, 46, 47) with differing specificity. The C2 domains of raphilin and Rab3-interacting molecule bind only to SNAP-25 (34, 35, 46), and the C2 domains of synaptotagmin, DOC2, and otoferlin bind to both SNAP-25 and syntaxins, although the Ca$^{2+}$ dependence and preference for distinct syntaxin isoforms vary (32, 33, 36, 47). PRIP-C2 interacted with both syntaxin 1 and SNAP-25, but the C2 domain of PLC-δ1, despite its structural similarity, did not bind to either syntaxin 1 or SNAP-25. CAPS and Munc13 contain C2 domains, but interactions with t-SNARE proteins are not mediated through the C2 domains (3, 28, 51). It remains to be clarified how the SNARE binding specificity of the C2 domains are accomplished because structures of any C2 domains as complexes with SNARE proteins are not available. The results from mutational analyses of synaptotagmin I (52, 53) and NMR analysis of raphilin-C2B (35) suggested that the structure surfaces for SNAP-25 binding differ. A structural analysis of the PRIP-C2 is underway to clarify the binding mode of PRIP-C2 with the SNARE component proteins.

In conclusion, the current results suggest that PRIP is a C2 domain-containing protein that regulates vesicular transport through C2 domain interactions with both SNAP-25 and syntaxin 1. SNARE binding by the C2 domain was required for PRIP to co-localize with t-SNARE proteins in cells and to inhibit SDS-resistant SNARE complex formation in vitro. Considering that membrane microdomains for exocytosis contain PtdIns(4,5)P$_2$ and the t-SNARE component proteins, syntaxin 1 and SNAP-25, PRIP would exert its inhibitory role by the combinatorial function of its PH and C2 domain, binding to PtdIns(4,5)P$_2$ and t-SNAREs, respectively.
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