Hetero-oligomerization of C2 domains of phospholipase C-related but catalytically inactive protein and synaptotagmin-1

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Hetero-oligomerization of C2 domains of phospholipase C-related but catalytically inactive protein and synaptotagmin-1

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A B S T R A C T

The C2 domain is a protein module often found in molecules that regulate exocytosis. C2 domains mediate interactions between the parental molecule and Ca^{2+}, phospholipids, and proteins. Although various molecules have been shown to interact with several C2 domains, no interactions between the C2 domains from different molecules have yet been reported. In the present study, we identified direct interactions between the C2 domain of PRIP (phospholipase C-related but catalytically inactive protein) and the C2 domains of other molecules. Among the C2 domains examined, those of synaptotagmin-1 (Syt1-C2A and Syt1-C2B) and phospholipase C δ-1 bound to the C2 domain of PRIP. We investigated the interactions between the C2 domain of PRIP (PRIP-C2) with Syt1-C2A and Syt1-C2B, and the mode of binding of each was Ca^{2+}-dependent and -independent, respectively. We further demonstrated that the Ca^{2+} dependence of the interaction between PRIP-C2 and Syt1-C2A was attributed to Ca^{2+} binding with Syt1-C2A, but not PRIP-C2, using a series of mutants prepared from both C2 domains. We previously reported that the interaction between PRIP-C2 and the membrane fusion machinery suggested a

Abbreviations: SNARE, soluble NSF (N-ethylmaleimide-sensitive factor)-attachment protein receptors; SNAP-25, synaptosome-associated protein of 25 kDa; Syt, synaptotagmin; PLC, phospholipase C; PRIP, phospholipase C-related but catalytically inactive protein; Rph, rabphilin-3A; GST, glutathione S-transferase.

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critical role for PRIP in exocytosis; therefore, the results of the present study further support the importance of PRIP-C2 in the inhibitory function of PRIP in regulating exocytosis.

Introduction

The secretion of neurotransmitters, neuropeptides, and peptide hormones, and also the exposure of membrane proteins such as receptors, channels, and transporters to the cell surface are mediated by the fusion of vesicles with the plasma membrane, namely the final step of exocytosis. The minimal machinery required for cellular membrane fusion are the hetero-trimeric complexes of SNARE [soluble NSF (*N*-ethylmaleimide-sensitive factor)-attachment protein receptors] proteins (Hong, 2005; Malsam et al., 2008; Weber et al., 1998), which consist of members of the VAMP (vesicle-associated membrane protein, also called synaptobrevin) family on the vesicular membrane (v-SNARE) as well as syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) families on the target plasma membrane (t-SNARE). A number of accessory proteins that directly interact with individual SNARE proteins and/or with assembled SNARE protein complexes are also indispensable for the tight control of SNARE-mediated membrane fusion triggered by increases in cytoplasmic Ca^{2+} (Bai and Chapman, 2004; Carr and Rizo, 2010; Deak et al., 2006; Friedrich et al., 2008; James et al., 2009; Walter et al., 2011). Synaptotagmin-1 (Syt1) is the best characterized accessory protein that confers Ca^{2+} sensitivity to vesicle exocytosis by modulating its own Ca^{2+}-dependent intermolecular interactions, including acidic phospholipids and fusion machinery, such as phosphatidylserine, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], and SNARE proteins (Brose et al., 1992; Chapman, 2002; Rizo et al., 2006; Sudhof, 2002). Synaptotagmin-1 is a member of synaptotagmin family, comprises an N-terminal transmembrane domain and two cytoplasmic C2 domains (membrane-proximal, C2A and membrane-distal, C2B), and attributes its Ca^{2+}-dependent properties to the C2 domains. These tandem C2 domains collaborate to exert Ca^{2+}-dependent and independent molecular interactions, however, Syt1-C2A mediates Ca^{2+}-dependent phospholipid binding (Chapman and Jahn, 1994; Davletov and Sudhof, 1993) while Syt1-C2B mediates Ca^{2+}-independent interactions with AP-2, as well as β-SNAP (Schiavo et al., 1995), inositol polyphosphates (Fukuda et al., 1994; Schiavo et al., 1996), and the syn-print region of N- and P/Q-type Ca^{2+} channels (Kim and Catterall, 1997; Sheng et al., 1997). Syt1-C2B has also been shown to mediate the homo-oligomerization of synaptotagmins in collaboration with the phospholipid binding of Syt1-C2A (Wu et al., 2003). Three and two Ca^{2+} ions bind to the C2A and C2B of Syt1, respectively, and are coordinated by five acidic residues (Asp) conserved in loops 1 and 3 of the eight-stranded β-sandwich (Fernandez et al., 2001; Fernandez-Chacon et al., 2001; Sutton et al., 1995, 1999; Ubach et al., 1998).

Phospholipase C-related but catalytically inactive protein (PRIP), which was named for its lack of catalytic activity in spite of its structural similarity to phospholipase C (PLC)-δ1 (Kanematsu et al., 1992, 1996), was originally isolated as an inositol 1, 4, 5-trisphosphate [Ins(1,4,5)P_3] binding protein in our laboratory. PRIP consists of a pleckstrin homology (PH) domain, EF-hand motifs, X and Y motifs, and a C2 domain, and has been shown to interact with a number of partners including GABARAP [γ-aminobutyric acid type A ($GABA_A$) receptor-associated protein] (Kanematsu et al., 2002; Uji et al., 2002), the β subunit of the $GABA_A$ receptor (Kanematsu et al., 2006, 2007), the catalytic subunit of protein phosphatase 1α (PP1α) and PP2A (Yoshimura et al., 2001; Kanematsu et al., 2006; Sugiyama et al., 2012; Sgiyama et al., 2013), and the phosphorylated (active) form of Akt (Fujii et al., 2010), in addition to Ins(1,4,5)P_3 and PtdIns(4,5)P_2 to the PH domain (Takeuchi et al., 1996, 2000). It was of particular interest that PRIP might participate in a phospho-regulation of variety of cellular functions by swapping phosphatases between PP1 and PP2A, depending on a phospho-state of PRIP itself (Sugiyama et al., 2012; Sgiyama et al., 2013). We generated knock-out (KO) mice lacking each or both of the known isoforms of PRIP (type 1 and type 2) in order to explore the biological roles in relation to these interacting proteins. Among the phenotypes we reported for PRIP-KO mice (Doira et al., 2001; Fujii

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et al., 2010; Kanematsu et al., 2002, 2006, 2007; Matsuda et al., 2009), an increase in the exocytosis of various peptide hormones such as gonadotropins and insulin (Doira et al., 2001; Matsuda et al., 2009) prompted us to focus on the inhibitory role of PRIP in exocytosis. Subsequent studies suggested that PRIP may be involved in the negative regulation of exocytosis through multiple molecular interactions. The interaction between the PH domain and PtdIns(4,5)P_2, which was shown to be required for vesicle exocytosis (Hay and Martin, 1993; Holz et al., 2000; James et al., 2008), supported PRIP being enriched near the site of the event. We recently reported that although the PRIP-C2 domain very weakly interacted with phospholipids, it interacted with t-SNARE proteins more strongly and 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 was found to have direct inhibitory effects on the formation of ternary SNARE complexes and on participation of synaptotagmin (Zhang et al., 2013). Thus, since membrane microdomains for exocytosis contain PtdIns(4,5)P_2 and the t-SNARE component proteins, syntaxin 1 and SNAP-25, we concluded that PRIP exhibited its inhibitory role by the combination of its PH and C2 domain binding to PtdIns(4,5)P_2 and t-SNAREs, respectively. In the course of these experiments, we also found that PRIP-C2 directly interacted with the C2 domains of Syt1.

In the present study, we investigated the underlying mechanisms for the direct interactions between PRIP-C2 and the isolated C2 domains of Syt1 in order to clarify the amino acid residues/interface involved in these interactions. We previously showed that PRIP-C2 competed with Syt1 for t-SNARE proteins in the previous report (Zhang et al., 2013); the results obtained herein may provide a deeper understanding of the relationship between PRIP, Syt1, and t-SNARE proteins.

Materials and methods

Materials

The antibodies to each protein in this study were as follows: SNAP-25 (Abcam, Cambridge, MA), syntaxin 1, GST (Santa Cruz Biotechnology, Santa Cruz, CA). An anti-PRIP-1 rabbit polyclonal antibody was prepared in this laboratory as described previously (Kanematsu et al., 2002).

Preparation of expression constructs and site-directed mutagenesis of the C2 domains

The preparation of all expression constructs for the C2 domains, SNAP-25, and syntaxin-1 has been described in detail elsewhere (Gao et al., 2012; Zhang et al., 2013). Mutants carrying substitutions of amino acid residues were produced using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). All constructs were fully sequenced to verify their integrity at the Research Support Center of the Graduate School of Medical Sciences at Kyushu University. More details on all constructs are available on request. GST (glutathione *S*-transferase)- or His-tagged proteins were prepared using a bacterial expression system as described previously (Takeuchi et al., 2000). The absence of contamination by oligonucleotides from bacteria in the isolated recombinant C2 domains used in this study was confirmed by measuring the UV spectrum of each preparation, which had a UV absorption maximum at 280 nm, not 260 nm.

GST pull-down assay

GST pull-down assays were performed as described previously (Zhang et al., 2013) with some minor modifications. Briefly, 10 pmol of the GST-fused protein and His-tagged protein or proteins prepared as GST-fusion proteins followed by thrombin digestion to remove the GST tag were mixed in 200 μl of the assay buffer, which consisted of 10 mM Hepes-KOH, pH 7.3, 100 mM KCl, 3.5 mM $MgCl_2$, 1 mM EGTA, 0.1% Nonidet P-40, and 1 mM dithiothreitol, and was incubated at 4 °C for 3 h. GST-fused proteins were then collected by incubating the mixture with glutathione-Sepharose 4B (GE Healthcare) at 4 °C for an additional hour. After being incubated, the beads were washed with the same buffer four times to remove unbound proteins and then subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting analysis. To examine the effects of Ca^{2+} on binding, the assays were performed in the same buffer as described above, but in the presence of a calculated amount of Ca^{2+} to give a free Ca^{2+}

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concentration of 10 μM or buffer containing 1 mM EGTA. An equivalent amount of the GST-fused protein in each assay mixture was confirmed by detection with the anti-GST antibody and Coomassie Brilliant Blue staining of the membrane.

Results and discussion

Direct interactions between the C2 domain of PRIP and other C2 domains

While determining the molecular mechanism underlying how PRIP regulated exocytosis through interactions between its multiple domains and other molecules following the finding that PRIP-C2 interacted with t-SNARE proteins (Zhang et al., 2013), we also found that the C2 domain of PRIP bound to the C2 domain of Syt-1. To confirm this specific interaction, we performed GST pull-down assays using isolated C2 domains from several molecules. His-PRIP-C2 clearly bound to GST-tagged Syt1-C2A, and Syt1-C2B, and to a lesser extent to PRIP-C2 and the C2B domain of rabphilin-3A (Rph-C2B), whereas no binding was observed to GST alone (Fig. 1A). Among these C2 domains, PRIP-C2 showed Ca^{2+} dependence when binding to Syt1-C2A only. Bacterial contaminants have been shown to tightly bind to the polybasic sequence of the C2 domain in order promote oligomerization of the C2 domain (Ubach et al., 2001). Therefore, we measured the UV spectrum of the isolated C2 domains used in the present study and confirmed that all preparations had UV absorption maxima at 280 nm, not 260 nm. Furthermore the ratio of absorption at 280 nm to that at 260 nm was

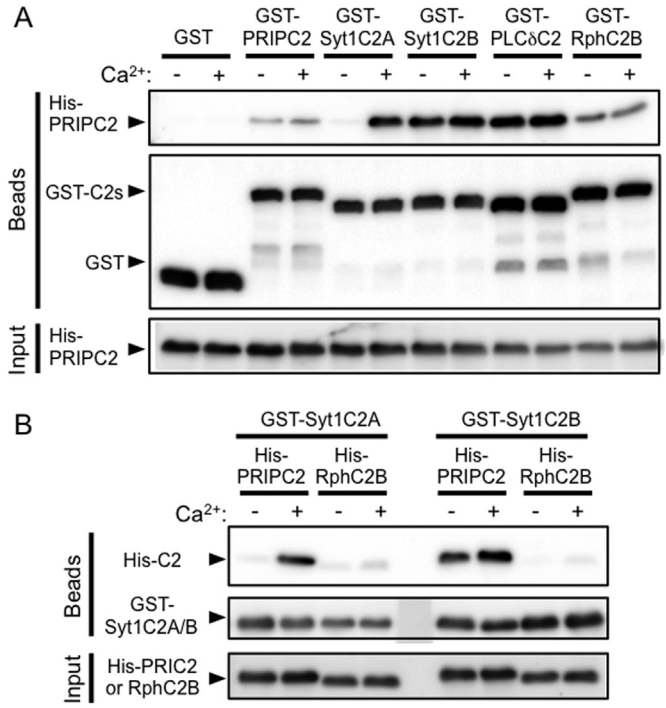

Fig. 1. Interaction between PRIP-C2 and various C2 domains. A: A mixture of 10 pmol each of His-tagged PRIP-C2 with GST or GST-fused C2 domains (PRIP-C2, Syt1C2A, Syt1C2B, PLCδC2, and RphC2B) was incubated in the presence or absence of Ca^{2+}, as described in the Materials and Methods, at 4 °C for 3 h and was then applied to glutathione beads. After being incubated for an additional hour, the beads were washed extensively, followed by Western blotting. His-tagged PRIP-C2 that remained on the beads (top panel) and in the mixture before the addition of the beads (bottom panel for input) was detected by an anti-PRIP rabbit polyclonal antibody that recognizes PRIP-C2. GST-fused proteins on the beads were detected by an anti-GST antibody. B: A GST pull-down assay was performed as in A. His-tagged C2 domains that remained on the beads were detected with an anti-His antibody.

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approximately 1.8, indicating negligible bacterial contamination. This was also confirmed in a subsequent experiment in which the binding of His-Rph-C2B to GST-Syt1-C2 was compared with that of PRIP-C2 (Fig. 1B). PRIP-C2 showed Ca^{2+}-dependent and -independent binding to Syt1-C2A and Syt1-C2B, respectively, while Rph-C2B bound to neither Syt1-C2A nor Syt1-C2B in the presence or absence of Ca^{2+}. The integrity of the preparation of His-Rph-C2B was previously demonstrated by its specific binding to SNAP-25 (Zhang et al., 2013); therefore the absence of interactions between Rph-C2B and Syt1-C2A or Syt1-C2B may not be attributed to the defective conformation of His-Rph-C2B. Thus, the results shown in Fig. 1B also showed that the ability to form hetero-oligomers with Syt1-C2A and Syt1-C2B was specific to PRIP-C2.

Comparison of the binding profile of PRIP-C2 to Syt1-C2A and Syt1-C2B

Increases in PRIP-C2 binding to Syt1-C2A and Syt1-C2B were examined using a GST pull-down assay (Fig. 2A and B). The binding of PRIP-C2 to each C2 domain increased in a dose-dependent manner. The EC_{50} (effective concentration required for a 50% effect) values for PRIP-C2 binding to Syt1-C2A in the presence or absence of Ca^{2+} were 100 nM and 4.5 μM, respectively (Fig. 2A). On the other hand, the binding of PRIP-C2 to Syt1-C2B was not significantly affected by the presence of Ca^{2+}, *i.e.*, the EC_{50} values for PRIP-C2 binding to Syt1-C2B in the presence or absence of Ca^{2+} were 80 nM and 200 nM, respectively (Fig. 2B). These binding profiles of PRIP-C2 to the C2 domains of Syt1 were compatible with the Ca^{2+} dependence of the binding observed in Fig. 1A and B. We previously reported that the EC_{50} values of PRIP-C2 to syntaxin-1 and SNAP-25 were 1.1 μM and 1.9 μM, respectively, using a similar assay procedure (Zhang et al., 2013). A simple comparison of these EC_{50} values indicated that PRIP-C2 had higher affinity to the C2 domains of Syt1 than to those of the t-SNARE proteins. Therefore, the inhibitory effects of PRIP-C2 on the binding of Syt1 to t-SNARE proteins may be mediated not only by PRIP-C2 binding to t-SNARE proteins competitively with Syt1, but also, or even rather by PRIP-C2 binding to Syt1 competitively with t-SNARE proteins. We also performed the same experiment using GST-Syt1-C2AB (tandem) and obtained EC_{50} values for PRIP-C2 binding of 0.8 μM and 2.3 μM in the presence and absence of Ca^{2+}, respectively, which was similar to Syt1-C2B, but with higher EC_{50} values (Fig. 2C). These EC_{50} values were similar with those to t-SNARE proteins, suggesting that the binding affinity of PRIP-C2 to Syt1-C2AB was similar to that to t-SNARE proteins.

Effects of site-directed mutagenesis on the relationship between pairs involving PRIP-C2

To investigate the relationships among PRIP, Syt1, and SNAP-25 and/or syntaxin 1, we attempted to identify the residues/interface essential for the interaction between each pair of these molecules. We prepared PRIP-C2 mutants with reference to the sequence alignment of the C2 domains (Fig. 3) and examined binding to Syt1-C2A and C2B (Table 1A). Since the acidic residues conserved in loops 1 and 3 of the C2 domains to accommodate Ca^{2+} ions were almost conserved in PRIP-C2, we prepared neutralized mutants in which these acidic residues were replaced with asparagine and examined binding to Syt1-C2 domains. However, even when all three residues in loop 3 were replaced (D801N/D803N/E808N), the binding of PRIP-C2 to Syt1-C2A and C2B was not reduced. We further neutralized additional acidic residues adjacent to those in loop 3 of PRIP-C2 (D801/802/803/807N/E808N) and examined binding to Syt1-C2A and C2B. This neutralization had a negligible effect on the Ca^{2+}-dependent interaction between PRIP-C2 and the C2 domain of Syt1 (Table 1A). Although only one out of two aspartate residues was conserved in loop 1, it was analogous to the C2 domain of PLC-δ1, which was shown to bind Ca^{2+} (Essen et al., 1997). Furthermore, in loop 3 of PRIP-C2, one of the three conserved aspartates was replaced by glutamate (E808, Fig. 4). However, a structural analysis of Rph-C2A, in which a glutamate was instead present and at the position of one of the three conserved aspartate residues similar to PRIP, revealed that glutamate could be directly involved in the recognition of Ca^{2+} by Rph-C2A (Coudevylle et al., 2008). Thus, the acidic residues in loops 1 and 3 of PRIP-C2, candidates for accommodating Ca^{2+} ions, may not have been involved in the Ca^{2+}-dependent binding of PRIP-C2 to Syt1-C2. Since the basic residues in the β4-strand of Syt1-C2 were shown to be involved in the interaction between Syt1 and the SNARE complex, the basic residues close to the

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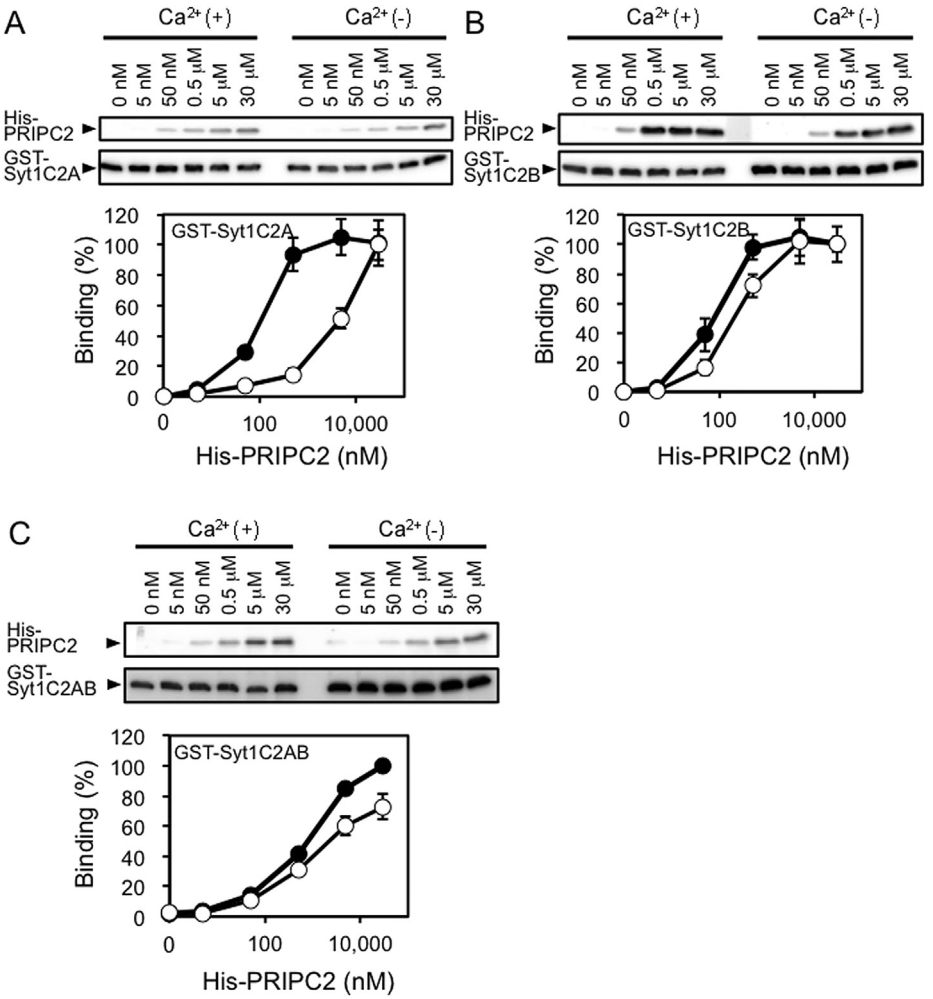

Fig. 2. Comparison of binding profiles of the PRIP-C2 domain to the C2 domains of Syt1. A GST pull-down assay was performed as described in Fig. 1, except that the mixture contained the indicated concentrations of His-PRIP-C2 and 10 pmol of GST-Syt1-C2 (A: GST-Syt1-C2A, B: GST-Syt1-C2B, C: GST-Syt1-C2AB). Each data point is the mean ± SE of at least 3 independent experiments with 2 immunoblots for each experiment.

corresponding site in PRIP-C2 were also mutated (R766Q/K768Q), indicating that PRIP-C2 may be recognized as an effector molecule by Syt1: however, the results obtained showed that the binding of PRIP-C2 to either Syt1-C2A or Syt1-C2B was unaffected.

We subsequently prepared several mutants of Syt1-C2A and C2B and examined binding to PRIP-C2. The binding of the mutants with the PRIP-C2 and SNARE proteins is summarized in Table 1B. The neutralization of acidic residues either in loop 1 (D178N) or loop 3 (D230/232A) completely abolished the Ca^{2+}-dependent binding of Syt1-C2A to PRIP-C2 (Fig. 4), suggesting that the Ca^{2+} dependence of the interaction between PRIP-C2 and Syt1-C2 (Fig. 2A) may be attributed to the conserved Ca^{2+} binding residues of Syt1-C2A. It currently remains unclear whether the PIRP-C2 interaction with Syt1-C2A affected the Ca^{2+}-dependent phospholipid binding of Syt1-C2A. On the other hand, any conserved aspartate residues in Syt1-C2B were not involved in binding to PRIP-C2 (Table 1B), which was consistent with the interaction between PRIP-C2 and Syt1-C2B being Ca^{2+}-independent (Fig. 2B). This

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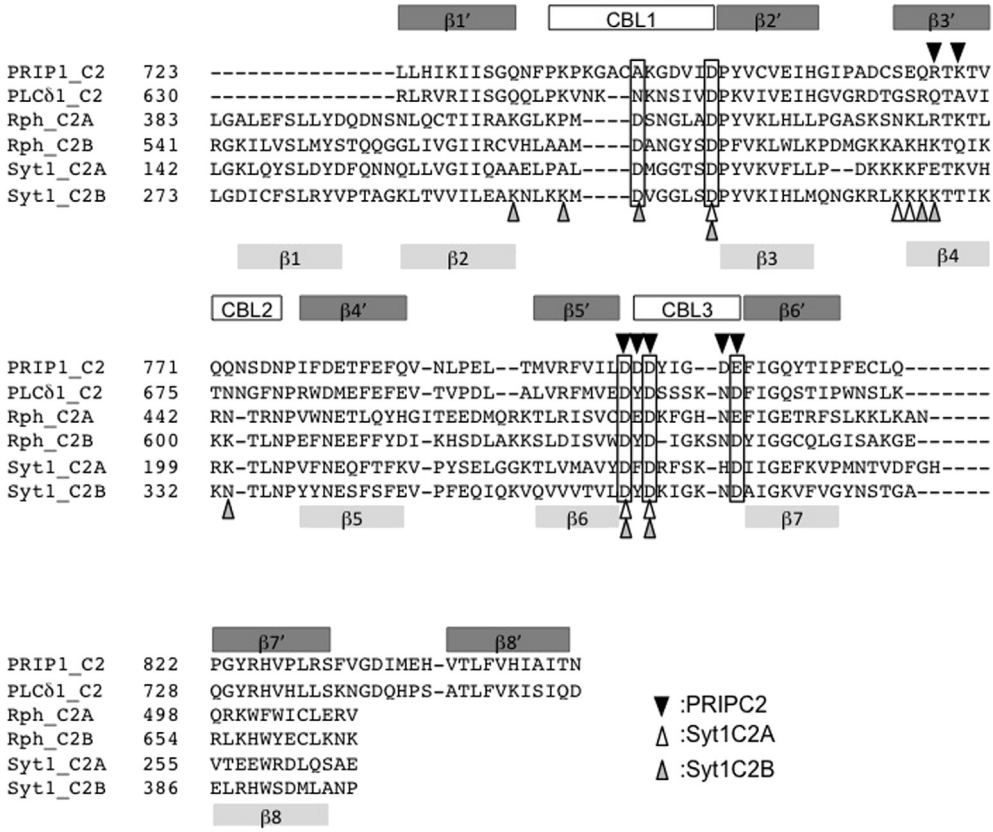

Fig. 3. Sequence alignment of C2 domains. The amino acid sequences of the C2 domains were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and minor corrections were applied manually. The positions of the conserved aspartate residues for coordinating Ca^{2+} are boxed. The secondary structures of type I and type II topologies (Nalefski, E. A., and Falke, J. J. (1996) Protein science 5, 2375–2390) are schematically shown above and below the sequence, respectively. The three loops involved in Ca^{2+} binding are shown above as CBL1, 2, and 3. The GenBank accession numbers of the parental proteins are rat PRIP-1; NP445908, rat PLC-δ1; NP058731, rat synaptotagmin I; NP001028852, rat rabphilin 3A; NP598202. The amino acid residues mutated in this study are indicated by arrowheads: PRIP-C2, arrowheads filled with black; Syt1-C2A, open arrowheads; Syt1-C2B, arrowheads filled with gray.

result was analogous to that from the mutagenesis experiment for Syt1-C2B in which the simultaneous neutralization of four (D303, 309, 363, 365N) acidic Ca^{2+} ligands resulted in Ca^{2+}-independent constitutive clustering activity, but did not affect binding to the effector molecules, AP-2 and synprint (Desai et al., 2000). We then replaced the other residues of Syt1-C2B, which were shown to be involved in binding with other protein targets, and all of them failed to abolish the interaction with PRIP-C2 (Table 1B). For example, lysines 326 and 327 were previously reported to be critical for the binding of Syt1 to AP-2 as well as the synprint region of Ca^{2+} channels, in addition to the homo-oligomerazation and substitution of both these lysines with alanine residues essentially abolishing all these interactions (Chapman et al., 1998). Although it was later shown that the lysine residues at positions 326 and 327 served as sites for strong interactions with bacterial contaminants (Ubach et al., 2001), which suggested that the contaminant may affect any behavior of the C2B, a UV spectrum analysis of the preparations used in the present study revealed that they did not contain any amount of nucleotides. It currently remains unclear how the Ca^{2+}-independent interaction between PRIP-C2 and Syt1-C2B is mediated, because none of the mutations introduced in the present study affected this interaction.

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Table 1
Summary of binding regarding the effects of amino acid substitutions of PRIP-C2 and Syt1-C2. A GST pull-down assay was performed in the presence or absence of Ca^{2+} as in Fig. 1, using combinations of His-PRIP-C2 mutants and GST-Syt1-C2A or C2B (A); GST-fused Syt1-C2A and Syt1-C2B mutants with the wild-type His-PRIP-C2 (B). The intensities of the bands indicating binding were analyzed by NIH ImageJ software. '+', binding was similar to that of the wild-type C2 in the presence of Ca^{2+}; '–', very weak or no detectable binding.

A

His-PRIP-C2	Syt1-C2A		Syt1-C2B	
Ca^{2+}:	–	+	–	+
WT	–	+	+	+
D748N	–	+	+	+
R766Q/K768Q	–	+	+	+
D801/803N	–	+	+	+
D808N	–	+	+	+
D801/803/808N	–	+	+	+
D801/802/803/807/E808N	–	+	+	+

B

	GST-Syt1A/B	PRIP-C2	
	Ca^{2+}:	–	+
GST-Syt1-C2A	WT	–	+
	D178N	–	–
	K191/192A	–	+
	D230/232A	–	–
GST-Syt1-C2B	WT	+	+
	K297/301Q	+	+
	D303N	+	+
	D309N	+	+
	K326/327A	+	+
	N333T	+	+
	D363/365A	+	+

Conclusions

We showed that PRIP-C2 formed a hetero-oligomer with the C2 domains of other molecules and investigated the binding profiles of PRIP-C2 with Syt1-C2A and Syt1-C2B. This is the first study to show a direct interaction between C2 domains of other molecules, except for hetero-oligomerization among the C2 domains of synaptotagmin family members (Chapman, 2002; Wu et al., 2003). The physiological role of the interaction between PRIP-C2 and Syt1-C2s currently remains unclear. However, a previous study strongly suggested that PRIP may play an inhibitory role in the participation of synaptotagmin in exocytosis, *i.e.*, Syt1 was incorporated in the immunocomplex containing t-SNARE proteins and PRIP, and the amount of Syt1 in the immunocomplex was increased when PRIP was omitted using a PRIP-KO

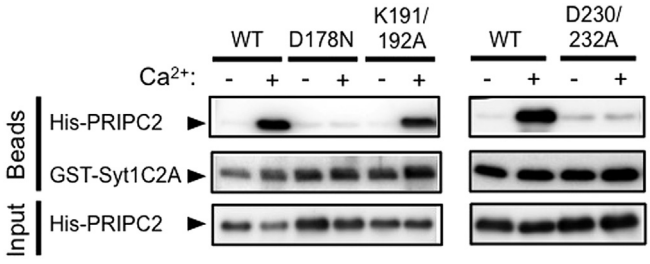

Fig. 4. Interaction between PRIP-C2 and Syt1-C2 mutants. A GST pull-down assay was performed as in Fig. 1 using GST-Syt1-C2A mutants.

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mouse brain as the source for immunoprecipitation (Zhang et al., 2013). Thus, further studies are required to clarify the molecular mechanism by which PRIP, Syt1, and t-SNARE proteins are coordinated to form a complex, which will, in turn, lead to the exact mechanism underlying the Ca^{2+}-triggered exocytosis regulated by these molecules being elucidated.

Conflict of interest

Authors have no conflict of interest.

Acknowledgments

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