The C2A domain of synaptotagmin-like protein 3 (Slp3) is an atypical calcium-dependent phospholipid-binding machine: comparison with the C2A domain of synaptotagmin I

Mitsunori FUKUDA[1]

Fukuda Initiative Research Unit, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

The synaptotagmin-like protein (Slp) family consists of an N-terminal Rab27-binding domain and C-terminal tandem C2 motifs, and although it has been suggested to regulate Rab27-dependent membrane trafficking, such as Ca^{2+}-regulated granule exocytosis in T-lymphocytes [Kuroda, Fukuda, Ariga and Mikoshiba (2002) J. Biol. Chem. **277**, 9212–9218], little is known about the Ca^{2+}-binding property of the Slp family. In this study, I demonstrated that the C2A domain of Slp3 exhibits Ca^{2+}-dependent phospholipid-binding activity similar to that of the C2A domain of synaptotagmin I (Syt I) with regard to phospholipid selectivity, bivalent cation selectivity and effect of ionic strength. This finding was surprising because the C2A domains of other C-terminal-type (C-type) tandem C2 proteins require five conserved acidic residues in the putative Ca^{2+}-binding loops 1 and 3 on the top of the β-sandwich structure for their Ca^{2+}-/phospholipid-binding activity, whereas the C2A domain of Slp3 contains only one conserved acidic residue in the putative Ca^{2+}-binding loop 1. Site-directed mutagenesis and chimaeric analysis of the C2A domains of Syt I and Slp3 showed that Glu-336 and Glu-337 in the putative Ca^{2+}-binding loop 1 and polybasic sequence (Lys-359, Lys-360 and Lys-361) in the β-4 strand of the C2 structure are crucial for Ca^{2+}-dependent phospholipid-binding activity of the Slp3 C2A domain, whereas the similar polybasic sequence in the C2A domain of Syt I is dispensable for Ca^{2+}-dependent phospholipid-binding activity. These results indicate that the C2A domain of Slp3 is an atypical Ca^{2+}-/phospholipid-binding machine, compared with other C-type tandem C2 proteins.

Key words: C-type tandem C2 protein, Griscelli syndrome, membrane trafficking, Rab27 binding, Slp homology domain.

INTRODUCTION

The synaptotagmin (Syt)-like protein (Slp) family was initially described as a new member of the C-terminal-type (C-type) tandem C2 proteins, which contain two C2 Ca^{2+}-binding motifs (named the C2A and C2B domains) [1] separated by a short linker (less than 50 amino acids) at the C-terminus [2]. To date, five different *slp* (or *sytl*) gene products (Slp1–5) have been described in mice and humans [3,4], and they are distinguished from other C-type tandem C2 protein families (rabphilin, Syts, Doc2s, B/K and Tac2-N) by their unique N-terminal sequences [5–12]. The Slp family contains a unique N-terminal Slp homology domain, but an alternatively spliced isoform that lacks the unique N-terminal domain has also been reported in some members of the Slp family [3] and the Syt family [13,14].

Although the exact role of Slps remains to be clarified, I recently found that the Slp homology domain specifically binds Rab27A and Rab27B both *in vitro* and *in vivo* [15,16], which belong to the small GTP-binding-protein superfamily believed to be essential to membrane trafficking of eukaryotic cells [17]. Since mutation of the *rab27A* gene causes defects in melanosome transport in melanocytes and Ca^{2+}-regulated granule exocytosis in T-lymphocytes of *ashen* mice and humans with Griscelli syndrome [18–21], by analogy to the function of rabphilin-3, a Rab3 effector, in synaptic vesicle exocytosis [22], the Slp family has been suggested to function as a novel Rab27 effector and regulate vesicular trafficking. Use of a functional block antibody against the C2A domain of Syt I [23–28] and analysis of knock-in mice [29] has shown the Ca^{2+}-dependent phospholipid-binding activity of the C2A domain of Syt I to be essential for Ca^{2+}-dependent transmitter secretion. However, little is known about the Ca^{2+}-dependent phospholipid-binding properties of the Slp family, despite such information being quite important to determining whether the Slp family is involved in Ca^{2+}-regulated processes.

In this study I investigated the phospholipid-binding properties (e.g. selectivity of bivalent cations and of phospholipids) of the C2A domain of Slp3 in comparison with the C2A domain of Syt I. Site-directed mutagenesis and chimaeric analysis between Syt I and Slp3 revealed that the Glu-336 and Glu-337 and polybasic sequence (Lys-359, Lys-360 and Lys-361) in the β-4 strand of the C2 structure are crucial to the Ca^{2+}-dependent phospholipid-binding activity of the Slp3 C2A domain. Based on my findings, I discuss the different mechanisms of Ca^{2+}-dependent phospholipid binding by the Syt I C2A and the Slp3 C2A domains.

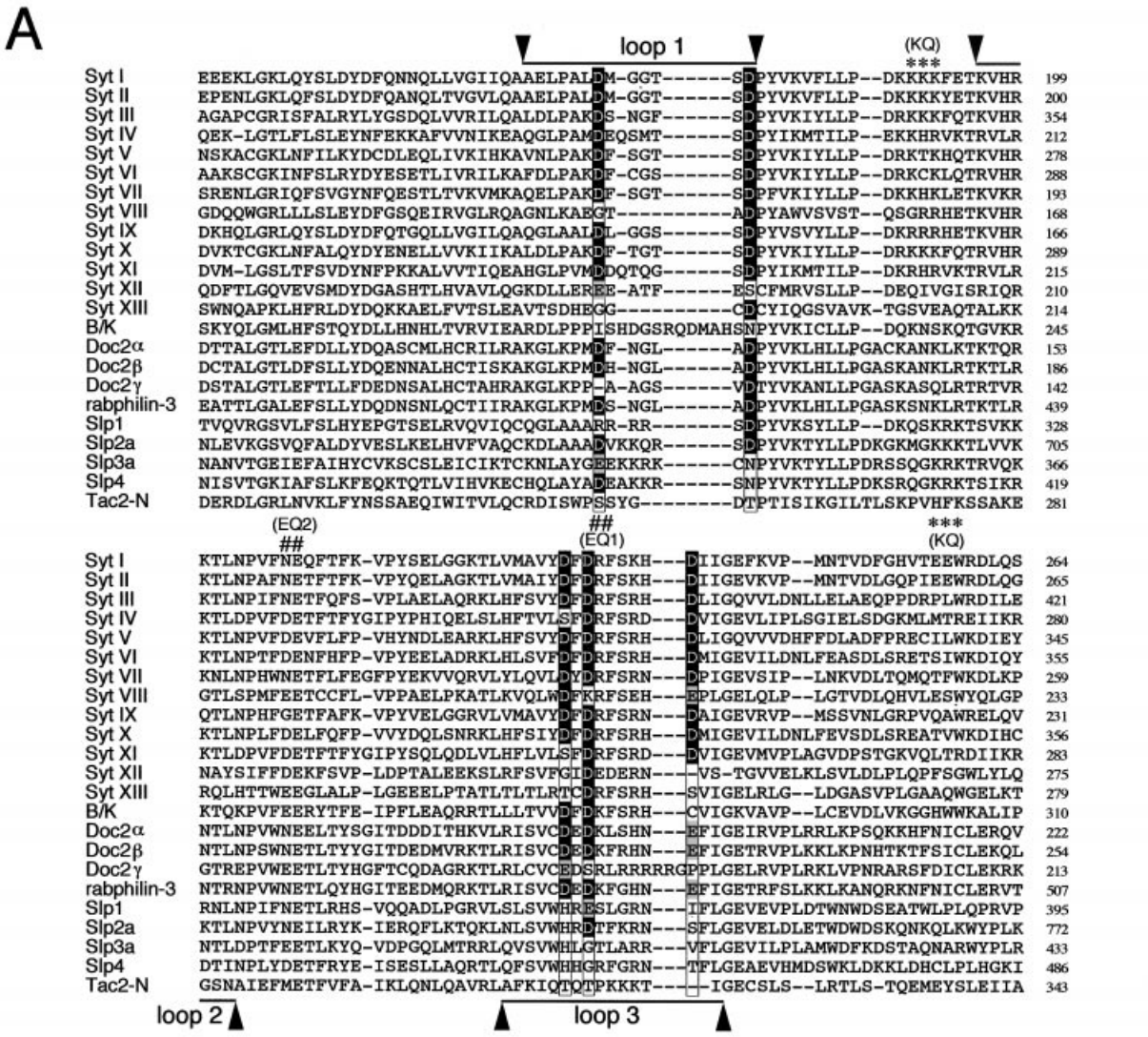
MATERIALS AND METHODS

Materials

Recombinant *Taq* DNA polymerase was obtained from Toyobo Biochemicals (Tokyo, Japan). Horseradish peroxidase-conjugated anti-T7 tag antibody was from Novagen (Madison, WI, U.S.A.). L-α-Phosphatidylcholine (PC), dipalmitoyl, L-α-phosphatidylserine (PS), dioleoyl and L-α-phosphatidylinositol (PI) were from Sigma (St. Louis, MO, U.S.A.). The 0.1 M EGTA and 0.1 M $CaCl_2$ standard solutions were from Nacalai Tesque

Abbreviations used: C-type, C-terminal-type; GST, glutathione S-transferase; PC, L-α-phosphatidylcholine; PI, L-α-phosphatidylinositol; PS, L-α-phosphatidylserine; Slp, synaptotagmin-like protein; Syt, synaptotagmin.

[1] E-mail mnfukuda@brain.riken.go.jp



No. of D/E in Ca^{2+}-binding loops	Name	PS/PC liposome binding	References
5	Syt I, II, III, V, VII, IX, X Doc2α, β rabphilin-3	Ca^{2+}-dependent	[34, 35, 39, 40, 43]
4	Syt IV*, XI	no binding (*binding to PS lonly)	[34, 41]
3	Syt VIII B/K Slp2	no binding	[2, 40, 44]
2	Syt XII, XIII Doc2γ Slp1	no binding	[2, 10, 42]
1	Slp3 Slp4	Ca^{2+}-dependent (Slp3) Ca^{2+}-independent (Slp4)	[2, 45]
0	Tac2-N	no binding	[12]

Figure 1 For legend see opposite page

(Kyoto, Japan). All other chemicals were commercial products of reagent grade. Solutions were made up in deionized water prepared with an Elix10 Water Purification System and Milli-Q Biocel A10 System (Millipore Corp., Bedford, MA, U.S.A.).

Site-directed mutagenesis

pGEX-4T-3-Slp3-C2A(EQ1) carrying E336Q and E337Q substitutions, pGEX-4T-3-Slp3-C2A(EQ2) carrying E374Q and E375Q substitutions, and pGEX-4T-3-Slp3-C2A(KQ) carrying K359Q, K360Q and K361Q substitutions were essentially produced by means of the two-step PCR techniques described previously [30] using the following pairs of oligonucleotides: C2A upper primer [2] and EQ1 primer 1, 5′-ACATATGGATTGCACTTTCTTTT-CTTTTGTTGTCC-3′, EQ2 primer 1, 5′-CTTAAGGGTTTGC-TGGAAAGTCGGGTC-3′, or KQ primer 1, 5′-GACTCGAG-TCTGCTGTTGTCCCTG-3′ (left half); EQ1 primer 2, 5′-TC-CATATGTCAAGACCTA-3′, EQ2 primer 2, 5′-ACCCTTAA-GTATCAGGTGGA-3′, or KQ primer 2, 5′-AAGACTCGAGT-CCAAAAG-3′, and C2A lower primer [2] (right half). The right and left halves were separately amplified by using pGEM-T-Slp3-a as a template [3], and the two PCR fragments obtained were digested with *Nde*I, *All*II or *Xho*I (the sites are underlined above), ligated to each other, and re-amplified with the C2A upper and lower primers. The PCR fragment obtained that encoded the mutant Slp3 was digested with *Bam*HI/*Eco*RI and inserted into the *Bam*HI/*Eco*RI site of pGEX-4T-3. pGEX-4T-3-Syt I-C2A(KQ) carrying K190Q, K191Q and K192Q substitutions and pGEX-4T-3-Syt I-C2A-loop mutants (replacement by the Slp3 loop domains; see Figure 5A, below) were similarly constructed by PCR using the following mutagenic oligonucleotides: Syt I-KQ primer 1, 5′-GTGCACTTTTGTCTCAAACTGC-TGCTGTTT-3′, and Syt I-KQ primer 2, 5′-GTGCACCGGA-AAACCCTCAA-3′; loop 1 primer 1, 5′-CATATGCAAGGTT-CTTAGCCTGGATGATTCCCACCA-3′, and loop 1 primer 2, 5′-CATATGGAGAAGAAAAGAAAAGAAAGTGCAAT-CCATACGTCAAAGTCT-3′; loop 2 primer 1, 5′-GACGCGT-GAGACCTGTGTCTCAAACTTCTTCTTT-3′, and loop 2 primer 2, 5′-TCACGCGTCCAAAAGAACACCCTCAATCC-AGTCTTCAA-3′; and loop 3 primer 1, 5′-GGTACCTAGGTG-CCACACCAGTGTCTTGCCACCTAA-3′, and loop 3 primer 2, 5′-GGTACCCTTGCCCGGAGGGTGTTCCTTGGAGAG-TTCAAAGTTCCTA-3′.

Phospholipid-binding assay

Glutathione S-transferase (GST) fusion proteins (GST-Syt I-C2A, GST-Syt X-C2A, GST-Syt XII-C2A and GST-Slp3-C2A) were expressed in *Escherichia coli* JM109 and purified as described previously [2,31–33]. Preparation of liposomes consisting of PC and PS (1:1, w/w), PS, PC or PI alone was also performed as described previously [34,35]. GST fusion proteins were incubated with liposomes corresponding to 160 μg of phospholipid (in 50 mM Hepes/KOH, pH 7.2) for 15 min at room temperature. After centrifugation at 12000 ***g*** for 10 min at room temperature, the phospholipid pellets were washed in 500 μl of the above equilibration buffer and then extracted with 300 μl of acetone at −20 °C for 30 min to remove excess lipid. The pellets obtained by centrifugation at 12000 ***g*** for 15 min at 4 °C were dissolved in SDS sample buffer and analysed by SDS/PAGE (10 % gel), followed by Coomassie Brilliant Blue R250 staining. The protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) using BSA as a reference. The gels shown in this paper are representative of at least two or three independent experiments.

RESULTS AND DISCUSSION

Phospholipid-binding properties of the C2A domain of the mouse C-type tandem C2 proteins

The C2 domain was initially proposed to be a Ca^{2+}-binding module [1], but various Ca^{2+}-independent C2 domains have subsequently been reported. These C2 domains function as a protein-interaction site (e.g. several Syt C2 domains; reviewed in [6]) or a nuclear localization signal [12,36]. The Ca^{2+}-dependent phospholipid-binding capacity of an uncharacterized C2 domain is often predicted by comparison of its primary amino acid sequence with the Syt I C2A domain, because conservation of five Glu/Asp residues is crucial for Ca^{2+} binding in the Ca^{2+}-binding loops of the Syt I C2A domain (see Figures 1A and 1C) [37,38]. For instance, the C2A domain of Syt X has five conserved Asp/Glu residues and the C2A domain of Syt XII has two. When their phospholipid-binding activities were tested, as shown in Figure 1(B), the Syt X C2A domain showed Ca^{2+}-dependent phospholipid-binding activity, the same as the Syt I C2A domain, but the Syt XII did not. The relationships between the number of Asp/Glu residues in the putative Ca^{2+}-binding loops and the phospholipid-binding activity of the C2A domains of the C-type tandem C2 proteins are summarized in Figure 1(C) [2,10,12,34,35,39–45]. I found a positive correlation between the number of Asp/Glu residues and the phospholipid-binding capacity of the C2A domains of other C-type tandem C2 proteins. All the C2A domains carrying five Asp/Glu residues in the putative Ca^{2+}-binding loops bound PS/PC liposomes in a Ca^{2+}-dependent manner, whereas other C2A domains carrying less than five Asp/Glu residues, except for the Slp3 and Slp4 C2A domains, did not, irrespective of the presence of Ca^{2+} (Figure 1C). Nevertheless, to my surprise, the C2A domain of Slp3 showed Ca^{2+}-dependent liposome (PS/PC)-binding activity despite having only one conserved Asp/Glu residue in the putative Ca^{2+}-binding loop 1 (Figure 1B, bottom panel), suggesting involvement of a different mechanism in its Ca^{2+}/phospholipid binding from that of the C2A domain of Syt I.

Phospholipid-binding property of the Slp3 C2A domain

In the next set of experiments I attempted to delineate the mechanism of the Ca^{2+}-dependent phospholipid (PS/PC liposome)-binding activity of the C2A domain of Slp3 and

Figure 1 Phospholipid-binding properties of the C2A domain of the mouse C-type tandem C2 proteins

(**A**) Sequence alignment of the C2A domain of the mouse C-type tandem C2 proteins [2,7,10,12,32,42–45,53,54]. The Asp and Glu residues, which are crucial for Ca^{2+} binding in the Syt I C2A domain, are shown on a black or a shaded background, respectively [37,38]. Amino acid numbers are shown on the right. Solid lines indicate the putative Ca^{2+}-binding loops [37,38]. The positions of EQ and KQ mutations in the Slp3 and Syt I C2A domains are represented by # and *, respectively. The arrowheads indicate the chimaeric points between the Syt I and Slp3 C2A domains (see also Figure 5A). (**B**) Ca^{2+}-dependent phospholipid-binding activity of Syt I C2A, Syt X C2A, Syt XII C2A and Slp3 C2A fused to GST. GST fusion proteins were incubated with and without (−) PS/PC liposomes in the presence of 2 mM EGTA or 1 mM Ca^{2+} for 15 min at room temperature. After centrifugation at 12000 ***g*** for 10 min, the pellets (phospholipid-binding fraction) were separated, subjected to SDS/PAGE (10% gels) and then stained with Coomassie Brilliant Blue R-250 as described previously [34,35]. Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample. (**C**) Relationship between the number of Asp/Glu (D/E) residues in the putative Ca^{2+}-binding loops and the phospholipid-binding activity of the C2A domain of the C-type tandem C2 proteins.

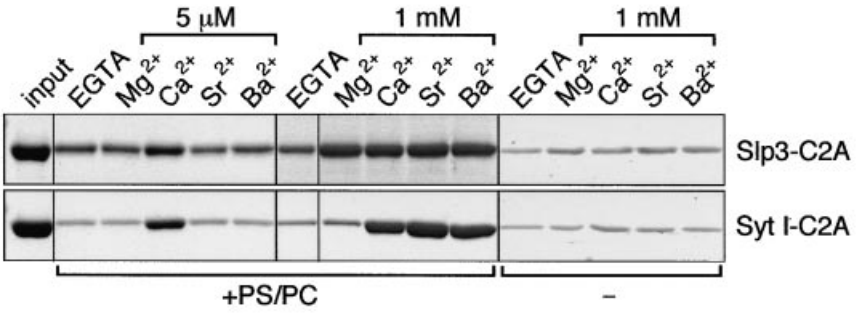


Figure 2 Bivalent cation regulation of the Ca^{2+}-dependent phospholipid-binding property of the Slp3 and Syt I C2A domains

Ca^{2+} ions selectively promoted phospholipid (PS/PC liposome)-binding activity of the Slp3 C2A and Syt I C2A domains. Phospholipid-binding assay was performed as described in the Materials and methods section. Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample.

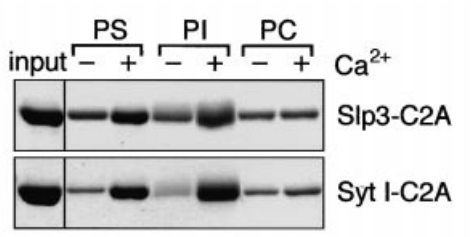


Figure 3 The Slp3 C2A domain selectively binds negatively charged phospholipids in a Ca^{2+}-dependent manner

Phospholipid (PS, PI or PC alone)-binding assay was performed in the presence and absence of 1 mM Ca^{2+} as described in the Materials and methods section. Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample. Note that both the Slp3 C2A and Syt I C2A domains bind negatively charged phospholipids (PS and PI), but not PC.

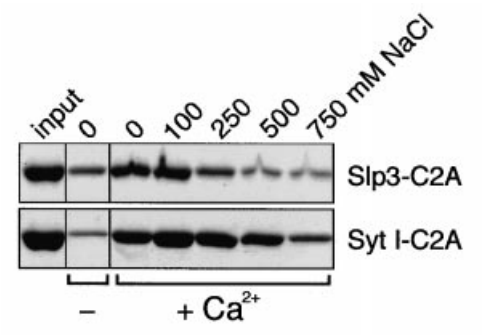


Figure 4 Effect of ionic strength on the Ca^{2+}-dependent phospholipid binding of the Slp3 C2A domain

Phospholipid (PS/PC liposome)-binding assay was performed in the presence of 1 mM Ca^{2+} and the concentrations of NaCl indicated, as described in the Materials and methods section. Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample. Note that the Ca^{2+}-dependent phospholipid-binding activity of the Slp3 C2A domain was inhibited by high concentrations of NaCl, as was that of the Syt I C2A domain.

compare it with the C2A domain of Syt I. First, I investigated the effect of bivalent cations (Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}) on the phospholipid-binding activity of the C2A domain of Slp3. Its Ca^{2+}-dependency was quite similar to that of Syt I: Ca^{2+} ions selectively promoted the phospholipid binding to the Slp3 C2A domain (Figure 2) [34,35,40,46]. The phospholipid-binding activity of both the Slp3 and Syt I C2A domains was also activated at high Sr^{2+} and Ba^{2+} concentrations (1 mM). Unlike the Syt I C2A domain, 1 mM Mg^{2+} activated phospholipid binding of the Slp3 C2A domain.

Second, I investigated the phospholipid selectivity of the Slp3 C2A domain. Both the C2A domains of Slp3 and Syt I bound negatively charged phospholipids (PS and PI), but not zwitterionic phospholipids (PC), in a Ca^{2+}-dependent manner (Figure 3) [35,46,47]. Since the C2A domain of Syt I is known to bind phospholipid by electrostatic interaction rather than hydrophobic interaction [47–49], I then investigated the effect of ionic strength (NaCl concentration) on the phospholipid-binding activity of the C2A domain of Slp3. As expected, the Ca^{2+}-dependent phospholipid (PS/PC liposome)-binding activity of the C2A domain of Slp3 was inhibited by mild concentrations of NaCl (250 mM), and it was more sensitive to ionic strength than the C2A domain of Syt I (Figure 4), suggesting that the interaction between the C2A domain of Slp3 and phospholipids is relatively weak compared with the C2A domain of Syt I.

Identification of the key amino acids responsible for Ca^{2+}-dependent phospholipid binding of the Slp3 C2A domain by site-directed mutagenesis and chimaeric analysis

To determine how the Slp3 C2A domain binds negatively charged phospholipids in a Ca^{2+}-dependent manner despite having only one conserved acidic residue in the putative Ca^{2+}-binding loops, I produced a chimaera between the putative Ca^{2+}-binding loops of the Slp3 and Syt I C2A domains (Figure 5), because the Ca^{2+}-binding loops 1 and 3 of the C2A domain of Syt I primarily determine the phospholipid-binding specificity and Ca^{2+} binding [49,50]. The Syt I-C2A-loop 1 (replacement by the Slp3 loop 1) exhibited Ca^{2+}-independent phospholipid-binding activity, and the Syt I-C2A-loop 3 (replacement by the Slp3 loop 3) mutants failed to exhibit Ca^{2+}-dependent phospholipid (PS/PC liposome)-binding activity (Figure 5B, second and fourth panels). By contrast, the Syt I-C2A-loop 2 mutant (replacement by the Slp3 loop 2) showed normal Ca^{2+}-dependent phospholipid-binding activity, the same as the wild-type Syt I C2A (Figure 5B, top and third panels), probably because loop 2 of Syt I is not involved in Ca^{2+}-binding [37,38] and thus replacement by the Slp3 loop 2

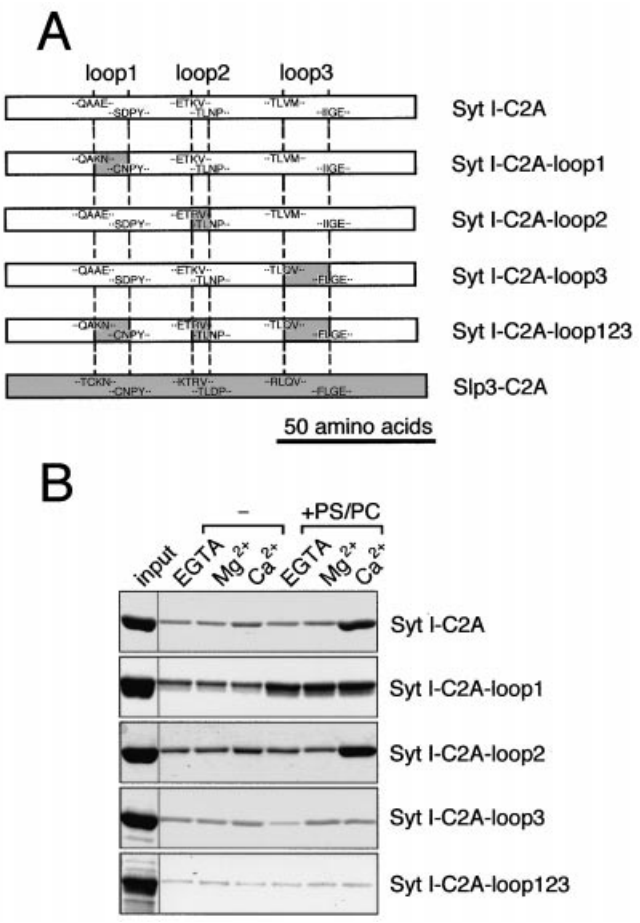


Figure 5 Chimaeric analysis of the putative Ca^{2+}-binding loops between the Slp3 and Syt I C2A domains

(**A**) Schematic representation of the chimaeric proteins between Syt I (open boxes) and Slp3 (shaded boxes). The letters in the boxes indicate the sequences around the chimaeric points. (**B**) Ca^{2+}-dependent phospholipid (PS/PC)-binding properties of the Syt I chimaera proteins. GST fusion proteins were incubated with and without (—) PS/PC liposomes in the presence and absence of bivalent cations. Input means the total proteins used for the phospholipid-binding assay. In the absence of liposomes, only 5–10% of the input proteins were recovered in the phospholipid-binding fraction. Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample.

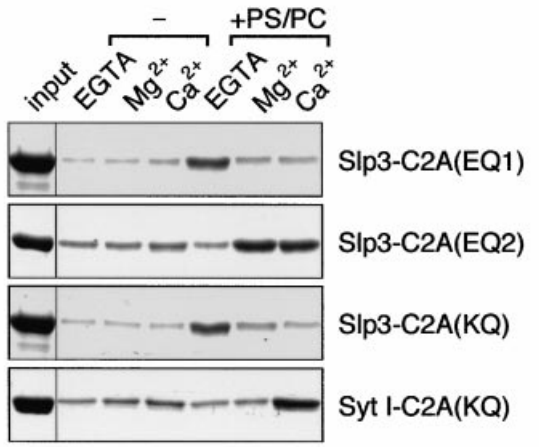


Figure 6 Mutational analysis of the Ca^{2+}-dependent phospholipid-binding site of the Slp3 C2A domain

Ca^{2+}-dependent phospholipid (PS/PC)-binding properties of the Slp3 or Syt I mutant proteins. Note that the Slp3-C2A(EQ1) and Slp3-C2A(KQ) mutants did not exhibit Ca^{2+}-dependent phospholipid-binding activity (top and third panels), but that the Syt I-C2A(KQ) mutant and Slp3-C2A(EQ2) did (second and bottom panels). Input means the total proteins used for the phospholipid-binding assay. The gels shown here represent the pelleted portion (phospholipid-binding fraction) of the input sample.

should be neutral for phospholipid-binding activity. Consistent with this, loop 1 and loop 3 of the Slp3 C2A domain lack one and three Glu/Asp residues, respectively, responsible for Ca^{2+} binding in the Syt I C2A domain (Figure 1A). Unexpectedly, however, replacement of the three loops (Syt I-C2A-loop 123) was not followed by Ca^{2+}-dependent phospholipid-binding activity, suggesting that three loops on top of the C2 β-sandwich structure alone are not sufficient for the Ca^{2+}-dependent phospholipid binding of the Slp3 C2A domain and that additional region(s) in the β-sandwich structure are required for the Ca^{2+}/phospholipid binding.

I then performed site-directed mutagenesis to identify the key amino acids responsible for the Ca^{2+}-dependent phospholipid-binding activity of the Slp3 C2A domain. I focused on the two Glu residues (Glu-336 and Glu-337) in the putative Ca^{2+}-binding loop 1 and polybasic sequence in the β-4 strands (Lys-359, Lys-360 and Lys-361), because a similar polybasic sequence in the Syt II C2B domain is essential for inositol polyphosphate binding [30]. When the two negative charges were neutralized by replacement of Gln [Slp3-C2A(EQ1)], the mutant protein did not show Ca^{2+}-dependent phospholipid-binding activity, but instead bound liposomes only in the absence of bivalent cations (Figure 6, top panel). By contrast, however, neutralization of two negative charges just near the putative Ca^{2+}-binding loop 2 [Slp3-C2A-(EQ2); see Figure 1A, #] had no effect on Ca^{2+}-dependent liposome binding activity (Figure 6, second panel). The Slp3-C2A(KQ) mutant carrying K359Q, K360Q, and K361Q substitutions also did not show Ca^{2+}-dependent phospholipid-binding activity, but it bound liposomes in the absence of bivalent cations (Figure 6, third panel). By contrast, the Syt I-C2A(KQ) mutant still exhibited Ca^{2+}-dependent phospholipid-binding activity (Figure 6, bottom panel), indicating that the polybasic sequence is dispensable for the Ca^{2+}-dependent phospholipid binding of the Syt I C2A domain. These results indicated that fundamental mechanism of Ca^{2+}/phospholipid binding in regard to critical residues responsible for Ca^{2+}-binding differs by the C2A domain of Slp3 and Syt I.

Conclusions

In the present study I compared the phospholipid-binding ability of the C2A domain of all known C-type tandem C2 proteins and found that five Asp/Glu residues identified in the Syt I C2A domain [37,38] in the top loops of the C2 β-sandwich structure primarily determines the Ca^{2+}-dependent phospholipid-binding ability (i.e. conservation of five Asp/Glu residues in loops 1 and 3 of C2 structure is a good marker for Ca^{2+}-dependent phospholipid-binding ability; see Figure 1C). The only exception is the Slp3 C2A domain, which contains only one conserved Glu residue in the putative Ca^{2+}-binding loop 1. Nevertheless, the Slp3 C2A domain binds negatively charged phospholipids via electrostatic interaction, which is selectively promoted by Ca^{2+}

ions, the same as the Syt I C2A domain. Site-directed mutagenesis (Figure 6) and chimaeric analysis (Figure 5) indicated that the Glu residues in loop 1 and the polybasic sequence in the β-4 strand in the Slp3 C2A domain are crucial for Ca^{2+}-dependent phospholipid binding, whereas the top loops of the Syt I C2A domain, but not the β-sandwich structure, primarily determine its Ca^{2+}-/phospholipid-binding ability [50]. Because of this different mechanism, the Syt I-C2A-loop 123 chimaera mutant did not exhibit phospholipid-binding activity (i.e. three loops of Slp3 alone are insufficient for the phospholipid-binding activity of Slp3 and Syt I-C2A-loop 123). Although the Syt I C2A domain also contains the similar polybasic sequence in the β-4 strand, its position was different from that of the Slp3 C2A domain. Since I previously showed that the positions and numbers of the basic residues in the β-4 strands of the C2B domain are essential for inositol polyphosphate binding [30], the polybasic sequence of the Syt I C2A domain is unlikely to contribute to Ca^{2+}/phospholipid binding of the Syt I-C2A-loop 123 mutant.

In summary, I have demonstrated that the C2A domain of Slp3 is an atypical Ca^{2+}-/phospholipid-binding machine compared with other C-type tandem C2 proteins. The Ca^{2+}-dependent phospholipid binding of the Slp3 C2A domain can be activated by at least 10 μM Ca^{2+}, which are comparable with the Ca^{2+} concentration that triggers Ca^{2+}-dependent exocytosis in endocrine cells or granule exocytosis in cytotoxic T-lymphocytes [51,52], although 1 mM Mg^{2+} can activate phospholipid binding of the Slp3 C2A domain. Further work is necessary to determine whether the phospholipid-binding activity of Slp3 is involved in Rab27-dependent Ca^{2+}-regulated exocytosis.

I thank Eiko Kanno and Yukie Ogata for technical assistance. This work was supported in part by grants from the Science and Technology Agency to Japan and grant 13780624 from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1 Nalefski, E. A. and Falke, J. J. (1996) The C2 domain calcium-binding motif: structural and functional diversity. Protein Sci. **5**, 2375–2390

2 Fukuda, M. and Mikoshiba, K. (2001) Synaptotagmin-like protein 1–3: a novel family of C-terminal-type tandem C2 proteins. Biochem. Biophys. Res. Commun. **281**, 1226–1233

3 Fukuda, M., Saegusa, C. and Mikoshiba, K. (2001) Novel splicing isoforms of synaptotagmin-like proteins 2 and 3: identification of the Slp homology domain. Biochem. Biophys. Res. Commun. **283**, 513–519

4 Kuroda, T. S., Fukuda, M., Ariga, H. and Mikoshiba, K. (2002) Synaptotagmin-like protein 5: a novel Rab27A effector with C-terminal tandem C2 domains. Biochem. Biophys. Res. Commun. **293**, 899–906

5 Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993) Rabphilin-3A, a putative target protein for smg p25A/rab3A p25 small GTP-binding protein related to synaptotagmin. Mol. Cell. Biol. **13**, 2061–2068

6 Marquèze, B., Berton, F. and Seagar, M. (2000) Synaptotagmins in membrane traffic: which vesicles do the tagmins tag? Biochimie **82**, 409–420

7 Fukuda, M., Kanno, E. and Mikoshiba, K. (1999) Conserved N-terminal cysteine motif is essential for homo- and heterodimer formation of synaptotagmins III, V, VI, and X. J. Biol. Chem. **274**, 31421–31427

8 Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H. and Takai, Y. (1995) Doc2: a novel brain protein having two repeated C2-like domains. Biochem. Biophys. Res. Commun. **206**, 439–448

9 Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H. and Takai, Y. (1995) Molecular cloning of an isoform of Doc2 having two C2-like domains. Biochem. Biophys. Res. Commun. **217**, 1053–1061

10 Fukuda, M. and Mikoshiba, K. (2000) Doc2γ, a third isoform of double C2 protein, lacking calcium-dependent phospholipid binding activity. Biochem. Biophys. Res. Commun. **276**, 626–632

11 Kwon, O. J., Gainer, H., Wray, S. and Chin, H. (1996) Identification of a novel protein containing two C2 domains selectively expressed in the rat brain and kidney. FEBS Lett. **378**, 135–139

12 Fukuda, M. and Mikoshiba, K. (2001) Tac2-N, an atypical C-type tandem C2 protein localized in the nucleus. FEBS Lett. **503**, 217–218

13 Fukuda, M. and Mikoshiba, K. (1999) A novel alternatively spliced variant of synaptotagmin VI lacking a transmembrane domain: implications for distinct functions of the two isoforms. J. Biol. Chem. **274**, 31428–31434

14 Craxton, M. and Goedert, M. (1999) Alternative splicing of synaptotagmins involving transmembrane exon skipping. FEBS Lett. **460**, 417–422

15 Kuroda, T. S., Fukuda, M., Ariga, H. and Mikoshiba, K. (2002) The Slp homology domain of synaptotagmin-like proteins 1–4 and Slac2 functions as a novel Rab27A binding domain. J. Biol. Chem. **277**, 9212–9218

16 Fukuda, M., Kuroda, T. S. and Mikoshiba, K. (2002) Slac2-a/melanophilin, the missing link between Rab27 and myosin Va: implications of a tripartite protein complex for melanosome transport. J. Biol. Chem. **277**, 12432–12436

17 Zerial, M. and McBride, H. (2001) Rab proteins as membrane organizers. Nat. Rev. Mol. Cell Biol. **2**, 107–117

18 Wilson, S. M., Yip, R., Swing, D. A., O'Sullivan, T. N., Zhang, Y., Novak, E. K., Swank, R. T., Russell, L. B., Copeland, N. G. and Jenkins, N. A. (2000) A mutation in *Rab27a* causes the vesicle transport defects observed in *ashen* mice. Proc. Natl. Acad. Sci. U.S.A. **97**, 7933–7938

19 Menasche, G., Pastural, E., Feldmann, J., Certain, S., Ersoy, F., Dupuis, S., Wulffraat, N., Bianchi, D., Fischer, A., Le Deist, F. and de Saint Basile, G. (2000) Mutations in *RAB27A* cause Griscelli syndrome associated with haemophagocytic syndrome. Nat. Genet. **25**, 173–176

20 Stinchcombe, J. C., Barral, D. C., Mules, E. H., Booth, S., Hume, A. N., Machesky, L. M., Seabra, M. C. and Griffiths, G. M. (2001) Rab27a is required for regulated secretion in cytotoxic T lymphocytes. J. Cell Biol. **152**, 825–834

21 Haddad, E. K., Wu, X., Hammer, III, J. A. and Henkart, P. A. (2001) Defective granule exocytosis in Rab27a-deficient lymphocytes from *Ashen* mice. J. Cell Biol. **152**, 835–842

22 Burns, M. E., Sasaki, T., Takai, Y. and Augustine, G. J. (1998) Rabphilin-3A: a multifunctional regulator of synaptic vesicle traffic. J. Gen. Physiol. **111**, 243–255

23 Fukuda, M. and Mikoshiba, K. (1997) The function of inositol high polyphosphate binding proteins. BioEssays **19**, 593–603

24 Mikoshiba, K., Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M. and Llinás, R. (1995) Role of the C2A domain of synaptotagmin in transmitter release as determined by specific antibody injection into the squid giant synapse preterminal. Proc. Natl. Acad. Sci. U.S.A. **92**, 10703–10707

25 Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K. and Kumakura, K. (1997) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells. Proc. Natl. Acad. Sci. U.S.A. **94**, 287–291

26 Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H. and Mikoshiba, K. (1997) Roles of synaptotagmin C2 domains in neurotransmitter secretion and inositol high-polyphosphate binding at mammalian cholinergic synapses. Neuroscience **77**, 937–943

27 Lang, J., Fukuda, M., Zhang, H., Mikoshiba, K. and Wollheim, C. B. (1997) The first C2 domain of synaptotagmin is required for exocytosis of insulin from pancreatic β-cells: action of synaptotagmin at low micromolar calcium. EMBO J. **16**, 5837–5846

28 Fukuda, M., Kowalchyk, J. A., Zhang, X., Martin, T. F. J. and Mikoshiba, K. (2002) Synaptotagmin IX regulates Ca^{2+}-dependent secretion in PC12 cells. J. Biol. Chem. **277**, 4601–4604

29 Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matos, M. F., Stevens, C. F., Brose, N., Rizo, J., Rosenmund, C. and Südhof, T. C. (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature (London) **410**, 41–49

30 Fukuda, M., Kojima, T., Aruga, J., Niinobe, M. and Mikoshiba, K. (1995) Functional diversity of C2 domains of synaptotagmin family: mutational analysis of inositol high polyphosphate binding domain. J. Biol. Chem. **270**, 26523–26527

31 Smith, D. B. and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**, 31–40

32 Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994) Inositol-1,3,4,5-tetrakisphosphate binding to C2B domain of IP4BP/synaptotagmin II. J. Biol. Chem. **269**, 29206–29211

33 Ibata, K., Fukuda, M. and Mikoshiba, K. (1998) Inositol 1,3,4,5-tetrakisphosphate binding activities of neuronal and non-neuronal synaptotagmins: identification of conserved amino acid substitutions that abolish inositol 1,3,4,5-tetrakisphosphate binding to synaptotagmins III, V, and X. J. Biol. Chem. **273**, 12267–12273

34 Fukuda, M., Kojima, T. and Mikoshiba, K. (1996) Phospholipid composition dependence of Ca^{2+}-dependent phospholipid binding to the C2A domain of synaptotagmin IV. J. Biol. Chem. **271**, 8430–8434

35 Fukuda, M., Kojima, T. and Mikoshiba, K. (1997) Regulation by bivalent cations of phospholipid binding to the C2A domain of synaptotagmin III. Biochem. J. **323**, 421–425
36 Fukuda, M., Saegusa, C., Kanno, E. and Mikoshiba, K. (2001) The C2A domain of double C2 protein γ contains a functional nuclear localization signal. J. Biol. Chem. **276**, 24441–24444
37 Sutton, R. B., Davletov, B. A., Berghuis, A. M., Südhof, T. C. and Sprang, S. R. (1995) Structure of the first C2 domain of synaptotagmin I: a novel Ca^{2+}/phospholipid-binding fold. Cell **80**, 929–938
38 Shao, X., Davletov, B. A., Sutton, R. B., Südhof, T. C. and Rizo, J. (1996) Bipartite Ca^{2+}-binding motif in C2 domains of synaptotagmin and protein kinase C. Science **273**, 248–251
39 Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K. and Takai, Y. (1993) Two functionally different domains of rabphilin-3A, Rab3A p25/smg p25A-binding and phospholipid- and Ca^{2+}-binding domains. J. Biol. Chem. **268**, 27164–27170
40 Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G. W., Brose, N. and Südhof, T. C. (1995) Ca^{2+}-dependent and -independent activities of neural and non-neural synaptotagmins. Nature (London) **375**, 594–599
41 von Poser, C., Ichtchenko, K., Shao, X., Rizo, J. and Südhof, T. C. (1997) The evolutionary pressure to inactivate: a subclass of synaptotagmins with an amino acid substitution that abolishes Ca^{2+} binding. J. Biol. Chem. **272**, 14314–14319
42 Fukuda, M. and Mikoshiba, K. (2001) Characterization of KIAA1427 protein as an atypical synaptotagmin (Syt XIII). Biochem. J. **354**, 249–257
43 Kojima, T., Fukuda, M., Aruga, J. and Mikoshiba, K. (1996) Calcium-dependent phospholipid binding to the C2A domain of a ubiquitous form of double C2 protein (Doc2 β). J. Biochem. (Tokyo) **120**, 671–676
44 Fukuda, M. and Mikoshiba, K. (2001) The N-terminal cysteine cluster is essential for membrane targeting of B/K protein. Biochem. J. **360**, 441–448
45 Wang, J., Takeuchi, T., Yokota, H. and Izumi, T. (1999) Novel rabphilin-3-like protein associates with insulin-containing granules in pancreatic beta cells. J. Biol. Chem. **274**, 28542–28548
46 Davletov, B. A. and Südhof, T. C. (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca^{2+}/phospholipid binding. J. Biol. Chem. **268**, 26386–26390
47 Nalefski, E. A., Wisner, M. A., Chen, J. Z., Sprang, S. R., Fukuda, M., Mikoshiba, K. and Falke, J. J. (2001) C2 domains from different Ca^{2+} signaling pathways display functional and mechanistic diversity. Biochemistry **40**, 3089–3100
48 Davletov, B., Perisic, O. and Williams, R. L. (1998) Calcium-dependent membrane penetration is a hallmark of the C2 domain of cytosolic phospholipase A2 whereas the C2A domain of synaptotagmin binds membranes electrostatically. J. Biol. Chem. **273**, 19093–19096
49 Chapman, E. R. and Davis, A. F. (1998) Direct interaction of a Ca^{2+}-binding loop of synaptotagmin with lipid bilayers. J. Biol. Chem. **273**, 13995–14001
50 Gerber, S. H., Rizo, J. and Südhof, T. C. (2001) The top loops of the C_2 domains from synaptotagmin and phospholipase A_2 control functional specificity. J. Biol. Chem. **276**, 32288–32292
51 Takayama, H. and Sitkovsky, M. V. (1987) Antigen receptor-regulated exocytosis in cytotoxic T lymphocytes. J. Exp. Med. **166**, 725–743
52 Poenie, M., Tsien, R. Y. and Schmitt-Verhulst, A. M. (1987) Sequential activation and lethal hit measured by $[Ca^{2+}]_i$ in individual cytolytic T cells and targets. EMBO J. **6**, 2223–2232
53 Fukuda, M., Kanno, E., Ogata, Y. and Mikoshiba, K. (2001) Mechanism of the SDS-resistant synaptotagmin clustering mediated by the cysteine cluster at the interface between the transmembrane and spacer domains. J. Biol. Chem. **276**, 40319–40325
54 Naito, A., Orita, S., Wanaka, A., Sasaki, T., Sakaguchi, G., Maeda, M., Igarashi, H., Tohyama, M. and Takai, Y. (1997) Molecular cloning of mouse Doc2α and distribution of its mRNA in adult mouse brain. Mol. Brain Res. **44**, 198–204

Received 27 March 2002/23 May 2002; accepted 5 June 2002
Published as BJ Immediate Publication 5 June 2002, DOI 10.1042/BJ20020484