Regulation by bivalent cations of phospholipid binding to the C2A domain of synaptotagmin III

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Synaptotagmins are Ca^{2+} - and phospholipid-binding proteins of synaptic vesicles that might function as Ca^{2+} receptors for neurotransmitter release via their first C2 (C2A) domain. Here we describe the effect of Mg^{2+} on phospholipid binding to the C2A domains of multiple synaptotagmins (II–VI), and demonstrate that only synaptotagmin III can bind negatively charged phospholipids [phosphatidylserine (PS) and phosphatidylinositol] in a Mg^{2+} -dependent manner. The Mg^{2+} -dependent interaction with PS was found to have an EC_{50} of approx. 30 μM

Mg^{2+} , which is comparable to that of Sr^{2+} and Ba^{2+} (EC_{50} values of approx. 10 μM). This binding property of the C2A domain is specific to synaptotagmin III, because none of the C2A domains of other proteins, such as rabphilin 3A, Doc2 α , Doc2 β or Gap1^m, showed phospholipid binding activity in the presence of 1 mM Mg^{2+} . Our results suggest that synaptotagmin III is involved in presynaptic functions different from those of synaptotagmins I and II.

INTRODUCTION

The C2 domain is a conserved protein module of approx. 120 amino acid residues found in many proteins; the number of C2 domains in one molecule varies from one to four [1]. This domain originates from the sequence motif of protein kinase C and was originally thought to be a Ca^{2+} -dependent phospholipid-binding site [2]. Recently, however, it has been found that some C2 domains lack this Ca^{2+} -dependent phospholipid-binding capacity [1,3], and others are involved in protein–protein interaction in addition to phospholipid binding [4–8]. The biochemical nature of the tandem C2 domains of synaptotagmin I (known as the C2A and C2B domains) has been particularly well characterized because synaptotagmin I is thought to be a Ca^{2+} sensor for neurotransmitter release (reviewed in [9]). The C2A domain of synaptotagmin I binds phospholipids [3,10,11] and syntaxin in a Ca^{2+} -dependent manner [6], whereas the C2B domain binds clathrin assembly protein AP2 [5], β -SNAP [8] and the inositol high polyphosphate series [Ins(1,3,4,5) P_4 , Ins(1,3,4,5,6) P_5 and Ins P_6] [3,12] irrespective of the presence of Ca^{2+} . Consistent with the difference in biochemical properties of the two C2 domains, they have different functions in synaptic vesicle trafficking, as demonstrated by the microinjection of domain-selective antibodies against each C2 domain into the squid giant presynapse [13,14], superior cervical ganglion cells [15] and chromaffin cells [16]: (1) the C2A domain functions as a Ca^{2+} sensor; (2) the inositol high polyphosphate series blocks synaptic transmission by binding to the C2B domain; and (3) the C2B domain is also involved in endocytosis, probably by binding AP2.

The synaptotagmins constitute a family of vesicle membrane proteins, and at least nine separate isoforms have been identified in rodents [6,17–21]. The existence of multiple isoforms raised the question of whether other isoforms also have the same function as that of synaptotagmin I. For that reason much attention has been focused on functional differences in these

isoforms [12,22–24]. In our previous study we demonstrated that the C2A domains of multiple synaptotagmins (II–VI) possess Ca^{2+} -dependent negatively charged phospholipid-binding capacity but that the C2A domain of synaptotagmin IV displays a dependence on phospholipid composition [24]. In this study we showed that Mg^{2+} also activates phospholipid binding to the C2A domain of synaptotagmin III. This unique Mg^{2+} -dependent interaction of phospholipids was not observed in the C2A domains of other synaptotagmin isoforms (II, IV–VI), rabphilin 3A, Doc2 α , Doc2 β or Gap1^m. We also analysed the bivalent cation specificity for phospholipid binding, and the functional diversity of synaptotagmin families are discussed on the basis of the results.

EXPERIMENTAL

Chemicals

Phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Sigma. All other chemicals were commercial products of reagent grade. Solutions were prepared in deionized water.

Preparation of glutathione S-transferase (GST) fusion proteins

pGEX-2T (Pharmacia Biotech) vectors carrying the C2A domains of synaptotagmins, rabphilin 3A, Doc2 β and Gap1^m were constructed as described previously [3,24–26]. GST fusion proteins were expressed and purified on glutathione-Sepharose (Pharmacia) by following the manufacturer's instructions. The GST fusion proteins of mouse synaptotagmins II–VI (GST-STII–VI-C2A), mouse rabphilin 3A (GST-Rph-C2A), mouse Doc2 β (GST-Dob-C2A), and mouse Gap1^m [GST-Gap-C2AB

Abbreviations used: GST, glutathione S-transferase; inositol high polyphosphate series, Ins(1,3,4,5) P_4 , Ins(1,3,4,5,6) P_5 and Ins P_6 ; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PKC, protein kinase C; PS, phosphatidylserine; STII–VI, synaptotagmins II–VI.

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(C2A + C2B)] used in this study were also prepared as described previously [3,24–26].

A cDNA encoding the C2A domain of Doc2 α was amplified from a human cerebellum cDNA library constructed in λ gt11 (Clontech Laboratories) by PCR (sense, 5'-CGGATCCGACTCGGATGATGCCACCGC-3'; anti-sense, 5'-GCAATTGTTGGCGCTCGAGGCAGATGT-3', design based on [27]). The cycling conditions were denaturation at 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 3 min, for 30 cycles. After digestion with *Bam*HI and *Mun*I, the PCR products were subcloned into the *Bam*HI–*Eco*RI site of pGEX-2T and sequenced by using a BcaBest dideoxy sequencing kit (Takara Shuzo). Comparison with previously published human cDNA sequences [27] revealed three amino acid changes: Lys-92 was changed to Thr (K92T), Val-108 to Cys (V108C) and Cys-109 to Ser (C109S). These are unlikely to have been PCR-induced errors because they were also conserved in Doc2 β and found in two independent clones (see Figure 5). GST–Doa-C2A contains residues 83–216 of human Doc2 α .

Site-directed mutagenesis of GST–STIII-C2A(A303S, L307D) was performed by means of PCR as follows. The C2A domain was amplified with oligonucleotides (mutagenic primer A, 5'-CTGATCAGGGGAAGCAGGGGACCCCTGTGGCCGCATCAGTTTTTCCCTCCGGTACGACT-3'; primer B, 5'-CGAATTCTCCAGGATGTCCCTCCAGA-3'). After digestion with *Fba*I and *Eco*RI, the PCR fragment encoding the mutant C2A domain of synaptotagmin III obtained (A303S, L307D) was subcloned into the *Bam*HI–*Eco*RI site of pGEX-2T and verified by DNA sequencing.

Four chimaeric proteins were produced from synaptotagmins II and III (see Figure 4) by two-step PCR that introduced an artificial *Spe*I or *Sac*I site into the C2A domain of the synaptotagmins [24]. GST–STII/III-a contained residues 139–177 of mouse synaptotagmin II followed by residues 332–421 of mouse synaptotagmin III (STII 139–177 + STIII 332–421). GST–STIII/II-a contained residues STIII 290–330 + STII 177–267; GST–STII/III-b, STII 139–219 + STIII 374–421; and GST–STIII/II-b, STIII 290–373 + STII 220–267.

Phospholipid binding assay

Liposomes were prepared in 50 mM Hepes/KOH (pH 7.2)/100 mM NaCl by sonication, collected by centrifugation and equilibrated with 50 mM Hepes/KOH, pH 7.2, in the presence of 2 mM EGTA and various concentrations of CaCl₂, MgCl₂, SrCl₂ or BaCl₂. To suppress the background due to contamination by Ca²⁺, 10 μ M EGTA (final concentration) was added to the reaction mixture when Mg²⁺, Sr²⁺ or Ba²⁺ was used, except in the experiments shown in Figure 1. GST fusion proteins (5–10 μ g) were incubated with liposomes corresponding to 100 μ g of phospholipid for 15 min at room temperature [3]. After centrifugation at 12000 *g* for 10 min at room temperature, the phospholipid pellets were washed in 500 μ l of the above equilibration buffer, then extracted with 300 μ l 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. The proteins in the supernatants were precipitated by adding an equal volume of 20% (w/v) trichloroacetic acid. After incubation for 15 min on ice the samples were centrifuged at 12000 *g* for 15 min at 4 °C and the precipitates were mixed with SDS sample buffer. Equal proportions of the supernatants and pellets were analysed by SDS/PAGE [10% (w/v) gel], followed by staining with Coomassie Brilliant Blue R-250. The proportion of the phospholipid-binding fractions was quantified by using a Bio Image (Millipore). The protein concen-

trations were determined with a Bio-Rad protein assay kit with BSA as the reference.

RESULTS

Mg²⁺-dependent liposome binding to the C2A domains of multiple synaptotagmins

The C2A domains of synaptotagmins are known to function as Ca²⁺- and phospholipid-binding domains. However, phospholipid binding to the C2A domain of synaptotagmin III, but not other isoforms, was activated by 1 mM Mg²⁺ as well as Ca²⁺ (Figure 1). GST–STIII-C2A bound to acidic phospholipids (PS and PI), but not PC or PE, in a Mg²⁺-dependent manner, as with Ca²⁺ [24]. This binding is not an artifact of contamination by Ca²⁺ or self-aggregation by Mg²⁺, because binding was still observed even in the presence of 10 μ M EGTA, and GST–STIII-C2A did not precipitate in the absence of PS liposomes (results not shown). This Mg²⁺/phospholipid binding to GST–STIII-C2A was less affected by phospholipid composition than synaptotagmin IV [24], because GST–STIII-C2A bound PS/PE, PS/PC and PS/PI liposomes (1:1, w/w) in the presence of 1 mM Mg²⁺ (results not shown).

Effect of bivalent cations on phospholipid binding to the C2A domain of synaptotagmins II and III

To compare the effects of bivalent cations, PS liposome binding to GST–STII-C2A and GST–STIII-C2A in the presence of various concentrations of Ca²⁺, Sr²⁺, Ba²⁺ or Mg²⁺ (0.1 μ M to 1 mM) was tested. GST–STII-C2A bound PS liposomes in a Ca²⁺, Sr²⁺ and Ba²⁺-dependent manner (Ca²⁺ > Sr²⁺ > Ba²⁺) but binding did not occur in the presence of Mg²⁺, at a concentration of 1 mM (Figure 2, right panel), as with the C2A domain of synaptotagmin I as previously described [10]. In contrast, GST–STIII-C2A bound PS liposomes in a Mg²⁺-dependent manner with a slightly lower affinity than that of Sr²⁺ and Ba²⁺

Figure 1 Mg²⁺-dependent phospholipid binding to the C2A domains of synaptotagmins II–VI fused to GST

Liposomes and GST fusion proteins were incubated in 50 mM Hepes/KOH, pH 7.2, in the presence of 2 mM EGTA or 1 mM Mg²⁺ for 15 min at room temperature. After centrifugation at 12000 *g* for 10 min, the pellets (P, phospholipid binding fraction) and supernatants (S, non-binding fraction) were separated as described in the Experimental section. Equal proportions of the supernatants and pellets were resolved by SDS/PAGE [10% (w/v) gel]. Note that only the C2A domain from synaptotagmin III bound negatively charged phospholipids (PS and PI), but not PE and PC. None of the other C2A domains bound PS or PI in the presence of 1 mM Mg²⁺.

Figure 2 Bivalent cation concentration dependence of PS liposome binding to the C2A domains of synaptotagmins II and III

Binding of PS liposome to GST-STII-C2A (right panel) and to GST-STIII-C2A (left panel) was examined as a function of the free Ca²⁺, Sr²⁺, Ba²⁺ or Mg²⁺ concentration. Phospholipid binding was measured as described in the Experimental section. Ca²⁺/EGTA buffers were used in the low Ca²⁺ concentration range (0.1–50 μM) [24]. For GST-STIII-C2A, 2 mM EGTA was used instead of 0.1 μM free Ca²⁺ for the PS liposome-binding assay (extreme left) because some fraction of GST-STIII-C2A bound PS liposomes even in the presence of 0.1 μM free Ca²⁺. GST-STII-C2A did not bind PS liposomes even in the presence of 1 mM Mg²⁺, whereas GST-STIII-C2A bound them in a Mg²⁺-dependent manner that was almost the same as the concentration dependence of Sr²⁺ and Ba²⁺. Note that Ca²⁺/phospholipid binding to GST-STII-C2A was suppressed in the presence of 1 mM Mg²⁺ (bottom row), whereas GST-STIII-C2A bound PS liposomes irrespective of the presence of Ca²⁺. Results are representative of three independent experiments.

Figure 3 Mg²⁺/phospholipid binding properties of C2A domains of molecules other than synaptotagmins

PS liposome binding to all of the C2 domains from rabphilin 3A (GST-Rph-C2A), Doc2α (GST-Doa-C2A), Doc2β (GST-Dob-C2A) and Gap1^m (GST-Gap-C2AB) was activated by 1 mM Ca²⁺ but not by 1 mM Mg²⁺ or in the absence of Ca²⁺ (2 mM EGTA). Because some fractions of GST-Doa-C2A and GST-Dob-C2A bound PS liposomes irrespective of the presence of Ca²⁺ (results not shown), PS/PC (2/5, w/w) liposomes were used instead of PS liposomes alone to reduce their Ca²⁺-independent phospholipid-binding activity. Abbreviations are the same as those in Figure 1.

(Ca²⁺ > Sr²⁺ ≥ Ba²⁺ > Mg²⁺; Figure 2, left panel). The EC₅₀ was estimated to be approx. 30 μM Mg²⁺. We further analysed the Ca²⁺-dependent PS liposome-binding capacity of GST-STII-C2A and GST-STIII-C2A in the presence of 1 mM Mg²⁺ (Figure 2, bottom row). GST-STII-C2A bound PS liposomes in a Ca²⁺-dependent manner but with lower affinity than in the absence of 1 mM Mg²⁺ (EC₅₀ approx. 1–5 μM free Ca²⁺), indicating that Mg²⁺ suppresses Ca²⁺/phospholipid binding to GST-STII-C2A. In contrast, GST-STIII-C2A bound PS liposomes in the presence of 1 mM Mg²⁺ irrespective of the presence of Ca²⁺.

Figure 4 Analysis of the Mg²⁺/phospholipid-binding site of the C2A domain of synaptotagmin III with chimaeric proteins

(A) Schematic representations of synaptotagmins II and III are shown at the top, where the transmembrane region (TM) and the two C2 repeats are shown. Constructs of the chimaeras of the GST fusion proteins are shown below. (B) Phospholipid-binding properties of chimaeric proteins in the presence of 2 mM EGTA or 1 mM Mg²⁺ containing 10 μM EGTA. GST-STIII/II-a and -b bound PS liposomes in a Mg²⁺-dependent manner, whereas GST-STII/III-a and -b did not bind them even in the presence of 1 mM Mg²⁺. Abbreviations are the same as those in Figure 1.

Effect of Mg²⁺ on phospholipid binding to the C2A domains of various molecules

C2A domains (or tandem C2 domains) are known to exist in other molecules, such as rabphilin 3A [28], Doc2α [27], Doc2β [29] and the Gap1 family [30]. To determine whether Mg²⁺-dependent phospholipid binding to the C2A domain is unique to synaptotagmin III, we examined the C2A domains of rabphilin 3A, Doc2α, Doc2β and Gap1^m by using GST fusion proteins. Figure 3 shows that these C2A domains did not bind PS liposomes in the presence of 1 mM Mg²⁺, although they did bind PS liposomes in a Ca²⁺-dependent manner as previously described [3,25–27,31]. Therefore the ability to bind Mg²⁺/phospholipid seems to be a specific property of synaptotagmin III.

Analysis of the Mg²⁺/phospholipid-binding site of synaptotagmin III

To determine which region causes the difference in Mg²⁺/phospholipid binding capacity between synaptotagmins II and III, four chimaeric proteins from synaptotagmins II and III, referred to as GST-STII/III-a and GST-STII/III-b, and GST-STIII/II-a and GST-STIII/II-b, were used (see the Experimental section and Figure 4A). GST-STIII/II-a and -b bound Mg²⁺/phospholipid but GST-STII/III-a and -b did not show significant binding activity (Figure 4B). Therefore one-third of the N-

terminus of the C2A domain of synaptotagmin III is required for Mg^{2+} /phospholipid binding.

DISCUSSION

In the present study we demonstrated that only the C2A domain of synaptotagmin III has Mg^{2+} -dependent phospholipid-binding capacity. This binding is also specific for negatively charged phospholipids (PS and PI) but not PC and PE, as for Ca^{2+} [10,11,24]. Although relatively high concentrations of Mg^{2+} (EC_{50} approx. $30 \mu M$) compared with that of Ca^{2+} are required for phospholipid binding to the C2A domain of synaptotagmin III [23,24], the physiological concentration of Mg^{2+} is thought to be 0.5–1 mM, which is at least 100-fold higher than the intracellular Ca^{2+} concentration in the resting state. Therefore Mg^{2+} probably functions as a physiological ligand of synaptotagmin III, although its biological significance has yet to be determined.

Chimaeric analysis indicated that one-third of the N-terminal region of the C2A domain of synaptotagmin III is responsible for Mg^{2+} -dependent phospholipid-binding capacity. According to X-ray [32] and NMR [33] studies of synaptotagmin I, this region forms two β -strands (βI and βII) before the first Ca^{2+} -binding loop. Although at this stage there is no direct evidence that Mg^{2+} occupies the same sites as Ca^{2+} , amino acid substitutions within the βI and βII strands of synaptotagmin III probably modulate the structure of the Ca^{2+} -binding loop to facilitate binding to Mg^{2+} . When compared with other molecules, certain amino acid substitutions were found in synaptotagmin III (Figure 5). For example, Ala-303 and Leu-307 are candidates for the cause of the unique Mg^{2+} /phospholipid binding. However, replacement of Ala-303 by Ser(A303S) and of Leu-307 by Asp (L307D) does not impair Mg^{2+} /phospholipid-binding capacity (results not shown). Further work will be necessary to identify the amino acid(s) responsible for Mg^{2+} /phospholipid binding.

In a study of knockout mice, synaptotagmin I was found to be involved in fast synchronous Ca^{2+} -dependent neurotransmitter release [34]. Synaptotagmin II is thought to be functionally equivalent to synaptotagmin I because of these two molecules' high degree of sequence similarities, complementary distribution

[22] and similar bivalent cation-dependent phospholipid-binding capacity ([10], and this study). However, because the biochemical nature of synaptotagmin III is apparently different from that of other isoforms, especially synaptotagmins I and II, we propose that synaptotagmin III has presynaptic functions different from those of synaptotagmins I and II. First, only the C2A domain of synaptotagmin III is activated by Mg^{2+} . Secondly, the C2B domain of synaptotagmin III lacks inositol high polyphosphate series binding, which results in a blocking of neurotransmitter release [3,12,13,15,16,35]. Thirdly, the interaction between synaptotagmin III and syntaxin is also activated by Sr^{2+} , which can mediate the slow component of neurotransmitter release [23].

In summary, this is the first time that the C2A domain of synaptotagmin III has been reported to be a Mg^{2+} -activated C2 domain and that the C2A domains of synaptotagmins are not always functionally equivalent with respect to phospholipid binding.

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REFERENCES

- 1 Brose, N., Hofmann, K., Hata, Y. and Südhof, T. C. (1995) *J. Biol. Chem.* **270**, 25273–25280
- 2 Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- 3 Fukuda, M., Aruga, J., Niinobe, M., Aimoto, S. and Mikoshiba, K. (1994) *J. Biol. Chem.* **269**, 29206–29211
- 4 Miyazaki, M., Shirataki, H., Kohno, H., Kaibuchi, K., Tsugita, A. and Takai, Y. (1994) *Biochem. Biophys. Res. Commun.* **205**, 460–466
- 5 Zhang, J. Z., Davletov, B. A., Südhof, T. C. and Anderson, R. G. W. (1994) *Cell* **78**, 751–760
- 6 Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G. W., Brose, N. and Südhof, T. C. (1995) *Nature (London)* **375**, 594–599
- 7 Chapman, E. R., Hanson, P. I., An, S. and Jahn, R. (1995) *J. Biol. Chem.* **270**, 23667–23671
- 8 Schiavo, G., Gmachl, M. J., Stenbeck, G., Söllner, T. H. and Rothman, J. E. (1995) *Nature (London)* **378**, 733–736
- 9 Südhof, T. C. (1995) *Nature (London)* **375**, 645–653
- 10 Davletov, B. A. and Südhof, T. C. (1993) *J. Biol. Chem.* **268**, 26386–26390
- 11 Chapman, E. R. and Jahn, R. (1994) *J. Biol. Chem.* **269**, 5735–5741
- 12 Fukuda, M., Kojima, T., Aruga, J., Niinobe, M. and Mikoshiba, K. (1995) *J. Biol. Chem.* **270**, 26523–26527
- 13 Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M., Mikoshiba, K. and Llinás, R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10708–10712
- 14 Mikoshiba, K., Fukuda, M., Moreira, J. E., Lewis, F. M. T., Sugimori, M., Niinobe, M. and Llinás, R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10703–10707
- 15 Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H. and Mikoshiba, K. (1997) *Neuroscience*, in the press
- 16 Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Murakami, T., Kawasaki, M., Mikoshiba, K. and Kumakura, K. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 287–291
- 17 Perin, M. S., Fried, V. A., Mignery, G. A., Jahn, R. and Südhof, T. C. (1990) *Nature (London)* **345**, 260–263
- 18 Geppert, M., Archer, III, B. T. and Südhof, T. C. (1991) *J. Biol. Chem.* **266**, 13548–13552
- 19 Mizuta, M., Inagaki, N., Nemoto, Y., Matsukura, S., Takahashi, M. and Seino, S. (1994) *J. Biol. Chem.* **269**, 11675–11678
- 20 Hilbush, B. S. and Morgan, J. I. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8195–8199
- 21 Craxton, M. and Goedert, M. (1995) *FEBS Lett.* **361**, 196–200
- 22 Ullrich, B., Li, C., Zhang, J. Z., McMahon, H., Anderson, R. G. W., Geppert, M. and Südhof, T. C. (1994) *Neuron* **13**, 1281–1291
- 23 Li, C., Davletov, B. A. and Südhof, T. C. (1995) *J. Biol. Chem.* **270**, 24898–24902
- 24 Fukuda, M., Kojima, T. and Mikoshiba, K. (1996) *J. Biol. Chem.* **271**, 8430–8434

Figure 5 Alignment of the N-terminal region of the first C2 domains from synaptotagmins (Syt) I–VI, rabphilin 3A (Rph3A), Doc2 α , Doc2 β and the C2 domain of protein kinase C α (PKC α)

Residues that are identical in the five sequences are boxed. Conserved residues in all sequences are shown in bold letters. Residue numbers are given at both sides. Asterisks indicate one of the essential aspartic residues for Ca^{2+} binding to the C2A domain of synaptotagmin I as shown by crystallographic and NMR analysis [32,33]; the locations of the β -strands are indicated by solid lines above the sequence; # indicates the residues that are conserved in almost all sequences except synaptotagmin III; and the arrowhead points to the chimaeric point between synaptotagmin IV and III (GST-STII/III-a or GST-STIII/II-a). The sequences are taken from the following sources: mouse synaptotagmins I and II [3], mouse synaptotagmin III [12], mouse synaptotagmin IV [20], rat synaptotagmin V [21], rat synaptotagmin VI [6], mouse rabphilin 3A [3], human Doc2 α (this work), mouse Doc2 β [26], and mouse PKC α [36].

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- 25 Fukuda, M. and Mikoshiba, K. (1996) *J. Biol. Chem.* **271**, 18838–18842
- 26 Kojima, T., Fukuda, M., Aruga, J. and Mikoshiba, K. (1996) *J. Biochem. (Tokyo)* **120**, 671–676
- 27 Orita, S., Sasaki, T., Naito, A., Komuro, R., Ohtsuka, T., Maeda, M., Suzuki, H., Igarashi, H. and Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* **206**, 439–448
- 28 Shirataki, H., Kaibuchi, K., Sakoda, T., Kishida, S., Yamaguchi, T., Wada, K., Miyazaki, M. and Takai, Y. (1993) *Mol. Cell. Biol.* **13**, 2061–2068
- 29 Sakaguchi, G., Orita, S., Maeda, M., Igarashi, H. and Takai, Y. (1995) *Biochem. Biophys. Res. Commun.* **217**, 1053–1061
- 30 Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokota, K., Imai, Y., Kohsaka, S., Nakamura, S. and Hattori, S. (1994) *Mol. Cell. Biol.* **14**, 6879–6885
- 31 Yamaguchi, T., Shirataki, H., Kishida, S., Miyazaki, M., Nishikawa, J., Wada, K., Numata, S., Kaibuchi, K. and Takai, Y. (1993) *J. Biol. Chem.* **268**, 27164–27170
- 32 Sutton, R. B., Davletov, B. A., Berghuis, A. M., Südhof, T. C. and Sprang, S. R. (1995) *Cell* **80**, 929–938
- 33 Shao, X., Davletov, B. A., Sutton, R. B., Südhof, T. C. and Rizo, J. (1996) *Science* **273**, 248–251
- 34 Geppert, M., Goda, Y., Hammer, R. E., Li, C., Rosahl, T. W., Stevens, C. F. and Südhof, T. C. (1994) *Cell* **79**, 717–727
- 35 Llinás, R., Sugimori, M., Lang, E. J., Morita, M., Fukuda, M., Niinobe, M. and Mikoshiba, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12990–12993
- 36 Rose-John, S., Dietrich, A. and Marks, F. (1988) *Gene* **74**, 465–471
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