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Calcium-Dependent Phospholipid Binding to the C2A Domain of a Ubiquitous Form of Double C2 Protein (Doc2β)[1]

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Rabphilin 3A and Doc2α are synaptic vesicle-associated proteins, and are thought to function as Ca^{2+} sensors in neurotransmitter release. If either rabphilin 3A or Doc2α plays a role in membrane trafficking, like the synaptotagmins, then non-neural forms should be present. Here we describe the isolation of a mouse cDNA which encodes a novel Doc2 homologue (Doc2β) that is present in all tissues. The encoded protein, which is highly homologous to human Doc2α (70% identity), is composed of 412 amino acids with a calculated relative molecular mass (M_r) of 45,837. The sequence identity is especially high in two C2 domains (74% in C2A and 84% in C2B). Northern and Western blot analyses have shown that Doc2β is expressed in all cell lines and tissues tested. Ca^{2+}-dependent phospholipid binding assaying of recombinant fusion proteins revealed that the single C2A domain, but not the C2B domain, of Doc2β binds phosphatidycholine and phosphatidylserine (2.5 : 1, w/w) liposomes. The binding is Ca^{2+}-dependent, with an EC_{50} value of approximately 1 μM and a Hill coefficient of approximately 3, which are comparable to those of synaptotagmins, rabphilin 3A and Doc2α. Our results suggest that Doc2β is involved in constitutive membrane trafficking.

Key words: C2 domain, Ca^{2+} sensor, Doc2, rabphilin 3A, synaptotagmin.

Many proteins which are involved in Ca^{2+}-regulated exocytosis in neurons and endocrine cells have homologues with roles in constitutive membrane trafficking (*1-8*). Of these proteins, synaptotagmin is thought to function as a Ca^{2+} sensor of neurotransmitter release, because it is a synaptic vesicle-specific protein and its C2A domain has Ca^{2+} binding capacity (*9-14*). The C2 domain was originally found in the C2 regulatory region of protein kinase C, and it has Ca^{2+}-dependent phospholipid binding ability (*9-11*). Recently, ubiquitous forms of synaptotagmin (named cellutagmins) were identified, and synaptotagmins are thought to play a general role in membrane trafficking, particularly in exocytosis (*8*).

Two C2 domains are also found in rabphilin 3A, a target protein of rab3A (*15*), and Doc2 (*16*). Rabphilin 3A and Doc2 are associated with synaptic vesicles, possibly *via* anchoring proteins (*16, 17*). Due to their Ca^{2+}-dependent phospholipid binding capacity (*11, 16, 18*), they are thought to be Ca^{2+} sensors. Recent studies supported that rabphilin 3A and Doc2α participate in the Ca^{2+} regulated exocytosis in squid giant axon preterminals (*19*), chromaffin cells (*20*), and PC12 cells (*21*). If rabphilin 3A and Doc2 are also involved in the fundamental process of membrane trafficking, then ubiquitous isoforms of these proteins should be present.

In the present study, we cloned a ubiquitous form of the mouse double C2-domain protein (Doc2β), and demonstrated that the C2A domain of Doc2β binds phosphatidylcholine (PC) and phosphatidylserine (PS) (2.5 : 1, w/w) liposomes in a Ca^{2+}-dependent manner. We discuss these results and the role of Doc2β in vesicular trafficking.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing—Total RNA was prepared from mouse (ICR) cerebellum as described by Chomczynski and Sacchi (*23*), and poly(A)$^+$ RNA was selected with Oligotex-dT 30 (Roche). First-strand cDNA was synthesized, with avian myeloblastosis virus reverse transcriptase, from the poly(A)$^+$ RNA. Two degenerate oligonucleotides were based on the nucleotide sequences encoding highly conserved regions in the C2 domains of protein kinase C (*24*), synaptotagmins (*7, 24-27*), and rabphilin

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Abbreviations: Doc2, double C2 protein; GST, glutathione *S*-transferase; IHPS, inositol high polyphosphate series (inositol 1,3,4,5-tetrakisphosphate, inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,2,3,4,5,6-hexakisphosphate); IP_4, inositol 1,3,4,5-tetrakisphosphate; M_r, relative molecular mass; PC, phosphatidylcholine; PCR, polymerase chain reaction; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

3A (*15*). The sense and antisense sequences used were 5′ CGGATCC(AG)TIT(AGT)(CGT)GA(CT)(GT)(AGT)IG-A(CT)(AC)(AG)ITT 3′ and 5′ CGAATTC(AG)T(CT)(AC-G)(ACT)AIT(AC)IGG(AG)TTIA(AG)IGT 3′, respectively. Using an LA PCR kit (Takara), PCR amplification was performed for 40 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 48°C for 2 min, and extension at 72°C for 3-4.5 min. The extension time was increased by 30 s every 10 cycles. The PCR products were subcloned into the pT7 Blue T-vector (Novagen) and then sequenced. Of 48 clones, 23 encoded a novel rabphilin 3A or Doc2-related protein. Using this insert as a probe, 2×10^7 plaques of a mouse cerebellum cDNA library were screened (*28*). Screening and sequence analyses of three overlapping clones were performed as described previously (*11*).

Northern Blot Analysis—Total RNAs, prepared from various mouse (ICR) tissues and several mammalian cell lines, were electrophoresed on 1.0% agarose-formaldehyde

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   1 GGCCGCCGCCGAGCAGGCAGCAGCCGCGTCAGGGCCGTCCGGGGGCCACGCTGGCGATGACCGCAGCCCCCGCAGCGCCCCGCGGGACCC   90
  91 GCTGACTTGCCCCCGGGCCGGGGTCACACCGGGCCGGGCCGGGCCGCGCGCCGCGGCGCTGCCTGCATGACCCTCCGGCGGCGCGGGGAG  180
                                                                 M  T  L  R  R  R  G  E     8

 181 AAGGCGACCATCAGCATCCAGGAGCATATGGCCATCGACGTGTGTCCCGGCCCCATTCGGCCTATCAAGCAGATCTCCGATTATTTTCCC  270
   9 K  A  T  I  S  I  Q  E  H  M  A  I  D  V  C  P  G  P  I  R  P  I  K  Q  I  S  D  Y  F  P    38

 271 CGCTTCCCGCGGGGCCTCCCCCCTACCGCCGCGCCCCGCGCCCCCGCGCCCCCGGACGCCCCCGCGCGCTCTCCCGCAGCCAGCGCCAGC  360
  39 R  F  P  R  G  L  P  P  T  A  A  P  R  A  P  A  P  P  D  A  P  A  R  S  P  A  A  S  A  S    68

 361 CCCCGCAGCCCCTCCGACGGCGCCCGCGACGACGACGAAGATGTGGACCAGCTCTTCGGAGCCTACGGAGCCAGCCCAGGCCCCAGCCCC  450
  69 P  R  S  P  S  D  G  A  R  D  D  D  E  D  V  D  Q  L  F  G  A  Y  G  A  S  P  G  P  S  P    98

 451 GGCCCCAGCCCCGCGAGGCCGCCCGCCAAGCCCCCCGAGGACGAGCCGGACGTCGACGGCTACGAGTCAGACGACTGCACCGCCCTGGGT  540
  99 G  P  S  P  A  R  P  P  A  K  P  P  E  D  E  P  D  V  D  G  Y  E  S  D  D  C  T  A  L  G   128

 541 ACGCTGGACTTCAGTCTGCTCTATGACCAGGAGAACAACGCACTGCACTGCACCATCAGCAAGGCCAAGGGCCTGAAGCCGATGGACCAC  630
 129 T  L  D  F  S  L  L  Y  D  Q  E  N  N  A  L  H  C  T  I  S  K  A  K  G  L  K  P  M  D  H   158

 631 AATGGACTGGCTGATCCCTACGTCAAACTACACCTGCTGCCTGGAGCCAGCAAGGCAAATAAGCTCAGAACAAAAACTCTTCGGAACACC  720
 159 N  G  L  A  D  P  Y  V  K  L  H  L  L  P  G  A  S  K  A  N  K  L  R  T  K  T  L  R  N  T   188

 721 CTGAACCCCTCGTGGAACGAGACCCTCACTTATTACGGAATCACGGATGAGGACATGGTCCGAAAGACCCTGAGGATCTCCGTGTGTGAT  810
 189 L  N  P  S  W  N  E  T  L  T  Y  Y  G  I  T  D  E  D  M  V  R  K  T  L  R  I  S  V  C  D   218

 811 GAGGACAAATTCCGCCACAATGAGTTCATTGGAGAGACTCGGGTGCCCCTGAAGAAGCTGAAGCCCAATCACACCAAGACATTCAGCATC  900
 219 E  D  K  F  R  H  N  E  F  I  G  E  T  R  V  P  L  K  K  L  K  P  N  H  T  K  T  F  S  I   248

 901 TGCCTGGAGAAGCAGCTGCCGGTGGACAAGGCAGAGGACAAGTCTCTGGAAGAGCGAGGCCGCATCCTCATCTCCCTCAAGTACAGCTCA  990
 249 C  L  E  K  Q  L  P  V  D  K  A  E  D  K  S  L  E  E  R  G  R  I  L  I  S  L  K  Y  S  S   278

 991 CAGAAGCAGGGCCTGCTGGTGGGCATCGTGCGCTGTGCACACCTGGCTGCCATGGATGCTAATGGCTACTCGGACCCCTATGTGAAAACA 1080
 279 Q  K  Q  G  L  L  V  G  I  V  R  C  A  H  L  A  A  M  D  A  N  G  Y  S  D  P  Y  V  K  T   308

1081 TATCTGAAGCCAGATGTAGACAAGAAATCCAAGCATAAGACAGCAGTGAAGAAGAAAACACTAAACCCAGAATTCAATGAGGAATTCTGT 1170
 309 Y  L  K  P  D  V  D  K  K  S  K  H  K  T  A  V  K  K  K  T  L  N  P  E  F  N  E  E  F  C   338

1171 TACGAGATCAAGCATGGAGACCTGGCCAAAAAGACTCTGGAGGTCACTGTCTGGGATTATGACATTGGAAAATCCAATGATTTCATCGGT 1260
 339 Y  E  I  K  H  G  D  L  A  K  K  T  L  E  V  T  V  W  D  Y  D  I  G  K  S  N  D  F  I  G   368

1261 GGTGTGGTTCTGGGCATCAACGCCAAGGGCGAGCGCCTGAAGCACTGGTTTGACTGCTTGAACAACAAGGACAAGAGGATTGAGCGTTGG 1350
 369 G  V  V  L  G  I  N  A  K  G  E  R  L  K  H  W  F  D  C  L  N  N  K  D  K  R  I  E  R  W   398

1351 CACACGCTCACCAATGAGCTCCCAGGGGCTGTACTCAGCGACTGACTGTCCCATCTGCTGCCACCCACCCTTGCCACCCGGCCCACACAG 1440
 399 H  T  L  T  N  E  L  P  G  A  V  L  S  D  *                                              412

1441 GTCCAACCCTGGGCTTTCTCAGCTGCCGCCAAGGGCAAGATCCAAGTTGTCTGCTCGGACATAGACAGTGCAGCCCCTGCTAGGAGGCCA 1530
1531 AGAGCACCCAGCCTCCCTCAGAGGGACAGGAAAACACAACATGATACCTGTTCCAGCTCCCTGGGCATCCAAGCTGGCCAGAGCTGGGGG 1620
1621 AATCCTCAGCCTCAGCTCCATCCAGAGGAAGGGCTATCTACAGTGAGGACCTATTGGAGGTGAGGGGTGGAGCCCTGCTGCAAGCCCAGA 1710
1711 AAATGGGGGGTACTCTGGCTGCCTGGAGACTACAGGGAGGACAATGGGCAGGCAGAGCCCAGGACTCCAGGACAGACAATGTAGCCAGCC 1800
1801 AGAGCTTCCAACTTGCTTGTGCATTGATGGGCAGATAGACTCCGTGAGCCTCTGGGACGGGGATTCCCACTCTAGCAGGGCATCTGGGTG 1890
1891 CACATATTGCTACAAAGGGGTGCAGCATACCAGAACCAGCCAGTTGTCTGCAGGGGCTGTGGGTACTGCTGACTGCTGAAGGGTGGAGGG 1980
1981 CAAGGAAGGCATCCCATGAGAGTCAGGGCTCAGCACAGTCTTGAAAACCCAGCTGTGGCTTGAAAGCTAGGAGAAAACCAAATACAGAAA 2070
2071 AGAGGAGGCCTGAAAGAGGGTACCAGGGGACAGGGAGCCATGGGGGCCTAGGGTTGCGCTAGGGCAGCATTGGGCTTTTAGGCAGGAGCT 2160
2161 AGGCTTAGGGATGTGTACAAGCACTTGGGAAACAATGTTCAAACCCAAGCTCTCCCATTTGATCTTGCTCCCAAGGGGTCTCAATGCACA 2250
2251 CTGCTCCTGTTCTCAGCGTAAATTCCCAGTCAGCTTCAGGGGAATGAGAAAGGGATCCTACTAATGTGGAAAGAAGCAAGCATCCCTCCT 2340
2341 GCCCACCTGTCCCTCCCTTCTTCATCCCCTCAGCTCAAGCTGGTGTTAGGTCCTTCACGGCTACCTATGGCATCCTGGCCCTATCAAGAC 2430
2431 AGTCAGGACCAGCAGTGGAAAATAGAAAGCCTTGTTTGGGGGAAGGTGGAGGGATGGGGGTTCTGCCCTCAGGAGCTGAGAACTCTGGCC 2520
2521 TCAGTTTCTAGGAAAGGGTAGGACACTGGTCAGTCTTGAAGCTCCCAACTGCCTCCTGCCAACTGGGATTCAGGAGCACAGGGGGAACAG 2610
2611 GGAACCAAGGAGTTCCACCATCATCTCAGAGCCCCATTTTCCTCAATCCATCACTGAGTGATAAGACACTGGGGTCCTGCCCTAAGCCTA 2700
2701 GAACCCTCCTACAGCTGAAATAAGAACTCGGGGTCCCAGAACATGATTCCAATCCACAGTGCTCACCAGCCTTCCCTAGTTGGCAGGGAA 2790
2791 AATAGGTCTCTAGCTCTCCACAGCACCAAGCCACCTGTTGCTTGTAAGAGACTGGGTTTCCCTCTTTAAAAGGTGTCGCCTGTTACTGAA 2880
2881 TTGGCAAACGCCTTCTTATTGAATCTTCACCATCCTGTGTGGTGGGAGGCATGGTCAACCCATTTTATAAATGAGATGGCTCCAGAGTTG 2970
2971 TTGATAAACTTCCAGGGCCACCACGGTGTGAAGGGCTGAAGCAAAAACTCCAAGCCTTTTGACTATCTGAGGACAAGGACTCTCAGGTCC 3060
3061 CTCTCTCCGGAGTTAATGGGGCTTAGTGAGGTCTCTTACAGAGAAGTCTTGTTATTGTCAGCCACCTCACCCTCTAGCTGGCTTTGAAAT 3150
3151 CATTTGTGCCATTTAGGATTTGCCAAAGGCTACAGCCAAGCCCTGGAACTCAGCTGTCCCACCCCCAGCCTGAGGTCAAGGAACTTGGGG 3240
3241 GACCGGCCTGATGTTTTCCCCATACAGCTAGAAACAGGAGCAGATATTTAAGTGCACCTGGTTGAGCTCTGAGTAACTCAGATTTTGCCT 3330
3331 TTTGGGGTGCTTTGTTCAGAGAGGAGTTTAGAAACCCCCG                                                    3371
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Fig. 1. **Nucleotide and deduced amino acid sequences of mouse Doc2β cDNA.** The deduced amino acid sequence is shown in the single letter code below the nucleotide sequence. Nucleotide and amino acid numbers are indicated at both sides of each line. The putative initiation codon of the Doc2β cDNA (CCTGCATGA) shows high similarity to the Kozak sequence [CCA/GCCATG(G)] (Ref. *36*). In-frame stop codons defining the 5′ and 3′ ends of the reading frame are indicated by an underline and an asterisk, respectively.

gels, transferred to Biodyne A (Pall), and then hybridized with ^{32}P-labeled cDNA probes, as previously described (*28*). A 469 bp fragment of Doc2β cDNA (nucleic acids 1-469) was used as a probe.

Preparation of Fusion Proteins—A cDNA fragment encoding the C2A and C2B domains of Doc2β (amino acids 123-257 and 257-375) was amplified by PCR (sense, 5′ CGGATCCGACTGCACCGCCCTGGGTAC 3′, and antisense, 5′ CGAATTCTTGTCCACCGGCAGCTGCTT 3′; and sense, 5′ CGGATCCGACAAGGCAGAGGACAAGTC 3′, and antisense, 5′ GCAATTGTTGATGCCCAGAACCACACC 3′, respectively). After digestion with *Bam*HI and *Eco*RI (or *Mun*I), the amplified fragments were subcloned into the *Bam*HI-*Eco*RI site of pGEX-2T and verified by DNA sequencing. Glutathione *S*-transferase (GST) fusion proteins of the C2A domain and the C2B domain of Doc2β (referred to as GST-C2A and GST-C2B, respectively) were expressed in *Escherichia coli* JM109, and then purified by glutathione-Sepharose 4B (Pharmacia) chromatography according to the manufacturer's recommendations.

Production of Polyclonal Antibodies—A New Zealand White rabbit was immunized with the purified GST-C2A using Freund's adjuvant system. Antiserum was collected after the third booster injection. After absorption with GST, the antiserum was affinity-purified with GST-C2A immobilized on Affi-Gel 10 (Bio-Rad) according to the manufacturer's recommendations.

Immunoblot Analysis—Proteins were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% skim milk, and then incubated with the purified anti-Doc2β C2A polyclonal antibodies. Primary antibody bind-

(A)

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rph  MTDTVVNRWM YPGDGPLQSN DKEQLQAGWS VHPGAQTDRQ RKQEELTDEE KEIINRVIAR AEKMEAMEQE  70

rph  RIGRLVDRLE TMRKNVAGDG VNRCILCGEQ LGMLGSACVV CEDCKKNVCT KCGVETSNNR PHPVWLCKIC 140

rph  LEQREVWKRS GAWFFKGFPK QVLPQPMPIK KTKPQQPAGE PATQEQPTPE SRHPARAPAR GDMEDRRPPG 210

dob                                                        MTL R-RRGEKATI SIQEHM---A  19
doa                                                          M :G::DRM:: N:::::---:  18
rph  QKPGPDLTSA PGRGSHGPPT RRASESRMST AARDSEGWDH AHGGGTGD:S :SPA:LRRAN :V:AARPAP: 280

dob  IDVCPGPIRP IKQISDYFPR FPRGLPPTAA PRAPAPPDAP ARSPAASASP RSPSDGARDD DEDVDQLFGA  89
doa  :N:::::::: :R:::::::: G:--------- ---------- ---------- -G:EG:GGSG G:APAH:VPL  59
rph  PVPS:A:PQ: VQPGPPGGS: ATP:PGRFPE QSTE:::SD: GYP------- -GAVAP::EE RTGPAGG:Q:  342

dob  YGASPGPSPG PSPARPPAKP PEDEPDVDGY ESDDCTALGT LDFSLLYDQE NNALHCTISK AKGLKPMDHN 159
doa  AL:P:AALL: -------:TT :::GAE::S: D::::A::::K :E:D::::RA SCT::VC:LR ::::::::F: 122
rph  APHTAA:YSQ AA:::Q:PPA E:E:EEANS: D::EA:T::A :E:::::::D :SN:Q:::IR ::::::::S: 412
                                                                  C2A
dob  GLADPYVKLH LLPGASKANK LRTKTLRNTL NPSWNETLTY YGITDEDMVR KTLRISVCDE DKFRHNEFIG 229
doa  :::::::::: :::::C:::: :K:::Q:::: ::V:::D::: S::::D:ITH :V:::A:::: ::LS:::::: 192
rph  :::::::::: :::::::S:: :::::::::R ::V:::::Q: H:::E:::Q: :::::::::: :::G:::::: 482

dob  ETRVPLKKLK PNHTKTFSIC LEKQLPVDKA EDKS------ ---------- ------LEER GRILISLKYS 277
doa  ::::::RR:: :SQK:H:N:: ::R:V:LASP SSM:AALRGI SCYLKDLEQA EQGQGL:::: ::::::::S:: 262
rph  :::FS::::: A:QR:N:N:: ::RVT:MKR: GTTGSARGMA LYEEEQVERI GD----I::: :K::V::M:: 548
                                                                  C2B
dob  SQKQGLLVGI VRCAHLAAMD ANGYSDPYVK TYLKPDVDKK SKHKTAVKKK TLNPEFNEEF CYEIKHGDLA 347
doa  :RRR:::::: L::::::::: V::::::::: :::R:::::: :::::C:::: :::::::::: F:::ELST:: 332
rph  T:QG::I::: I::V:::::: :::::::F:: LW::::MG:: A::::QI::: :::::::::: F:D:::S::: 618

dob  KKTLEVTVWD YDIGKSNDFI GGVVLGINAK GERLKHWFDC LNNKDKRIER WHTLTNELPG A---VLSD  412
doa  T::::::::: :::::::::: :::S::PG:R ::AR:::S:: :QQR:AAL:: :::::S:::P :AGALS:A  400
rph  ::S:DIS::: ::::::::Y: ::CQ:::S:: :::::::YE: :K::::K::: ::Q:Q::NH- ----:S::  681
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(B)

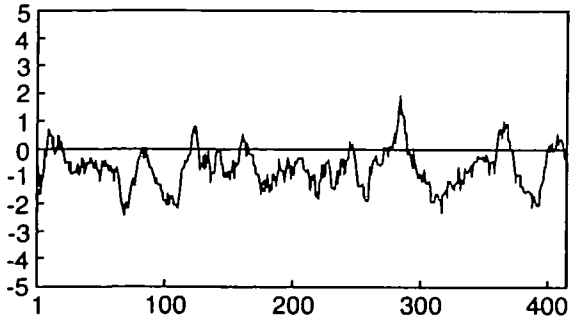

Fig. 2. **Alignment of the mouse Doc2β (dob) amino acid sequence with those of human Doc2α (doa) and mouse rabphilin-3A (rph), and the hydropathy profile of mouse Doc2β.** (A) Amino acids are shown in the single-letter code. Residues that are identical with those in Doc2β are indicated by colons. The C2 domains are boxed and shaded, the C2A domain being marked C2A and the C2B domain marked C2B. Asterisks indicate the conserved four aspartyl residues implicated in the Ca^{2+}-binding of synaptotagmin I C2A (see the text). Sequences are numbered on the right. (B) Hydropathy profile of mouse Doc2β, obtained according to Kyte and Doolittle (Ref. *29*). Note that the mouse Doc2β has no transmembrane region. The numbers indicate the positions of the amino acid residues of the protein.

ing was detected using a goat anti-rabbit IgG peroxidase-conjugated antibody. Immunoreactive bands were visualized with an enhanced chemiluminescence detection system (ECL kit; Amersham).

Phospholipid Binding Assay—^{3}H-labeled liposomes were prepared from either PC or PC mixed with PS, phosphatidylethanolamine (PE), or phosphatidylinositol (PI) (2.5 : 1, w/w) (Sigma), as described by Davletov and Südhof (*9*) with slight modifications (*12*). All buffers were made up in Ca^{2+}-free water using a 0.1 M $CaCl_2$ standard solution (Nacalai Tesque, Kyoto). GST-C2A or GST-C2B (10 μg) bound to glutathione-Sepharose beads (10 μl wet volume) was equilibrated with 50 mM Tris-HCl, pH 7.2, 0.1 M NaCl (buffer 1). The beads were resuspended in 500 μl of buffer 1 containing ^{3}H-labeled liposomes (3.5 μg of phospholipids; approximately 70,000 cpm), and various concentrations of Ca^{2+} buffered with EGTA (incubation buffer). Each mixture was incubated at room temperature for 15 min with vigorous shaking. The beads were briefly centrifuged in a tabletop centrifuge and then washed four times with 500 μl of the incubation buffer without liposomes. Liposome binding was quantified by liquid scintillation counting of the beads.

RESULTS AND DISCUSSION

Molecular Cloning of a Novel Double C2-Domain Protein cDNA—In a search for novel two C2-domain proteins, we used a PCR strategy involving amplification of segments including the junction domain between the C2A and C2B domains. Primers encoding parts of the C2 domains, which are highly conserved in protein kinase C, synaptotagmins and rabphilin 3A, were chosen. Forty-eight PCR products were cloned, sequenced, and classified into three groups, which included rabphilin 3A and two novel sequences. Of the two novel sequences, one encoded an amino acid sequence similar to that of the C2 domain. Using this clone as a probe, a mouse cerebellum cDNA library was screened and three overlapping clones were isolated. The 3,371 bp nucleotide sequence of these clones is shown in Fig. 1. The sequence contains an open reading frame of 1,236 bp nucleotides encoding a putative protein of 412 amino acids with a calculated M_r of 45,837. Homology search analysis revealed that this protein showed the highest homology to human Doc2α (70% identity) (*16*). At the time of the present study, the sequence for human Doc2β was reported (*22*). Human Doc2β is 94% identical to the protein deduced from our cDNA, thus we propose that our cDNA clone encodes the mouse homologue of the human Doc2β protein. As judged on hydrophobicity analysis (*29*), the mouse Doc2β protein, like the human Doc2β one, contains no hydrophobic region sufficient for a signal sequence or transmembrane region (Fig. 2B and Ref. *22*). Amino acid sequence alignment of Doc2α, Doc2β, and rabphilin 3A showed high sequence homology between Doc2α and Doc2β, especially in their C2 domains (sequence identity 74% in C2A and 84% in C2B; Fig. 2A). The major differences between Doc2α and Doc2β are that Doc2β has a shorter junction domain and a longer N-terminal sequence. The length of the junction domain between the C2A and C2B domains is more similar to that in the synaptotagmins than to that in either rabphilin 3A or Doc2α.

Tissue Distribution of Doc2β—Northern blotting revealed that the mRNA for Doc2β is expressed in a variety of murine tissues and mammalian cell lines (Fig. 3A). Doc2β mRNA appeared as a single band corresponding to approximately 5.2 kb. An antibody was raised against GST-C2A and used to determine if the mRNA was translated in murine tissues. As shown in Fig. 3B, a strong immunoreactive band corresponding to a M_r of approximately 44,000, which agrees with the M_r, 45,837, calculated from the Doc2β cDNA sequence, was detected in all tissues. This antibody may also detect Doc2α, however, this expression pattern is quite different from that of the Doc2α protein, which is only expressed in brain (*16*). Thus, these findings indicate that Doc2β is ubiquitously expressed.

Phospholipid Binding Properties of Doc2β—Most two

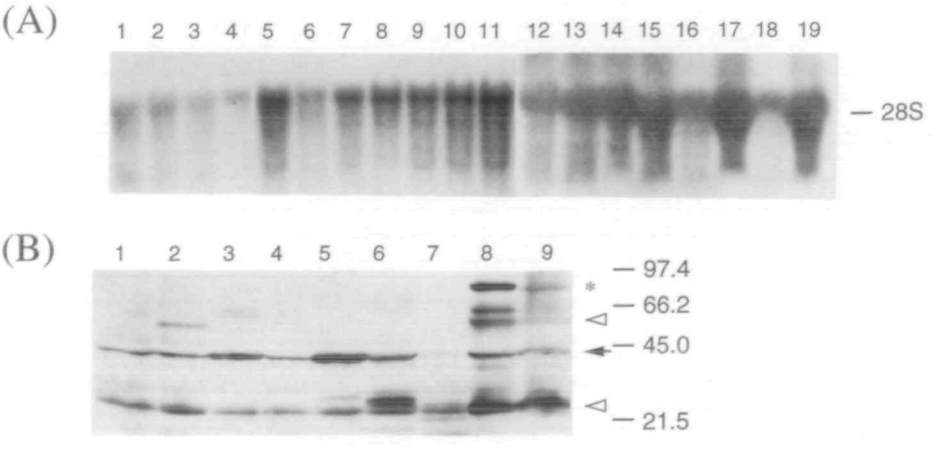

Fig. 3. **Tissue distribution of mouse Doc2β.** (A) Northern blot analysis of Doc2β mRNAs in various mouse tissues and cultured cell lines. Total RNAs from the indicated tissues and cells (20 μg/lane) were analyzed on 1.0% agarose-formaldehyde gels. A ^{32}P-labeled fragment (469 bp) of Doc2β was used as a probe. Lanes 1-11, mouse tissues: 1, skeletal muscle; 2, ovary; 3, testis; 4, kidney; 5, liver; 6, lung; 7, heart; 8, thymus; 9, brain stem; 10, cerebellum; 11, cerebral cortex; Lanes 12-19, cultured cell lines: 12, ONS76 (human medulloblastoma); 13, NB1 (human neuroblastoma); 14, T98G (human glioblastoma); 15, PC12 (rat pheochromocytoma); 16, N18 (mouse neuroblastoma); 17, B104 (mouse neuroblastoma); 18, NIH3T3 (mouse fibroblast); 19, C6 (rat glioma). (B) Immunoblot analysis of the Doc2β protein. Homogenates of the indicated mouse tissues (40 μg/lane) were analyzed by immunoblotting using affinity-purified antibodies and ECL detection as described under "EXPERIMENTAL PROCEDURES." 1, ovary; 2, testis; 3, kidney; 4, lung; 5, heart; 6, liver; 7, spleen; 8, cerebellum; 9, whole brain. The arrow indicates the Doc2β protein band. The bands indicated by an asterisk or arrowheads are thought to be that of rabphilin 3A or non-specific bands, respectively. This was confirmed by the absorption of the anti GST-C2A antibody by the recombinant GST-rabphilin 3A C2A fusion protein or GST-C2A (data not shown). Numbers on the right indicate the molecular weights of the size markers.

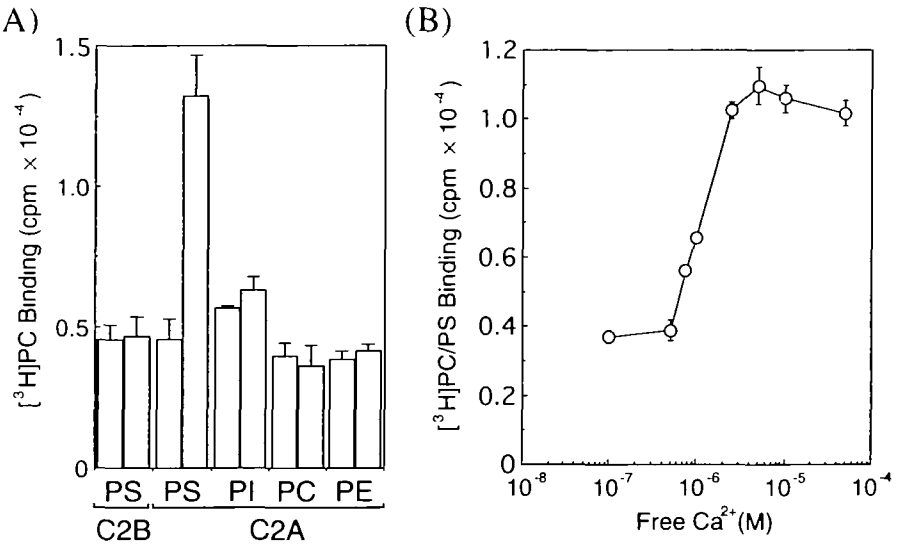

Fig. 4. **Phospholipid binding to the C2A domain and the C2B domain of Doc2β.** GST-C2A or GST-C2B was attached to glutathione-Sepharose beads and then incubated with ^{3}H-labeled liposomes in the buffers containing the indicated concentrations of free Ca^{2+}. The beads were washed and bound radioactivity was counted. Error bars indicate the standard deviation for triplicate determinations. (A) Phospholipid dependence of Ca^{2+}-dependent liposome binding by GST-C2A and GST-C2B. GST-C2A or GST-C2B was incubated in the absence (2 mM EGTA; open bars) or presence (10 μM; shaded bars) of Ca^{2+}. The liposomes used were composed of only PC, or of PC mixed with PE, PS or PI. For GST-C2B, only PS/PC liposomes were used. (B) Ca^{2+} concentration dependence of phospholipid binding to GST-C2A. The binding of ^{3}H-labeled PS/PC liposomes to GST-C2A was studied as a function of the Ca^{2+} concentration. The experiment shown is representative of three independent experiments, half-maximal binding being observed at 1.0 μM with a Hill coefficient of 3.3.

C2-domain proteins have been shown to be Ca^{2+}/phospholipid binding ones [synaptotagmins (I-VII), rabphilin 3A, and Doc2α] (*8-11, 16, 18, 30*). Four aspartyl residues are implicated in the Ca^{2+}-binding of synaptotagmin I C2A (*31*); these residues are highly conserved in proteins with C2A domains and in Doc2β (asterisks in Fig. 2A). To determine whether or not Doc2β is also a Ca^{2+}/phospholipid binding protein, binding measurements were carried out using GST-C2A or GST-C2B. The incubation of either GST-C2A or GST-C2B conjugated glutathione-Sepharose with ^{3}H-labeled PS/PC liposomes resulted in Ca^{2+}-dependent binding activity only toward GST-C2A (Fig. 4A). Liposomes consisting of only PC, or of PE/PC or PI/PC (1 : 2.5, w/w) did not bind to GST-C2A in a Ca^{2+}-dependent manner.

We have determined the Ca^{2+} concentration range in which phospholipid binding to the C2A domain of Doc2β is concentration-dependent (Fig. 4B). In three independent experiments, using PS/PC liposomes (1 : 2.5, w/w), half-maximal binding at approximately 1 μM free Ca^{2+} and a Hill coefficient of approximately 3 were observed. These values are comparable to those obtained for the C2A domains of synaptotagmins (*8, 9, 30, 32*), suggesting that the C2A domains of Doc2β and synaptotagmins have similar physiological roles. Since Doc2β is expressed ubiquitously, it is expected to function in constitutive vesicle transport pathways. Ca^{2+} is involved in the transport of protein between the ER and the Golgi apparatus in mammalian cells (*33*). The optimal free Ca^{2+} concentration required for transport was reported to be from 0.01 to 0.1 μM. This range corresponds well with the EC_{50} values we obtained on Ca^{2+}/phospholipid binding measurements of Doc2β. Thus, Doc2β may function as a Ca^{2+} sensor in protein transport.

We have previously shown that the C2B domains of synaptotagmins bind to inositol high polyphosphate series [IHPS; inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,3,4,5,6-pentakisphosphate, and inositol 1,2,3,4,5,6-hexakisphosphate], and that this binding inhibited the vesicular fusion process required for neurotransmitter release (*11, 34, 35*). However, like that of rabphilin 3A, the C2B domain of Doc2β did not bind IP_4 (*11* and data not shown), indicating that the Doc2β function is not regulated by IHPS.

In this study, we cloned a cDNA encoding the mouse Doc2β protein and have shown its ubiquitous expression. The C2A domain of Doc2β is a Ca^{2+}/phospholipid binding domain having similar characteristics to those of synaptotagmins; however, the C2B domain does not serve as an IHPS-binding domain. Our results suggest that Doc2β serves as a Ca^{2+} sensor which is involved in vesicle transport pathways.

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REFERENCES

1. Bennett, M.K. and Scheller, R.H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA* **90**, 2559-2563
2. Ferro-Novik, S. and Jahn, R. (1994) Vesicle fusion from yeast to man. *Nature* **370**, 191-193
3. Söllner, T. and Rothman, J.E. (1994) Neurotransmission: Harnessing fusion machinery at the synapse. *Trends Neurosci.* **17**, 344-347
4. Bennett, M.K., García-Arrarás, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D., and Scheller, R.H. (1993) The syntaxin family of vesicular transport receptors. *Cell* **74**, 863-873
5. Elferink, L.A., Trimble, W.S., and Scheller, R.H. (1989) Two vesicle-associated membrane protein genes are differentially expressed in the rat central nervous system. *J. Biol. Chem.* **264**, 11061-11064
6. McMahon, H.T., Ushkaryov, Y.A., Edelmann, L., Link, E., Binz, T., Niemann, H., Jahn, R., and Südhof, T.C. (1993) Cellubrevin is a ubiquitous tetanus-toxin substrate homologous to a putative synaptic vesicle fusion protein. *Nature* **364**, 346-349
7. Perin, M.S., Fried, V.A., Mignery, G.A., Jahn, R., and Südhof, T.

C. (1990) Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. *Nature* **345**, 260-263
8. 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* **375**, 594-599
9. 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
10. Chapman, E.R. and Jahn, R. (1994) Calcium-dependent interaction of the cytoplasmic region of synaptotagmin with membranes: Autonomous function of a single C_2-homologous domain. *J. Biol. Chem.* **269**, 5735- 5741
11. 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
12. 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. USA* **92**, 10703-10707
13. Mochida, S., Fukuda, M., Niinobe, M., Kobayashi, H., and Mikoshiba, K. (1995) Binding of inositol polyphosphate to synaptotagmin block neurotransmitter secretion at synapses formed in culture. in *Satellite Symposium 15th ISN Meeting, June 27-30, Hamamatsu, Japan*, p. 124 (abstr.)
14. Ohara-Imaizumi, M., Fukuda, M., Niinobe, M., Misonou, H., Ikeda, K., Kumakura, K., and Mikoshiba, K. (1995) Distinct roles of C2A and C2B domains of synaptotagmin in the regulation of exocytosis in adrenal chromaffin cells in *Satellite Symposium 15th ISN Meeting, June 27-30, Hamamatsu, Japan*, pp. 125-126 (abstr.)
15. 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
16. 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 C_2-like domains. *Biochem. Biophys. Res. Commun.* **206**, 439-448
17. Shirataki, H., Yamamoto, T., Hagi, S., Miura, H., Oishi, H., Jin-no, Y., Senbonmatsu, T., and Takai, Y. (1994) Rabphilin-3A is associated with synaptic vesicles through a vesicle protein in a manner independent of rab3A. *J. Biol. Chem.* **269**, 32717-32720
18. 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
19. Burns, M.E., Fukui, K., Sasaki, T., Takai, Y., and Augustine, G.J. (1995) Rabphilin3A regulates neurotransmitter release at the squid giant synapse. *Soc. Neurosci.* **21**, p. 326 (abstr.)
20. Chung, S.-H., Takai, Y., and Holz, R.W. (1995) Evidence that the rab3a-binding protein, rabphilin3a, enhances regulated secretion: Studies in adrenal chromaffin cells. *J. Biol. Chem.* **270**, 16714-16718
21. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996) Doc2 enhances Ca^{2+}-dependent exocytosis from PC12 cells. *J. Biol. Chem.* **271**, 7257-7260
22. 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
23. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
24. Rose-John, S., Dietrich, A., and Marks, F. (1988) Molecular cloning of mouse protein kinase C (PKC) cDNA from Swiss 3T3 fibroblasts. *Gene* **74**, 465-471
25. Geppert, M., Archer, B.T., III, and Südhof, T.C. (1991) Synaptotagmin II: A novel differentially distributed form of synaptotagmin. *J. Biol. Chem.* **266**, 13548-13552
26. Wendland, B., Miller, K.G., Schilling, J., and Scheller, R.H. (1991) Differential expression of the p65 gene family. *Neuron* **6**, 993-1007
27. Perin, M.S., Johnston, P.A., Özcelik, T., Jahn, R., Francke, U., and Südhof, T.C. (1991) Structural and functional conservation of synaptotagmin (p65) in *Drosophila* and humans. *J. Biol. Chem.* **266**, 615-622
28. Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P_{400}. *Nature* **342**, 32-38
29. Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157**, 105-132
30. 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
31. 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
32. Li, C., Davletov, B.A., and Südhof, T.C. (1995) Distinct Ca^{2+} and Sr^{2+} binding properties of synaptotagmins: Definition of candidate Ca^{2+} sensors for the fast and slow components of neurotransmitter release. *J. Biol. Chem.* **270**, 24898-24902
33. Beckers, C.J.M. and Balch, W.E. (1989) Calcium and GTP: Essential components in vesicular trafficking between the endoplasmic reticulum and Golgi apparatus. *J. Cell Biol.* **108**, 1245-1256
34. 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
35. Fukuda, M., Moreira, J.E., Lewis, F.M.T., Sugimori, M., Niinobe, M., Mikoshiba, K., and Llinás, R. (1995) Role of the C2B domain of synaptotagmin in vesicular release and recycling as determined by specific antibody injection into the squid giant synapse preterminal. *Proc. Natl. Acad. Sci. USA* **92**, 10708-10712
36. Kozak, M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12**, 857-872