The C2A Domain of Double C2 Protein γ Contains a Functional Nuclear Localization Signal*

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The C2 domain was originally defined as a homologous domain to the C2 regulatory region of Ca2+-dependent protein kinase C and has been identified in more than 50 different signaling molecules. The original C2 domain of protein kinase Cα functions as a Ca2+-binding module, and the Ca2+-binding to the C2 domain allows translocation of proteins to phospholipid membranes. By contrast, however, some C2 domains do not exhibit Ca2+-binding activity because of amino acid substitutions at Ca2+-binding sites, and their physiological meanings remain largely unknown. In this study, we discovered an unexpected function of the Ca2+-independent C2 domain of Doc2γ in nuclear localization. Deletion and mutation analyses revealed that the putative Ca2+-independent C2 domain of Doc2γ contains six Arg residues and that this basic cluster is both necessary and sufficient for nuclear localization (30, 31). Unlike other members of the Doc2 family, the C2A domain of Doc2γ lacks Ca2+-dependent phospholipid binding activity, probably because of the amino acid substitutions of the key amino acids (Glu or Asp) responsible for Ca2+-binding (30, 32–34). Interestingly, six Arg residues are clustered at one of the putative Ca2+-binding loops in the Doc2γ C2A domain (see Fig. 3, #). Our deletion and mutation analyses indicate that these basic residues are essential for nuclear localization of Doc2γ instead of Ca2+-binding.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pEF-T7- Doc2γ, -Doc2γ-A2C2AB (amino acid residues 1–80), -Doc2γ-A2C2B (amino acid residues 1–217), -Doc2γ-A2C2AB (amino acid residues 80–217), Doc2γ-A2B (amino acid residues 234–358), and Doc2γ-A2B (amino acid residues 1–418) (32) were constructed by polymerase chain reaction (PCR) using the following sets of primers with appropriate restriction enzyme sites (underlined) and/or termination codons (bold letters), as described previously (19, 35): 5′-GGATCCATGACCCTCCGGCGGCGGGG-3′ (Met primer; sense), 5′-GGATCCATGACCCTCCGGCGGCGGGG-3′ (Met primer; antisense), 5′-GGGTGATGCATCCGAGACGAGCGGCGGCGGGG-3′ (C2B upper primer; sense), 5′-GGGTGATGCATCCGAGACGAGCGGCGGCGGGG-3′ (C2B upper primer; antisense), 5′-GGGTGATGCATCCGAGACGAGCGGCGGCGGGG-3′ (C2B lower primer; sense), and 5′-GGGTGATGCATCCGAGACGAGCGGCGGCGGGG-3′ (C2B lower primer; antisense).

Briefly, purified PCR products digested with BamHI and SspI were cloned into the BamHI/SspI site of a modified pEF-BOS vector with a T7 tag (19, 35, 36) and verified by DNA sequencing with a Hitachi SQ-5500 DNA sequencer. Plasmid DNA was prepared by using Waza-
Site-directed Mutagenesis of Doc2—A mutant Doc2γAR (deletion of amino acids 180–183 (four Arg residues) in the C2A domain) was essentially produced by means of two-step PCR techniques, as described previously (21), using the following pairs of oligonucleotides: Met primer and 5′-GGGGGGCCCCCGCAGCCGTGAGTCCTC-3′ (AR-5′ primer; sense) and C1 primer (right half); and 5′-GGGGGGCCCCCGCAGCCGTGACTCATCACACACGG-3′ (AR-3′ primer; antisense) (left half), and 5′-GGGGGGCCCCCGCAGCCGTGACTCATCACACACGG-3′ (AR-3′ primer; sense) and C1 primer (right half). Briefly, the right and left halves were separately amplified by using pGEM-T-Doc2 (30) as a template, and the two resulting PCR fragments were digested with ApaI (underlined above), ligated to each other, and reamplified with the Met and C1 primers. The PCR fragment obtained that encoded the mutant Doc2γAR was digested with BamHI/SpeI, inserted into the BamHI/SpeI site of the pEF-T7 tag vector (19, 35), and verified by DNA sequencing. A mutant Doc2β(R6) was similarly constructed by using the following mutagenic oligonucleotides: 5′-GGGCCCCGAGCCGCGCCGGCCGCGGCTGACTCATACACACCGGAGAT-3′ (Doc2β(R6)-3′ primer; antisense) and 5′-GGGGGGCCCCCGCAGCCGTGACTCATACACACCGGAGAT-3′ (Doc2β(R6)-5′ primer; sense) and C1 primer (right half).

Cell Culture, Transfections, and Immunocytochemistry—Transfection of pEF-T7-Doc2 into PC12 cells (0.5–1 × 10^6 cells, the day before transfection/35-mm dish; MatTek Corp., Ashland, MA) or into COS-7 cells (5 × 10^5 cells, the day before transfection/10-cm dish) was performed as described previously (19, 35, 37). After washing twice with phosphate-buffered saline, the PC12 cells were fixed, incubated with anti-T7 tag monoclonal antibody (1/5000 dilution; Novagen, Madison, WI) and anti-p300 rabbit polyclonal antibody (1/5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and then visualized with anti-mouse Alexa 488 and anti-rabbit Alexa 568 antibodies (1/5000 dilution; Molecular Probes, Eugene, OR), respectively. In some cases, Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was added after immunostaining with anti-T7 tag antibody. Immunoreactivity was analyzed with a Fluorescence microscope (TE2000 Nikon, Tokyo, Japan) attached to a laser confocal scanner unit CSU 10 (Yokogawa Electric Corp., Tokyo, Japan) and HiSCA CCD camera (C6790; Hamamatsu Photonics, Hamamatsu, Japan). Images were pseudo-colored and superimposed with Adobe Photoshop software (Version 4.0).

Phospholipid Binding Assay—Glutathione S-transferase (GST) fusion proteins were expressed and purified on glutathione-Sepharose

RESULTS AND DISCUSSION

Nuclear Localization of Doc2γ Proteins in PC12 Cells—The Doc2 family consists of three isoforms (α, β, and γ) in rats and mice (30, 32, 34, 39) and shares a highly conserved amino-
terminal Munc13–1 interacting domain (Mid domain; amino acid residues 13–37 of Doc2α) (40) and two C2 domains at the carboxyl terminus (the C2A domain and the C2B domain) (Fig. 1A). Although this carboxyl-terminal tandem C2 domain structure is also found in the synaptotagmin family and rabphilin-3A, the Doc2 family is distinguished from other tandem C2 protein families by the presence of two C2 domains of the mouse C-type tandem C2 protein family. The number signs (#) indicate the basic (six Arg) residues that are only conserved in the C2A domain of Doc2. The location of the β-strands is indicated by arrows (44, 48). Amino acid numbers are indicated on the right. The amino acid sequences of the mouse C-type tandem C2 proteins were from Syt I, Syt II, and rabphilin-3A (12), Syts III and IV (21), Syt V (32), Syt VI (35), Syt VII (40), Syt IX (39), Syt X (38), Syt XI (36), Syt XII (37), Sytx V-XI (35), Sytx XIII (29), granuphilin-a (46), Doc2α (53), Doc2β (32), Doc2γ (30), Slp1–3 (45), and Syt XII.2

with p300 transcription factor and DAPI (Fig. 1B, top panels, and data not shown). The Doc2γ proteins seemed to be uniformly present throughout the nucleoplasm. By contrast, Doc2β proteins are mainly present in the cytosol, the same as Doc2α proteins (Fig. 1B, bottom panels) (42).

Mapping of the Domain Responsible for the Nuclear Localization of Doc2γ Proteins—To determine which domain is essential for the nuclear localization of Doc2γ, we produced four deletion mutants, each of which involves a different domain of Doc2γ (Doc2γΔC2AB, Doc2γΔC2B, Doc2γ–C2A, and Doc2γ–C2B; see Fig. 2A). First, we checked the size of the mutants by immunoblotting and confirmed that they were expressed correctly, with no degradation (Fig. 2B). Each deletion mutant was then expressed in PC12 cells, and its subcellular localization

FIG. 3. Alignment of the putative Ca2+ binding loop 3 of the two C2 domains of the mouse C-type tandem C2 proteins. * Asterisks indicate the conserved Asp or Glu residues, which may be crucial for Ca2+ binding by analogy with the Syt I-C2A domain (44, 48). The number signs (#) indicate the basic six Arg residues that are only conserved in the C2A domain of Doc2γ. The location of the β-strands is indicated by arrows (44, 48). Amino acid numbers are indicated on the right. The amino acid sequences of the mouse C-type tandem C2 proteins were from Syt I, Syt II, and rabphilin-3A (12), Syts III and IV (21), Syt V (32), Syt VI (35), Syt VII (40), Syt IX (39), Syt X (38), Syt XI (36), Syt XII (37), Sytx V-XI (35), Sytx XIII (29), granuphilin-a (46), Doc2α (53), Doc2β (32), Doc2γ (30), Slp1–3 (45), and Syt XII.2

with p300 transcription factor and DAPI (Fig. 1B, top panels, and data not shown). The Doc2γ proteins seemed to be uniformly present throughout the nucleoplasm. By contrast, Doc2β proteins are mainly present in the cytosol, the same as Doc2α proteins (Fig. 1B, bottom panels) (42).

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FIG. 4. A basic cluster in the Doc2γ C2A domain is essential for nuclear localization signal. A, schematic representation of Doc2γ deletion mutants (Doc2γΔR6) and chimera between Doc2β and Doc2γ (Doc2γΔR6) (B). Doc2γΔR lacks four Arg residues (dashes) between the β6 and β7 strands of the C2A domain. Doc2γΔR6 contains basic residues of Doc2γ (SRLRRRGGP, underlined) between the β6 and β7 strands of the C2A domain. B, Ca2+-dependent phospholipid binding properties of the Doc2γ–C2A domain. PS/PC 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 Ca2+. After centrifugation at 12,000 × g for 10 min, the supernatants and pellets were subjected to 10% SDS-polyacrylamide gel electrophoresis and then stained with Coomassie Brilliant Blue R-250. Note that GST-Doc2γΔR6–C2A completely lost phospholipid binding activity. The results shown are representative of three independent experiments. C, subcellular localization of T7-Doc2γ–A and T7-Doc2γ–R6. PC12 cells expressing pEF′-T7-Doc2γ and Doc2γ mutants were fixed, permeabilized, and co-stained with anti-T7 tag antibody (green in left panels) and anti-p300 (red in middle panels) as described under “Experimental Procedures.” The right panels represent an overlay (in yellow) of left and middle panels. Note that the insertion of SRLRRRGGP sequence into Doc2γ is sufficient for nuclear localization. Scale bar indicates 10 μm.

A Novel Function of the Doc2γ C2A Domain
was determined by immunocytochemistry, as described above (Fig. 2C). Interestingly, both the Doc2γΔC2B and Doc2γ-C2A proteins showed nuclear localization in PC12 cells, whereas the amino-terminal Mid domain was localized in the cytosol, and the Doc2γ-C2B protein was localized in both the nucleus and the cytosol. We therefore concluded that only the C2A domain contains a functional nuclear localization signal.

The Doc2γ C2A Domain Contains a Functional Nuclear Localization Signal—Various nuclear localization signals have been determined in many proteins localized in nucleus, and they have often consisted of clusters of basic residues (Arg and Lys; reviewed in Ref. 43). Consistent with this, we found that the Doc2γ-C2A domain contains a cluster of basic residues (RLRRRRRRRR) in the putative Ca2⁺ binding loop 3, between the β6 and β7 strands (Fig. 3, #) (44). Interestingly, the loop 3 domain of the Doc2γ-C2A domain is three amino acids longer than in other carboxy-terminal type (C-type) tandem C2 protein families, including Syts I-IX (9, 29), Sp1–3 (synaptotagmin-like protein) (45), granophilin-α (46), rabphilin-3A (47), and other members of the Doc2 family (31). It is also noteworthy that other C-type tandem C2 domains do not contain an Arg cluster at this position (Fig. 3). Consistent with this, there have been no reports of tandem C2 proteins that specifically localized in nucleus. Although three Asp residues between the β6 and β7 strands in the C2A domain of Syt I (asterisks in Fig. 3) are known to bind Ca2⁺ ions (48), the C2A domain of Doc2γ lacks two Asp residues (Ser-176 and Pro-185), and because of these amino acid substitutions, the Doc2γ-C2A domain does not display any clear Ca2⁺-dependent phospholipid (PS/PC liposome) binding activity (Fig. 4B) (30).

To determine whether the basic cluster of the Doc2γ C2A domain is the sole nuclear localization signal of this protein, we produced a deletion mutant lacking four of six Arg residues (named Doc2γAR; see Fig. 4A). As expected, the Doc2γAR proteins were mainly localized in the cytosol of PC12 cells and mostly absent in the nucleus (Fig. 4C, top panels). Finally, we investigated whether the basic cluster alone of Doc2γ is a sufficient nuclear localization signal by producing chimera proteins between Doc2β and Doc2γ in which the loop 3 domain of Doc2β was replaced by that of Doc2γ (named Doc2β(R6); see Fig. 4A). As a result of this substitution, the Doc2β(R6) C2A domain completely lost its Ca2⁺-dependent phospholipid binding activity (Fig. 4B), whereas the Doc2β(R6) proteins acquired the ability to localize in the nucleus of PC12 cells (Fig. 4C, bottom panels). These findings indicate that the basic cluster of Doc2γ is both necessary and sufficient for nuclear localization of Doc2γ protein.

Conclusions—This study revealed the novel function of the Ca2⁺-independent type of the Doc2γ C2A domain in nuclear localization. It is noteworthy that the basic cluster (RLRRRRRRRR) is present in the putative Ca2⁺ binding loop 3, which is located at the apex of β-sandwich structure of the Doc2γ C2A domain (i.e. loop 3 functions as a nuclear localization signal rather than a Ca2⁺-binding site). Thus, the function of the loop domains of the C2 domain is more diversified than we expected. The function of Doc2γ in the nucleus remains unclear, but because Doc2γ is involved in secretory vesicle exocytosis (42, 49, 50), and vesicle traffic is thought to be regulated by a conserved protein family, such as SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, C-type tandem C2 protein families, and rab family (51, 52), Doc2γ might be involved in nuclear envelope assembly. As far as we know, Doc2γ is the only isoform of the C-type tandem C2 protein family that is localized in the nucleus. Further work is necessary to elucidate whether Doc2γ regulates nuclear envelope assembly.

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