DOC2 Proteins in Rat Brain: Complementary Distribution and Proposed Function as Vesicular Adapter Proteins in Early Stages of Secretion

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Summary

DOC2 proteins constitute a novel protein family that may function in secretion and contain a double C2 domain. We have cloned and characterized two DOC2 isoforms in rat brain and studied their interactions with other proteins implicated in secretion. DOC2A was virtually brain specific, DOC2B ubiquitous. Within brain, the isoforms were expressed nonuniformly and complementary within neurons, not astroglia, and co-purified with synaptic vesicles. Affinity purification, yeast two-hybrid analysis, and coimmunoprecipitation revealed that DOC2 binds munc18, a protein also implicated in secretion. The first DOC2 C2 domain and most of munc18 are involved in direct interactions. Munc18 may regulate formation of 'core complexes' during vesicle docking, by interacting with syntaxin. We show that DOC2 and syntaxin compete for munc18. Other core complex components shifted the equilibrium between syntaxin-munc18 versus DOC2-munc18. These data suggest that DOC2 proteins are vesicular adapter proteins regulating munc18-syntaxin complexes and herewith synaptic vesicle docking.

Introduction

The secretion of neurotransmitters from nerve terminals in the brain appears to be regulated by several families of Ca^{2+}-binding proteins. Several of these families are characterized by two copies of a Ca^{2+}-binding domain, called C2 domain, first characterized as a conserved sequence in protein kinase C (Nishizuka 1988). This double C2 domain is usually found within the carboxy terminus of these secretory proteins. Outside this domain, the different families diverge to yield transmembrane proteins (synaptotagmins) or membrane-associated proteins (rabphilin3A, munc13s) (for alignments, see Brose et al., 1995). The crucial role of synaptotagmin1 in the Ca^{2+}-dependent triggering of secretion has been characterized in detail (Geppert et al., 1994), but the exact role of the other proteins in secretion has remained elusive.

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Recently, a new family of double C2 domain proteins were cloned from human MOLT4 cells (Orita et al., 1995; Sakaguchi et al., 1996) and named DOC2 (for double C2 domain). The present studies aim to gain insight into the function of DOC2 proteins. Since most studies of mammalian synaptic transmission are performed in rodents, we have cloned and characterized DOC2 proteins in rat. This allows a variety of biochemical studies to relate DOC2 proteins to known protein complexes, cellular processes, and subcellular locations within nerve terminals in order to understand their precise function. We have performed systematic studies of DOC2 participation in protein–protein interactions in docking–fusion of synaptic vesicles, which were previously described in rat brain. We propose here a model for the cellular functions of DOC2 by demonstrating, by three independent methods, their interaction with another protein involved in regulated secretion, namely munc18.

Results

Structure and Evolutionary Conservation of DOC2-Related Proteins

We have characterized rat DOC2 isoforms by isolating clones from a rat brain cDNA library. More than 40 clones were isolated and characterized; 16 overlapping clones were sequenced to obtain the nucleotide sequences of two rat DOC2 isoforms. Figure 1 shows the deduced amino acid sequences and alignments to the human isoforms and rabphilin3A isoforms in different species. The characteristics of these two rat sequences and their alignment with published sequences allowed formulation of a three domain model for DOC2 proteins. The amino terminal domain is a nonpolar, alanine, proline-rich domain that is highly conserved among rat and human isoforms (conserved area is shaded in Figure 1). This domain is unique to DOC2 proteins, i.e., no significant homology was found with domains in any other protein in the databases. The two carboxy terminal domains are repeats of a C2 domain, first characterized in protein kinase C and also found in other presynaptic proteins, such as synaptotagmins, rabphilin3A, and munc13s (for alignments, see Brose et al., 1995). The available structural information from synaptotagmin1 first C2 domain (Sutton et al., 1995) and the high homology of DOC2 proteins within this domain suggest that DOC2 C2 domains are also Ca^{2+}-binding domains (see also Orita et al., 1995; Südhof and Rizo, 1996). In between these three domains, DOC2 proteins contained spacer sequences with low homologies and different sizes among isoforms.

Homology analysis of the deduced amino acid sequences with other presynaptic proteins that contain the C2 domain repeat indicated that rabphilin3A is most closely related to the DOC2 protein family. The homologies among the first and second C2 domains suggest that the duplication of this domain occurred earlier in evolution than the branch between rabphilin3A and DOC2 proteins. Because rab proteins are considered to
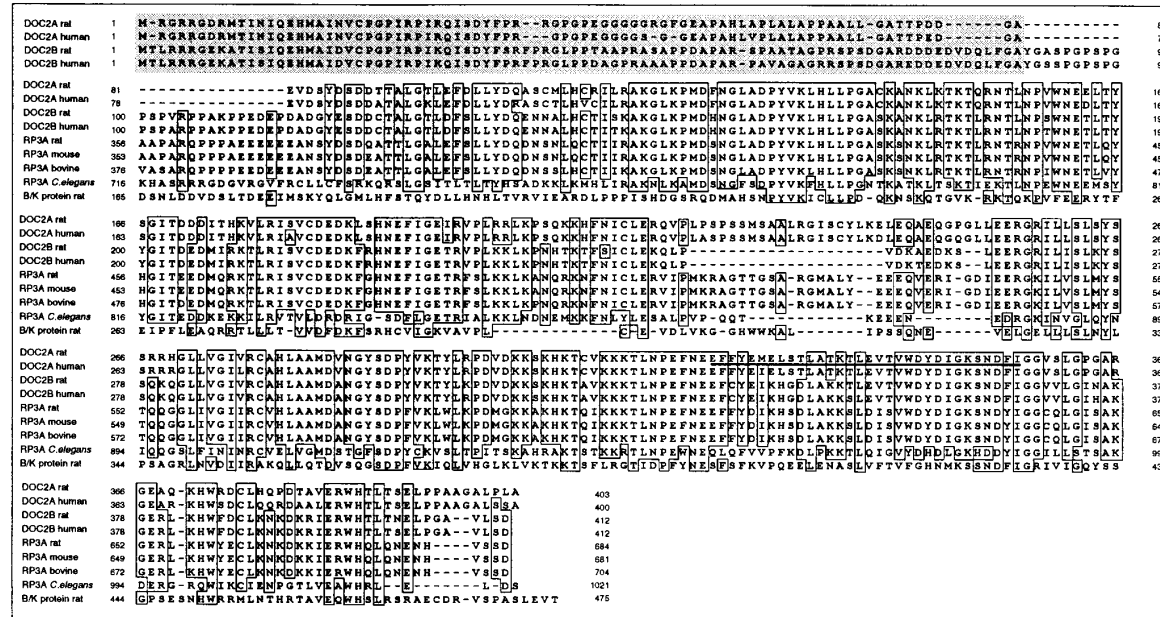
Figure 1. Structure and Evolutionary Conservation of DOC2 Proteins and Other Double C2 Domain–Containing Proteins

Alignment of rat and human DOC2 proteins and the most closely related structures, i.e., the carboxy termini of rabphilin3A (RP3A) from different species and rat B–K protein. Residue numbers are indicated in both margins. Conserved residues are indicated by boxes (>60% identity). Sequences are shown in single letter amino acid code with hyphens indicating gaps. The deduced amino acid sequence of the original genomic clone of rat DOC2A is indicated by a horizontal line. The dashed area indicates the conserved amino terminal domain of DOC2 proteins. The rat DOC2 sequences were determined from the nucleotide sequences of multiple overlapping cDNA clones. Human DOC2A was taken from Orita et al., 1995; human DOC2B from Sakaguchi et al., 1996; rabphilin3A isoforms from Li et al., 1994 (rat) Inagaki et al., 1994 (mouse); Shirataki et al., 1993 (bovine); Fulton and Waterston, GenBank U00032 (*C. elegans*); and B–K protein from Kwon et al., 1996.

add specificity and increase efficacy of the interactions between proteins, the amino terminal rab-binding domain in rabphilin3A may represent a evolutionary adaptation of the function of DOC2–rabphilin ancestors.

Complementary Expression of DOC2 Proteins in Rat Brain

The distribution of DOC2 proteins was studied by Northern blot analysis, in situ hybridization, and subcellular fractionation. RNA blots of equal amounts of total mRNA from different rat tissues were hybridized with ^{32}P-labeled cDNA probes. DOC2A mRNA was highly enriched in brain (as previously demonstrated, Orita et al., 1995). We have also detected a low level of DOC2A expression in lung and kidney and higher expression in testis (data not shown). DOC2B was found to be expressed ubiquitously, with the highest expression in brain, heart, and lung. Notably, DOC2B was also highly expressed in adrenal (data not shown).

Since both DOC2 isoforms are expressed in brain, we performed in situ hybridization of rat brain sections to study their distribution within brain (Figure 2). Throughout the brain, expression of the two DOC2 isoforms was highly nonuniform and complementary. DOC2A was highly expressed in cortex and areas of the brain stem, whereas DOC2B expression was very low in these areas. Conversely, DOC2B was highly expressed in caudate putamen, limbic structures, and the cerebellum, where DOC2A expression was very low. Within the hippocampus, DOC2A was highly expressed only in the CA3–CA4

areas, whereas DOC2B was highly expressed only in CA1–CA2 areas. Both isoforms were found in the dentate gyrus. In addition, DOC2A was enriched in ventral hypothalamic nuclei, while DOC2B was enriched in amygdala and enthorhinal cortex. Rabphilin3A distribution was more homogenous throughout the brain, with detectable expression in all brain areas. Interestingly, a few specific locations showed no detectable rabphilin3A expression (such as the CA1–CA2 region of the hippocampus). Together, the expression patterns suggest that most neurons within the brain express rabphilin3A together with a single DOC2 isoform.

Subcellular Localization of DOC2 Proteins

To analyze the localization of DOC2 isoforms within neurons, subcellular fractions were isolated and analyzed by immunoblotting (Figure 3). DOC2 proteins appeared to be exclusively associated with membranes, as indicated by their absence in the supernatant (cytosolic) fractions S2 and LS2, in contrast to the cytosolic markers calbindin 28K and GDP-dissociation inhibitor. Neither isoform was detected in astroglia (Figure 3, right lane). In the case of DOC2B, this is striking, given its ubiquitous expression throughout the organism. Within neurons, both DOC2A and DOC2B copurified with synaptic vesicle markers, such as synaptotagmin and rabphilin3A in the LP2 fraction. In addition, DOC2 proteins were also found to a lesser extent in synaptosomal plasma membranes (SPM), i.e., copurified with SPM-marker dynamin I.
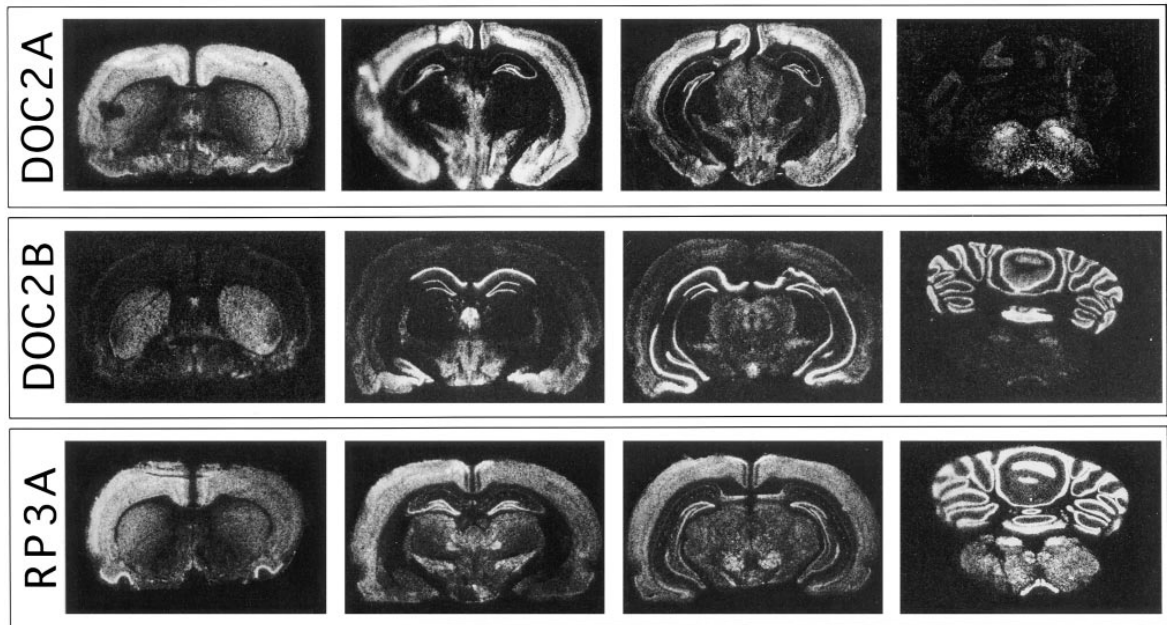
Figure 2. Differential Distribution of DOC2 Proteins and rabphilin3A (RP3A) in Rat Brain

Transversal sections of rat brain were hybridized with labeled probes and exposed to film (see Experimental Procedures for details).

DOC2 proteins appeared to be exclusively associated with membranes, but their sequence does not predict a hydrophobic segment (Figure 1). Therefore, we tested the association of DOC2 proteins with membranes with salt washes and detergent extraction. Native DOC2 proteins were dissociated effectively from SPM by washing
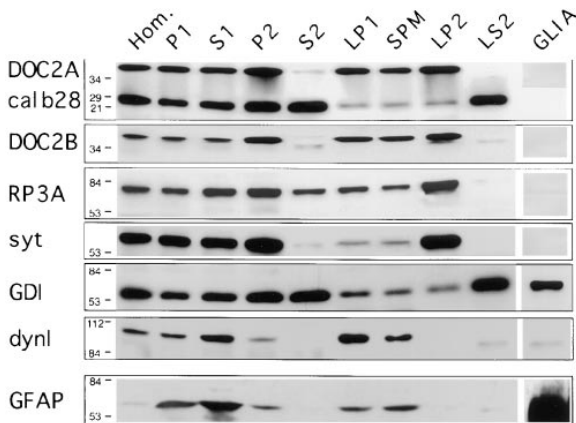

Figure 3. Distribution of DOC2 and Other Proteins in Subcellular Fractions of Total Rat Brain and Rat Astroglial Homogenates

Crude synaptosomes were sedimented from rat brain homogenate (Hom.) by differential centrifugation (P2), lysed hypo-osmotically, and separated into fractions enriched in heavy membranes (LP1), synaptosomal plasma membranes (SPM), synaptic vesicles (LP2), and in presynaptic cytosol (LS2). DOC2 purification was analyzed by SDS–PAGE and Western blotting and compared to markers for synaptic vesicles (RP3A, rabphilin3A; and syt, synaptotagmin), synaptosomal plasma membranes (dynl and dynaminl), cytosol (calb28, calbindin 28K; and GDI, GDP dissociation inhibitor), and astroglia (GFAP, glial fibrillary acidic protein). Numbers on the left indicate positions of molecular weight markers (in kilobases).

with 0.5 M NaCl, suggesting a weak, ion-sensitive interaction of DOC2 proteins with membranes, potentially through another, unidentified protein. Washing with 1–5 mM Ca^{2+} was ineffective in removing DOC2 proteins from membranes. The association of DOC2 proteins to membranes may also involve a posttranslational hydrophobic modification of the proteins. Both isoforms contain two cysteine residues in their amino terminus, which may serve as an acceptor for such modifications (Figure 1). To test this, SPM were extracted with Triton-X-114 (Bordier, 1981). In contrast to rab3A, which is known to contain a hydrophobic modification (see Li et al., 1994), native DOC2 proteins were exclusively extracted to the membrane fraction (data not shown), suggesting that such modifications are not responsible for the association of DOC2 proteins to membranes. To identify which domains of DOC2 proteins may be responsible for the association to membranes, small quantities of the amino terminal domain and both isolated C2 domains of DOC2A were translated–transcribed in vitro and mixed with SPM. All three domains were found largely in the membrane fraction, the C2 domains to a larger extent than the amino terminal domain (data not shown). This suggests that all three domains can in principle account for the association of DOC2 to membranes.

DOC2 Proteins Bind munc18

To gain insight into the cellular functions of DOC2 proteins, we screened for proteins interacting with DOC2 isoforms by three independent methods: affinity purification from brain using glutathione-S-transferase (GST) fusion proteins, the yeast two-hybrid system, and immunoprecipitation.
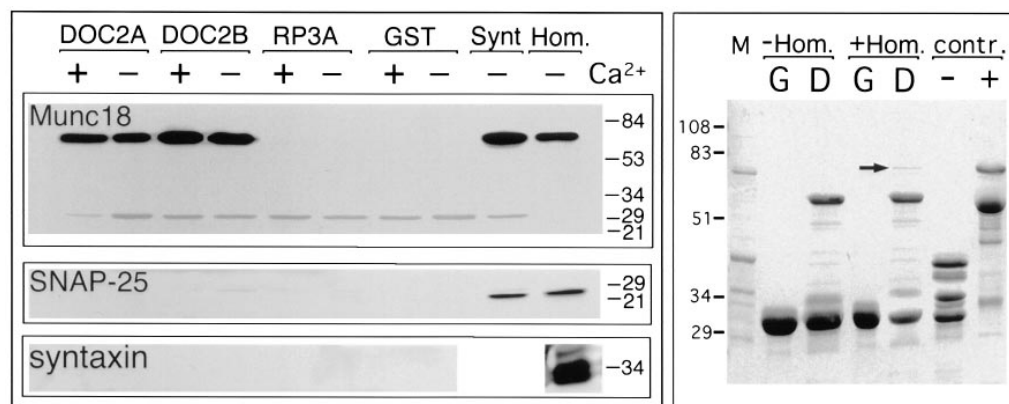
Figure 4. Affinity Purification of munc18 from Brain Using GST–DOC2 Fusion Proteins

DOC2 interacting proteins were identified by incubating GST–DOC2 fusion proteins (G-DOC2A-B and G-DOC2B-C) coupled to beads with solubilized rat brain proteins. Beads were washed extensively; sedimented proteins were separated by SDS–PAGE and subjected to silver staining (right panel) or identified by Western blotting (left panel) using munc18 antibodies (J370, Hata et al., 1993). Western blotting: negative controls, GST-control protein and GST-rabphilin3A (RP3A), do not purify munc18 from brain. Syntaxin1A (synt) was used as a positive control. The specificity of the antibody was confirmed by blotting total brain homogenate (Hom, 33 μg). GST–DOC2 proteins do not bind syntaxin or another syntaxin-interacting protein, SNAP25. A weak low molecular signal probably originates from cross-reactivity of the antibody with endogenous GST molecules purified on the beads. Silver staining: GST alone (G) or GST–DOC2B fusion protein (D) with and without rat brain homogenate (Hom.). Positive control: GST–syntaxin1A; negative control: GST coupled to the amino terminal of DOC2A. Position of munc18 is indicated by 5 μg 6HIS–Munc18 in the first lane (M). Both the 6HIS–Munc18 protein and the GST-DOC2A-N-terminal construct showed significant degradation (lower bands in lanes 1 and 6). Numbers in the margins indicate positions of molecular weight markers (in kilobases).

Different domains of both DOC2 isoforms were expressed as GST-fusion proteins in bacteria and incubated with solubilized rat brain homogenate (Figure 4). After extensive washing, putative interacting proteins were analyzed using silver staining and Western blotting, using an array of antibodies directed against approximately 30 proteins implicated in presynaptic functions. These analyses did not detect any of the known constituents of docking–fusion protein complexes, except for munc18 (Figure 4, left panel). Several of the DOC2 constructs were found to bind munc18-1, a protein known to form a stable dimer with syntaxin1 in vitro and therefore also implicated in neuronal secretion (Hata et al., 1993, also called rbSec1 or n-sec1; Garcia et al., 1994; Pevsner et al., 1994a). DOC2B constructs bound munc18-1 more effectively than DOC2A constructs; Ca^{2+} had little effect.

Within the C2 domain repeat, the identity between rabphilin3A and DOC2A is 61%, and between rabphilin3A and DOC2B, 73%. Despite this high homology, corresponding GST–rabphilin3A fusion proteins did not bind munc18, neither did GST control proteins. DOC2 fusion proteins did not bind syntaxin nor other known syntaxin-binding molecules, SNAP25 (Figure 4, left panel) and synaptobrevin–VAMP (not shown). Hence, DOC2 fusion proteins appear not to affinity purify munc18 from brain by sedimenting syntaxin-containing protein complexes but presumably directly through molecular interactions between DOC2 and munc18. A number of other GST-coupled proteins implicated in the docking–fusion complexes failed to bind munc18 or DOC2 proteins (synaptotagmin1, synapsin1, synaptophysin, synaptobrevin–VAMP, and SNAP25; data not shown). Accordingly, immunoprecipitations using specific antibodies against a number of secretory proteins (syntaxin, SNAP25, and rabphilin3A) did not coprecipitate DOC2 proteins, indicating that DOC2 proteins are absent from the docking–fusion protein complexes previously published (reviewed by Südhof, 1995). Conversely, GST–DOC2 fusion proteins did not bind such proteins (see above). Finally, the binding between DOC2 proteins and munc18 was not modulated by ATP, ATPγS, or GTPγS (data not shown). Using large excess of rat brain homogenate, DOC2-purified munc18 could also be visualized directly using silver staining (Figure 4, right panel). Coomassie–silver staining produced no evidence for additional proteins binding to GST–DOC2 fusion proteins.

Mapping of DOC2–munc18 Interacting Sequences

To map DOC2 domains involved in the interaction with munc18 and to test whether these proteins interact directly with each other, we performed a series of experiments using the in vitro transcription–translation of munc18 in rabbit reticulocyte lysate and several GST-fusion proteins of DOC2B. The GST–DOC2B fusion protein as used in Figure 4 expressed the two C2 domains, implicating this region in the interaction with munc18-1. Indeed, the double C2 domain of DOC2B bound munc18 in cell-free experiments (Figure 5), indicating that the two proteins interact directly. The isolated amino terminal domain of DOC2B did not bind munc18. Different deletions in the double C2 domain fusion proteins revealed that the truncated first C2 domain is sufficient for the interaction with munc18. The first 23 amino acids of the first C2 domain appeared not to be essential for the interaction with munc18 (Figure 5A). This is in contrast to interactions involving other double C2 domain proteins, i.e., synaptotagmin, where such deletions are believed to delete the first β strand and induce a loss of affinity (see Südhof and Rizo, 1996).

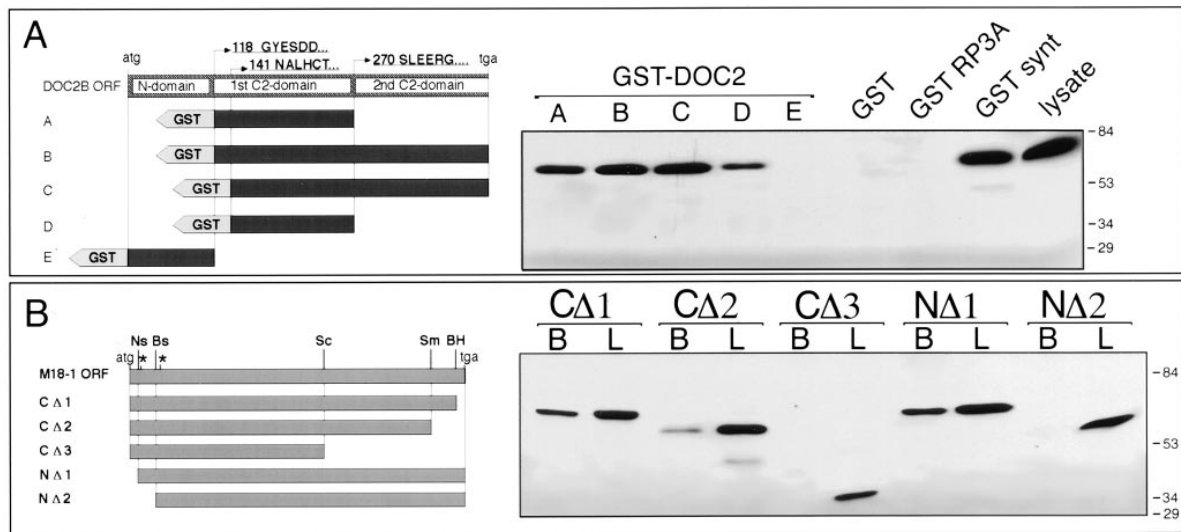In contrast to DOC2 proteins, munc18 does not exhibit

Figure 5. Domain Mapping of the DOC2–munc18 Interaction In Vitro

(A) Effects of DOC2 deletions on the interaction with ³⁵S methionine–labeled munc18 expressed in rabbit reticulocyte lysate. Deletion mutants of DOC2B (A–E) are schematically represented in the left panel. The sequences of the deletion constructs are indicated by the first residue number of the construct followed by the first amino acids of these constructs in single letter code.

(B) Effects of munc18 deletions on the interaction with DOC2. ³⁵S methionine–labeled munc18 deletion constructs were expressed in rabbit reticulocyte lysate (lanes denoted L), incubated with GST–DOC2B on beads, and washed extensively (lanes denoted B). Deletion mutants of munc18 are schematically represented in the left panel. Alternate transcription initiation sites (ATG) are indicated by the asterisks. Restriction sites exploited to obtain deletion constructs are indicated: Ns, NsiI; Bs, BspHI; Sc, SacI; Sm, SmaI; and BH, BamHI.

an obvious domain structure. Previous studies (Hata and Südhof, 1995) suggested that the complete molecule is necessary for the interaction with syntaxin1. In contrast, the interaction between munc18-1 and DOC2B was preserved after deletions of the munc18-1 protein, when GST–DOC2B fusion proteins were incubated with reticulocyte lysate in which munc18 deletion constructs were synthesized (Figure 5B). Small deletions of both the amino and the carboxy termini of munc18 still allowed the interaction with DOC2, whereas larger deletions did not. These data suggest that different domains of munc18-1 are involved in the interactions with syntaxin and DOC2.

Yeast Two Hybrid Analysis of the DOC2–munc18 Interaction

The yeast two-hybrid system (Fields and Song, 1989) provides an alternative, independent method to study protein–protein interactions and to test the validity of the biochemical data suggesting that DOC2 and munc18 interact. In addition, the yeast two-hybrid system provides a cellular context, i.e., mimics the in vivo situation. Yeast L40 strain was cotransfected with munc18 bait constructs and DOC2 prey constructs (pBTM116 and pVP16, respectively; Vojtek et al., 1993). After growing the transfectants for 3 days in restrictive media, the β-galactosidase activity was assayed (Figure 6). These data essentially confirmed the biochemical evidence that DOC2 and munc18 interact, independent of other presynaptic proteins. Also, the higher apparent affinity of the munc18–syntaxin interaction, compared to the munc18–DOC2 interaction, was reproduced in these experiments. We observed an apparent difference between the two DOC2 isoforms in the interaction with

munc18 carboxy terminal deletion constructs. Whereas DOC2B still interacted with the first two carboxy terminal deletions of munc18 (see also Figure 5), the interaction between DOC2A and munc18 was lost. In addition, we tested the affinity of the ubiquitous munc18 isoform, munc18-2, for DOC2 proteins. Cotransfection of munc18-2 and DOC2 vectors produced β galactosidase–positive clones. The β-galactosidase assay suggested that the apparent affinity of DOC2B for both munc18 isoforms is comparable. Furthermore, DOC2A appeared to bind only munc18-1 effectively in this assay and with a slightly lower apparent affinity than DOC2B. This observation is in line with the tissue distribution of both protein families, i.e., DOC2A and munc18-1 are essentially brain specific, whereas DOC2B and munc18-2 are ubiquitous.

Coimmunoprecipitation of munc18 with DOC2-Specific Antibodies

Interaction analysis using GST fusion proteins as well as the yeast two-hybrid system may benefit from artificially high concentrations of one or both proteins involved in an interaction. To confirm the validity of the DOC2–munc18 interaction using physiologically relevant concentrations of both proteins, we generated new DOC2 antibodies and performed coimmunoprecipitation from an isolated nerve terminal preparation (Verhage et al., 1991), where both proteins are naturally enriched (see also Figure 3). Figure 7A shows immunostaining of the DOC2 precipitates with munc18 antibody, confirming a specific interaction between DOC2 and munc18. Again, the interaction appeared to be Ca²⁺ independent. Immunostaining for syntaxin was negative in these precipitates (data not shown), confirming the observation that
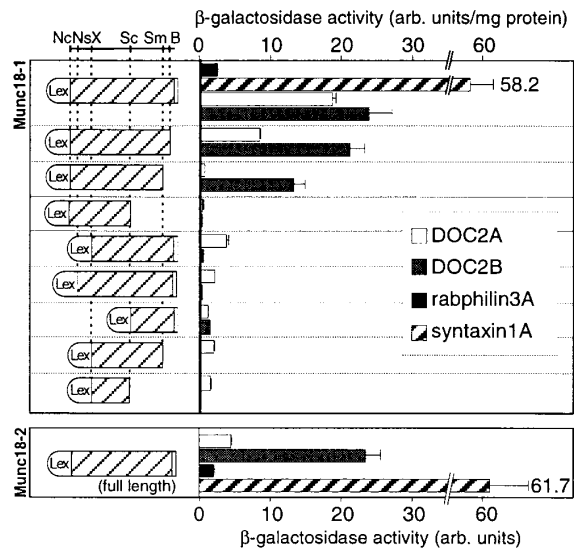
Figure 6. Analysis of the DOC2–munc18 Interaction Using the Yeast Two-Hybrid System

The double C2 domains of both DOC2 isoforms were cloned into the LexA-activating domain vectors and cotransfected with a number of munc18 sequences ligated in LexA-binding domain vectors. The inserted munc18 sequences are indicated in the left panel. The complete munc18-1 open reading frame is indicated on the top; Nc indicates an NcoI restriction site, Ns, NsiI; Sc, SacI; Sm, SmaI; and B, BamHI. Transfectants were allowed to grow in restrictive media for 72 hr, lysed, and analyzed for β-galactosidase activity. Enzyme activity was expressed in arbitrary units per mg protein +SEM of three independent transfections. Correct expression of noninteracting constructs in yeast was confirmed by immunoblotting yeast lysates from parallel cultures in complementing media using LexAb- and munc18-specific antibodies (data not shown).

syntaxin was not purified using GST–DOC2 affinity purifications of munc18 (Figure 4).

DOC2 and Syntaxin Compete for munc18 Binding

The observation that different domains of munc18 are involved in the interaction with syntaxin and with DOC2 (Figures 5 and 6) appears to conflict with the observation that DOC2 and syntaxin can bind munc18 from brain but do not cosediment each other (Figure 4), i.e., DOC2-munc18-syntaxin trimers were never observed. To investigate this issue further, we performed a series of competition experiments (Figure 7b). The original affinity purification of munc18 from brain using DOC2 fusion proteins (Figure 4) was now repeated in the presence of a 4-fold excess of exogenous, bacterially expressed proteins. Addition of excess syntaxin1A cytoplasmic domain prevented the affinity purification of munc18 on DOC2 beads. Hence, the munc18 pool within brain homogenate that was available for interaction with DOC2 could completely be absorbed by exogenous syntaxin, i.e., syntaxin and DOC2 compete for these munc18 molecules. Conversely, a 4-fold excess of 6His–DOC2B was unable to prevent munc18–syntaxin interactions (data not shown). Thus, the interaction between munc18 and syntaxin appeared to have a higher affinity than the interaction between munc18 and DOC2. The presence

of other exogenous proteins, such as SNAP25, which interacts with syntaxin, and, to a lesser extent, the double C2 domains of rabphilin3A, made the absorption of munc18 on exogenous syntaxin less efficient, in a way that DOC2 constructs could now again bind a significant amount of munc18. Hence, SNAP25 appeared to reduce the syntaxin–munc18 affinity and allowed munc18 to interact with DOC2 constructs.

Discussion

In this paper, we present the isolation and cloning of two DOC2 proteins from rat and describe their structure and their nonuniform and complementary distribution within brain and show their copurification with synaptic vesicle markers. We present clues to the cellular function of DOC2 proteins, i.e., using several independent approaches, we show that DOC2 proteins interact directly with munc18 and compete with syntaxin in binding to munc18. The interacting sequences within DOC2 and munc18 proteins were mapped.

DOC2 proteins are members of the C2 domain-containing protein family. Several members have a double C2 domain and appear to be involved in Ca^{2+}-dependent secretion in the brain (reviewed by Südhof and Rizo, 1996). Based on homology with crystallized C2 domains (Sutton et al., 1995), DOC2 proteins may also bind Ca^{2+} and phospholipids (Orita et al., 1995; Südhof and Rizo, 1996). However, we found no indication for a Ca^{2+}-dependent function. The interaction between DOC2 and munc18 is essentially Ca^{2+} independent, and no other Ca^{2+}-dependent interactions were observed with either affinity purifications or coimmunoprecipitations. Apparently, the binding of Ca^{2+} and munc18 to the first C2 domain of DOC2 represent two independent interactions. Similar Ca^{2+}-independent interactions were observed for synaptotagmin C2 domains. A number of different molecules interact with these domains without apparent relation to the binding of two Ca^{2+} ions to the same domain. Taking the available structural information into account, this has been explained by a proposed 'Janus-faced' structure of C2 domains (Südhof and Rizo, 1996). In this model, one side of the structure binds Ca^{2+} and takes part in Ca^{2+}-dependent interactions, whereas the other is involved in physiologically relevant interactions that are not Ca^{2+} regulated. Our findings may be explained by a similar model for DOC2 C2 domains.

In addition to the C2 domains, DOC2 proteins contain an amino terminal domain of high homology among DOC2 isoforms, but with no apparent homology to any other sequence in the data banks. We have not detected any interactions of the isolated amino terminal domains, expressed as fusion proteins. Their function remains to be resolved.

As previously reported (Orita et al., 1995; Sakaguchi et al., 1996), DOC2A was found almost exclusively in brain, whereas DOC2B was found in many tissues, including brain. For a number of protein families implicated in neuronal secretion, nonneuronal isoforms were cloned, including a nonneuronal isoform of munc18, munc18-2 (Hata and Südhof, 1995). These isoforms show low expression in brain and are considered to have
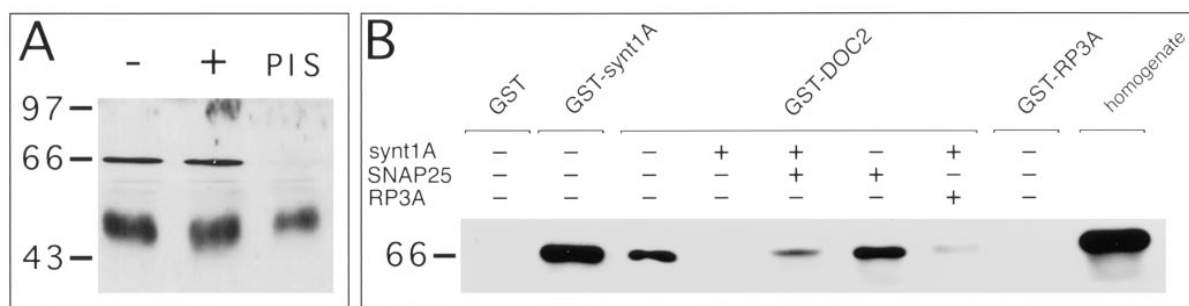
Figure 7.

(A) Coprecipitation of munc18 using DOC2-specific antibody P011 from synaptosomal lysate. The presence of Ca^{2+} during the incubation with primary antibody is indicated with plus and minus signs. Negative control was preimmune serum (right lane), indicated as PIS. Precipitates were blotted and immunostained for munc18 using munc18-specific antibody. The lower broad band (approximately 50 kDa) represents nonspecific staining of P011 immunoglobulin heavy chain.

(B) Syntaxin-interacting proteins shift the equilibrium between syntaxin–munc18 and DOC2–munc18 complexes in vitro. Affinity purification of munc18 from brain using GST–DOC2 beads was repeated in the presence of excess exogenous MBP–syntaxin1A cytoplasmic domain either alone or together with excess exogenous MBP–SNAP25 and MBP–rabphilin3A (double C2 domain).

a function devoted to nonsynaptic membrane traffic. However, DOC2B appears to differ from such isoforms. Within brain, DOC2B is highly expressed and complementary to DOC2A, suggesting that most neurons within the brain express either DOC2A or DOC2B. Furthermore, in adrenal, i.e., endocrine tissue with profound regulated secretion, DOC2B is the only isoform highly expressed. Hence, the two isoforms may play similar roles in different brain areas. In line with this, both DOC2 isoforms bind the brain-specific munc18 isoform, and only the ubiquitous DOC2 isoform binds the ubiquitous munc18 isoform effectively.

A systematic study of DOC2 interactions with other proteins implicated in secretion, using affinity purifications, the yeast two-hybrid system, and coimmunoprecipitations, produced no positive results, except the interaction with munc18. Given the suggested localization on synaptic vesicles, this was an unexpected finding. Other synaptic vesicle proteins appear to be involved in larger multimeric complexes (Bennett et al., 1992; Söllner et al., 1993a, 1993b; McMahon et al., 1995; Südhof and Rizo, 1996). These complexes are considered to regulate priming and fusion of the vesicles. The absence of DOC2 proteins from these complexes suggested that DOC2 proteins may have a function in other stages of the synaptic vesicle cycle. Furthermore, DOC2 proteins were also detected in synaptosomal plasma membrane. Hence, the association of DOC2 to synaptic vesicles may not be stable and/or exclusive.

Munc18-1 was first characterized (Hata et al., 1993) by its high affinity interaction with syntaxin, a component of the trimeric 'core complex' of syntaxin, SNAP25, and synaptobrevin–VAMP (see Söllner et al., 1993a). One model considers munc18 a negative regulator of syntaxin and therefore a negative regulator of core complex formation and vesicle docking (Pevsner et al., 1994b, Schulze et al., 1994). We show here that DOC2 proteins compete with syntaxin for the interaction with munc18, i.e., DOC2-munc18-syntaxin trimers were never observed. According to the above model, DOC2 may thus regulate the availability of munc18 to suppress synaptic vesicle docking.

The interaction between syntaxin and munc18 is consistently stronger than between DOC2 proteins and munc18. However, other vesicle and core complex proteins modulated the affinity of the syntaxin–munc18 interaction, shifting the equilibrium in favor of a DOC2–munc18 interaction. Hence, DOC2 proteins appear to compete with syntaxin more successfully in the presence of other constituents of docking–fusion complexes, i.e., DOC2 proteins can remove munc18 from syntaxin as soon as core complexes are ready to form. Thus, a plausible model for the function of DOC2 proteins is that they represent vesicular adapter proteins in early stages of secretion, by conditionally removing munc18 from syntaxin and herewith regulating core complex formation and synaptic vesicle docking.

Experimental Procedures

Genomic DNA and cDNA Cloning and Sequencing
A partial DOC2A sequence (underlined in Figure 1) was first cloned from a mouse genomic library (λ-FIX, Stratagene) hybridized with a 0.97 kb fragment encoding the two C2 domains of rat rabphilin3A cDNA (pr-85-Ia, PCR product T943-T944; see Li et al., 1994). More than 100 clones out of 107 plaques hybridized to this probe. Most of these encoded rabphilin3A. One clone contained a small exon encoding a rabphilin3A-like protein (residue 324–355 of the rat DOC2A protein, indicated by the horizontal line in Figure 1). An oligonucleotide derived hereof (GCGTCTAGACAGCAAGAGGAG CACCTTC) was used to screen rat brain libraries (λ-ZAPII, Stratagene). Further screens were performed using DOC2A fragments obtained from this initial screen. Together, >40 overlapping clones were obtained and characterized, and 14 clones were selected and sequenced using the dideoxynucleotide chain termination method with fluorescent primers. Fluorescent products were analyzed on an ABI370A automatic DNA sequencer.

RNA Blotting and Analysis
Total RNA was isolated from different rat tissues and blotted onto nylon membranes according to standard procedures (Sambrook et al., 1990). In addition, commercial RNA blots were used (multiple tissue Northern blot, Clontech).

In Situ Hybridization
Rat brains were frozen in isopentane on dry ice, sectioned on a cryostat (16 μm), and immobilized on poly-lysine-coated slides with 4% paraformaldehyde in PBS for 5 min. Slides were washed twice

with PBS for 5 min, placed in 0.25% acetic anhydride in 0.1 M triethanolamine HCl (pH 8.0) for 10 min at room temperature, and washed with PBS for 5 min and with 0.83% NaCl for 5 min. Tissue was dehydrated with ethanol: 30% (1 min), 50% (1 min), 70% (5 min), 85% (1 min), 95% (2 min), 100% (1 min), chloroform (1 min), and ethanol 100% (1 min). Sections were hybridized with ^{35}S-labeled cRNA probes of DOC2–rabphilin3A open reading frames in both orientations in 80 μl hybridization buffer (50% deionized formamide, 2 × SSC, 10% dextran sulphate, 1 × Denhardt's solution, 5 mM EDTA, 0.1 M DTT, and 0.5 mg/ml yeast tRNA in 10 mM phosphate buffer [pH 8.0]) overnight at 55°C. Slides were washed for 30 min in 50% formamide, 2 × SSC, and 10 mM DTT at 65°C and with NTE buffer (0.5 M NaCl, and 5 mM EDTA in 10 mM Tris–HCl [pH 8.0]) 3 × 10 min at 37°C. Sections were subsequently treated with 20 μg/ml RNAse A in NTE buffer for 15 min at 37°C, washed again in NTE buffer for 15 min at 37°C, and again in 50% formamide, 2 × SSC, 10 mM DTT, in 2 × SSC, and in 0.1 × SSC for 15 min each at room temperature. Sections were then dehydrated by quickly passing them through 30%, 60%, 80%, and 95% ethanol, all including 0.3 M ammonium acetate, followed by 100% ethanol twice. Sections were exposed to film (Hyperfilm β-max, Kodak) at −80°C for 1–3 weeks.

Subcellular Fractionation

Subcellular fractionation was performed largely as described by Huttner et al., 1983. The LP2 fraction was obtained after centrifugation at 165.000 g instead of 100.000 g. Astroglia cells were obtained from neonatal rat brains. Brains were dissected and chopped in Hanks balanced salt solution and incubated with 0.25% trypsin and 0.1% DNAse I (Sigma) for 15 min at 37°C. Cells were recovered by adding DMEM with 5% foetal calf serum and washed once by centrifugation at 500 g for 10 min and resuspension in the same medium. Glial cells were cultured for 2–3 weeks and harvested using 0.25% trypsin, washed twice, and sonicated for 3 × 10 s on ice. Proteins were solubilized in 1% Nonidet P-40 and 100 mM NaCl, and insoluble fragments were sedimented at 100,000 g for 1 hr at 4°C.

Bacterial Expression Vectors and DOC2 Fusion Proteins

Bacterial expression vectors for DOC2–GST fusion proteins were constructed in pGEX–KG (Guan and Dixon 1991) by amplifying DOC2 cDNAs with specific primers containing appropriate restriction enzyme sites for subcloning, except G-DOC2B-C and -D, where an endogenous NcoI site was used for in-frame cloning. The GST–DOC2 fusion proteins used in this study contain the following amino acid residues: G-DOC2A-A, 122-end; G-DOC2A-B, 84-end; G-DOC2A-C: 1–90; G-DOC2B-A, 118–276; G-DOC2B-B, 118-end; G-DOC2B-C, 141-end; G-DOC2B-D, 141–276; G-DOC2B-E, 1–124 (constructs are indicated in Figure 6). GST–syntaxin1 (Hata et al., 1993) and GST–rabphilin3A (Fykse et al., 1995) were described previously. GST fusion proteins were expressed in bacteria and purified as described (Smith and Johnson, 1988).

Affinity Purification of DOC2-Interacting Proteins

The GST fusion proteins and GST control protein were attached to glutathione agarose beads and incubated with solubilized total rat brain homogenate in solubilization buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 g/l phenylmethylsulfonyl fluoride, and 1 mg/l leupeptin and pepstatin). In some experiments, one of the following additions was made: 4 mM Mg^{2+}, 4 mM Ca^{2+} and 4 mM Mg^{2+}, 50 μM GTPγS, and 1 mM ATP. After overnight incubation at 4°C, the beads were washed three to five times with the same buffer. Interacting proteins were analyzed using PAGE and Coomassie staining or immunoblotting. In competition experiments, bacterially expressed proteins were included in the experiments described above: 50 μg MBP–SNAP25A, 50 μg MBP–syntaxin1A (cytoplasmic domain), or 50 μM MBP–rabphilin3A (double C2 domain), eluted from amylose beads with 10 mM maltose.

Antibodies

Polyclonal antibodies against DOC2 proteins, N321, N320, M452, I378, and P011, are described in detail elsewhere (Verhage et al, unpublished data). For Western blotting, specific DOC2A and DOC2B antibodies, M452 and N321, respectively, were prepared with affinity purification using the respective GST-fusion proteins and preabsorbing with the other isoform. For immunoprecipitation, antibody P011 was used, which recognizes both DOC2 isoforms. Other antibodies used in these studies have been described before; munc18, J370 (Hata et al., 1993), commercial monoclonals (Figure 8a; Transduction Laboratories, Lexington, KY), SNAP25, and syntaxin (I733 and I378, respectively; McMahon et al., 1995).

In Vitro Transcription–Translation and Labeling

In vitro expression of DOC2 proteins and munc18 was performed using rabbit reticulocyte lysate TNT-kit, Promega) using ^{35}S-methionine according to the manufacturers' protocols. DOC2 and munc18 cDNAs were cloned into pBluescript (Stratagene) and transcribed using T7 or T3 promoters. Residues transcribed in these deletion mutants are: CΔ1, 1–567; CΔ2, 1–528; CΔ3, 1–341; NΔ1, 14-end; and NΔ1, 51-end. The lysate was incubated with fusion proteins on beads for 2 hr at room temperature, then subsequently washed five times and subjected to SDS–PAGE.

Yeast Two-Hybrid System

The validity of protein–protein interaction involving DOC2 was tested by cloning DOC2 (sequences from G-DOC2B-B and -C) and munc18 constructs (as indicated in Figure 7) into bait and prey yeast expression vectors pBTM116 and pVP16–3 (gift of Dr. S. Hollenberg, University of Washington, Seattle; Vojtek et al., 1993). Yeast L40 strain (Vojtek et al., 1993) was transfected with bait and prey vectors and plated on selection plates lacking uracil, tryptophan, and leucine. After 3 days at 30°C, colonies were inoculated into supplemented minimal medium lacking uracil, tryptophan, and leucine and incubated in a shaker at 30°C again for 3 days. β-galactosidase activity was determined on yeast extracts as described (Rose et al., 1990).

Immunoprecipitation

Synaptosomes were prepared as described (Verhage et al., 1991), lysed with solubilization buffer (see above), incubated with P011 polyclonal antibody and subsequently with protein A Sepharose (Pharmacia, Uppsala, Sweden), washed extensively, and analyzed by PAGE and Coomassie staining–Western blotting using monoclonal munc18 antibody (see above).

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GenBank Accession Numbers