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DOC2B, C2 Domains, and Calcium: A Tale of Intricate Interactions

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Abstract Ca^{+2}-dependent exocytosis involves vesicle docking, priming, fusion, and recycling. This process is performed and regulated by a vast number of synaptic proteins and depends on proper protein–protein and protein–lipid interactions. Double C2 domain (DOC2) is a protein family of three isoforms found while screening DNA libraries with a C2 probe. DOC2 has three domains: the Munc13-interacting domain and tandem C2s (designated C2A and C2B) connected by a short polar linker. The C2 domain binds phospholipids in a Ca^{2+}-dependent manner. This review focuses on the ubiquitously expressed isoform DOC2B. Sequence alignment of the tandem C2 protein family in mouse revealed high homology (81%) between rabphilin-3A and DOC2B proteins. We created a structural model of DOC2B's C2A based on the crystal structure of rabphilin-3A with and without calcium and found that the calcium-binding loops of DOC2B move upon calcium binding, enabling efficient plasma membrane penetration of its C2A. Here, we discuss the potential relation between the DOC2B bioinformatical model and its function and suggest a possible working model for its interaction with other proteins of the exocytotic machinery, including Munc13, Munc18, and syntaxin.

Keywords DOC2B · Calcium · C2 domain · Exocytosis · Munc13

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Introduction

Neurons and neuroendocrine cells are secretory machines dedicated to repeated rounds of exocytosis—a process by which membrane-bounded vesicles fuse with the plasma membrane (PM), releasing their contents to the extracellular space (the synaptic cleft in neurons). Many proteins have been identified as regulators of Ca^{+2}-triggered exocytosis; among them are the calcium-dependent protein families of synaptotagmin, double C2 domains (DOC2), and rabphilin-3A that share sequence homology in their tandem C2 domains [1]. Sequence alignment of the three families has revealed that rabphilin-3A and DOC2 proteins belong to the same branch on the phylogenetic tree—the homologies between the first and second C2 domains suggesting that the duplication of this domain occurred earlier in evolution than the branching between rabphilin-3A and DOC2 proteins [2].

DOC2 is a family of synaptic proteins first described by Orita et al. in 1995 [3], and their name reflects their structure—double C2 domains. DOC2 family proteins consist of three isoforms designated DOC2A, DOC2B, and DOC2C, and they are approximately 400 amino acids in length (45–47 kDa) with no predicted transmembrane domain. All three isoforms have a Mid (Munc13-interacting) domain, a spacer (the region between the Mid domain and the C2A domain), a C2A domain, an interdomain linker, and a C2B domain (Fig. 1a; [1, 4]). DOC2 is expressed in the brain and other tissues [5], including mast cells [6], chromaffin cells [7], and osteoblasts [8], suggesting a role in secretion. This review focuses on the ubiquitously expressed isoform DOC2B [2, 7] and in particular on its C2A and Mid domains, in relation to vesicle fusion.

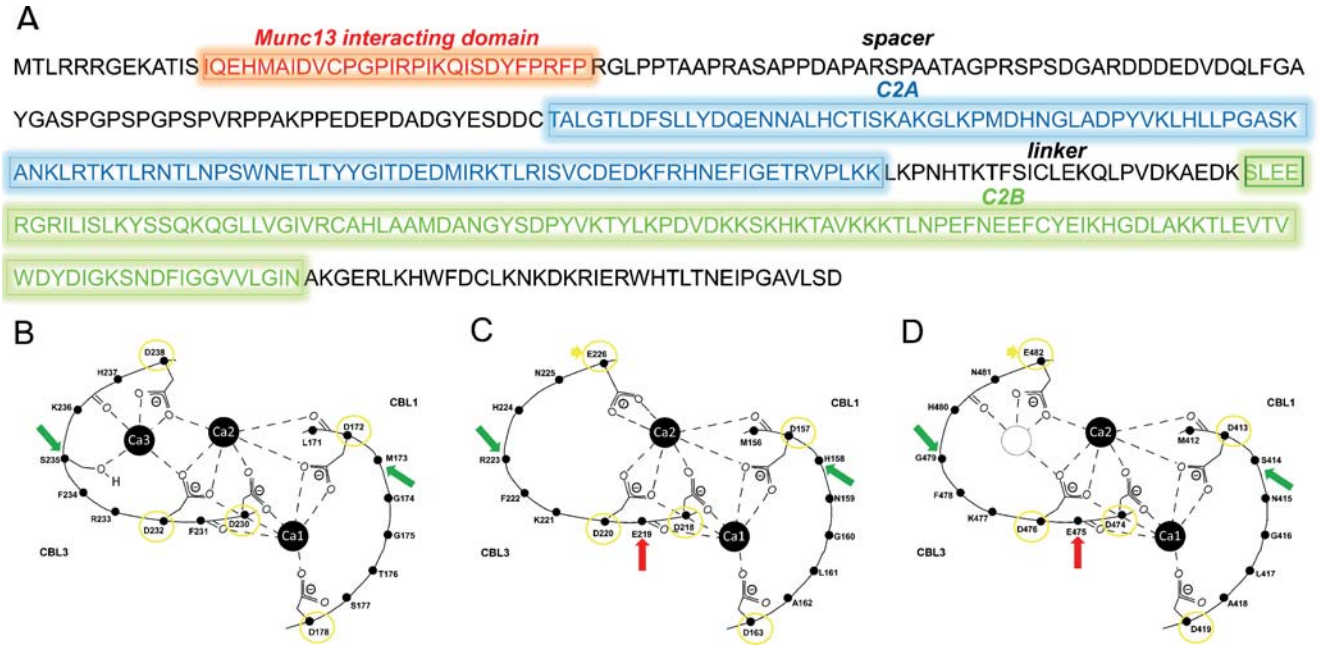

Fig. 1 Schematic representation of DOC2B. **a** The sequence of DOC2B with its domains: Mid domain represented in *red*, C2A domain represented in *blue*, and C2B domain represented in *green*. The spacer and the linker are indicated above the sequence. **b** Schematic representation of the potential Ca^{2+}-binding sites of the C2A domain of synaptotagmin1 [23]. *Solid circles* represent the three Ca^{2+}-binding sites. *Yellow circles* surround the calcium-binding motifs. *Green arrows* point to the variations in C2A between synaptotagmin1 and DOC2B **c** Schematic representation of the potential Ca^{2+}-binding sites of the C2A domain of DOC2B. Labeling as in **b**. *Red arrow* points to the additional negative charge in the middle of the calcium-binding pocket. **d** Schematic representation of the potential Ca^{2+}-binding sites of the C2A domain of rabphilin-3A [17]. Labeling as in **b**. *Dotted circle* represents the position of a hypothetical third calcium-binding site. *Green arrows* point to the variations in C2A between rabphilin-3A and DOC2B. *Red arrow* points to the additional negative charge in the middle of the calcium-binding pocket

The C2A Domain of DOC2B Binds Phospholipids in a Calcium-Dependent Manner

C2 domains are ubiquitous protein modules originally identified in protein kinase C (PKC) [9]. They all consist of 130 residues [10] and share a common fold composed of two four-stranded β-sheets arranged in a compact β-sandwich and surrounded by variable loops and helices (Fig. 2a, top right). Two main topologies have been described for these domains, namely type I topology, first described for the synaptotagmin C2A domain, and type II topology, originally found in the phosphoinositide-specific phospholipase Cδ1 [11]. The general structure of the C2 domain consists of calcium-binding motifs that contain five aspartate residues (D1–D5, Fig. 1b–d, yellow circles). C2 domains bind phospholipids in a calcium-dependent manner and cause calcium-dependent protein–membrane associations [12]. Indeed, DOC2B translocates to the PM upon elevation of internal calcium concentration ($[Ca^{2+}]_i$) in many cell types, including HEK293, PC12, chromaffin, and neurons [13–15]. We suggest that this translocation is mediated by its C2A domain.

The C2A domain of DOC2B binds to liposomes composed of phosphatidylcholine (PC) and phosphatidylserine (PS) in a Ca^{2+}-dependent manner with an EC50 of 1 μM [7, 16]. In vivo experiments have revealed that DOC2A and B associate with the PM in a calcium-dependent fashion, with EC50 values of 450 and 175 nM $[Ca^{2+}]_i$, respectively [14]. The high calcium sensitivity of both DOC2A and DOC2B may arise from the local organization of amino acid residues that stabilize the interaction of Ca^{2+} ions and the PM with the predicted calcium-binding pocket of the C2A domain (Fig. 1c). DOC2A and DOC2B present high homology with rabphilin-3A's C2A domain (73% and 81%, respectively) [14], while homology of DOC2B with synaptotagmin1 is lower (38%) [14].

To learn about the behavior of the C2A of DOC2B following calcium binding, we performed homology modeling for DOC2B C2A based on the structure of rabphilin-3A with [17] or without [18] calcium (see Methods for details). The calcium-binding motifs of rabphilin-3A and DOC2B contain five aspartate residues (D1–D5, Fig. 1c, d, yellow circles) that provide a negative charge to the calcium-binding surface of the C2 motif as in synaptotagmin [11]. A slight change exists in *both* rabphilin-3A and DOC2A and B compared with synaptotagmin1: The fifth aspartate motif is replaced with glutamic acid (Fig. 1b–d). An additional negative charge

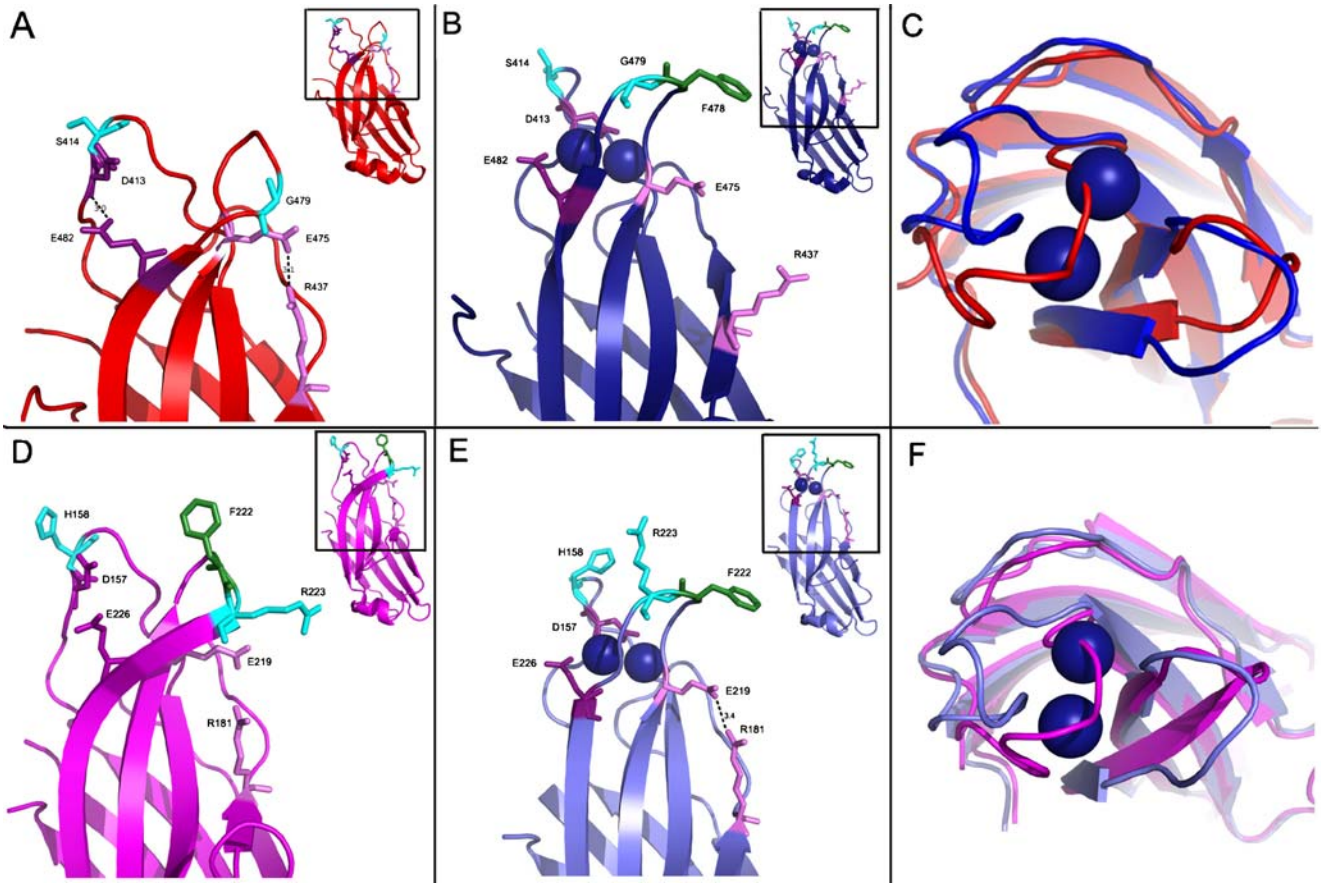

Fig. 2 Homology modeling of the C2A of DOC2B. **a** Crystal structure of rabphilin-3A without calcium in *red* (PDB: 2CHD [18]). The whole structure is shown in the *top right corner*, and the CBLs are scaled up in the *middle*. Residues in stick presentation: *S414, G479* (*cyan*), *D413*, *E482* (*purple*), *E475*, *R437* (*pink*). *Dashed lines* represent hydrogen bonds. Bond distances are indicated on the figure. **b** NMR structure of rabphilin-3A with calcium in *blue* (PDB: 2K3H [17]). The whole structure is shown in the *top right corner*, and the CBLs are scaled up in the *middle*. Calcium ions are represented by *blue spheres*. Residues in stick presentation: *S414*, *G479* (*cyan*), *D413*, *E482* (*purple*), *E475*, *R437* (*pink*), *F478* (*green*). **c** Superposition of the two structures of rabphilin-3A: without calcium (*red*) and with calcium (*blue*). Calcium ions are represented by *blue spheres*. **d** Structural model of DOC2B without calcium (*magenta*) based on crystal structure of rabphilin-3A C2A without calcium (2CHD). The whole structure is shown in the *top right corner*, and the CBLs are scaled up in the *middle*. Residues in stick presentation: *H158*, *R223* (*cyan*), *D157*, *E226* (*purple*), *E219*, *R181* (*pink*), *F222* (*green*). *Dashed lines* represent hydrogen bonds. Bond distances are indicated on the figure. **e** Structural model of DOC2B with calcium (*light blue*) based on NMR structure of rabphilin-3A (2K3H). The whole structure is shown in the *top right corner*, and the CBLs are scaled up in the *middle*. Residues in stick presentation: *H158*, *R223* (*cyan*), *D157*, *E226* (*purple*), *E219*, *R181* (*pink*), *F222* (*green*). *Dashed lines* represent hydrogen bonds. Bond distances are indicated on the figure. **f** Superposition of the two model structures of DOC2B without calcium (*magenta*) and with calcium (*light blue*). Calcium ions are represented as *blue spheres*

(glutamic acid) is found between the third and fourth aspartate motifs in both rabphilin-3A (E475, Fig. 1d, red arrow) and DOC2A (E187) and B (E219, Fig. 1c, red arrow). In the calcium-free form of rabphilin-3A, these changes suggest a possible mechanism for the formation of hydrogen bonds (between E482 and D413, 3 Å, and between E475 and R437, 3.1 Å [17], Figs. 1d and 2a, b), increasing the rigidity of calcium-binding loops (CBLs) 1 and 3. The nuclear magnetic resonance (NMR) structure of the Ca^{2+}-bound form of rabphilin-3A revealed that Ca^{2+} binding induces large conformational changes within CBL 1 and CBL 3 (Fig. 2c) that lead to the rupture of the two hydrogen bonds. These conformational changes are probably associated with a high activation energy, which explains the low affinity of rabphilin-3A's C2A domain for Ca^{2+} [17]. According to our model of DOC2B, without calcium, these hydrogen bonds do not exist (between E226 and D157, 4.8 Å, and between E219 and R181, 4.5 Å, Fig. 2d). However, in the calcium-bound model of DOC2B, one hydrogen bond is formed between E219 (the additional negative charge residue in the middle of the Ca^{2+}-binding pocket) and R181 (3.4 Å, Fig. 2e). The formation of this putative hydrogen bond stabilizes the calcium-bound conformation, making it energetically favorable [19–22], and this provides a possible explanation for the high affinity of DOC2B for calcium [14].

Synaptotagmin1 binds three calcium ions [23], whereas rabphilin-3A binds only two (Fig. 1b, d); the NMR study of rabphilin-3A could not uncover a third Ca^{2+} ion bound to the third CBL [17]. We found a Hillslope of 2 for DOC2 calcium-dose-dependent translocation (1.75 for DOC2A and 1.89 for DOC2B [14]), suggesting that the C2A of DOC2A and B binds two calcium ions as in rabphilin-3A and not three calcium ions as in synaptotagmin1. It is also important to note that the serine (S235, Fig. 1b) in synaptotagmin1 is replaced with glycine in rabphilin-3A (G479, Fig. 1d, green arrow) and with arginine in DOC2B (R223, Fig. 1c, green arrow). This positively charged amino acid also contradicts the notion of a third calcium ion in DOC2B's predicted calcium-binding pocket.

In synaptotagmin, Ca^{2+} binding allows the C2A and C2B domains to interact with negatively charged phospholipids such as PS [24], and both C2A and C2B can penetrate the membrane bilayer in response to calcium binding [25–30]. This interaction results in the insertion of amino acids that are located at the tips of the membrane-binding loops into the PM to one-third of the lipid monolayer's depth (M173 and F234 of C2A, and V304 and I367 of C2B) [29–32]. Such perturbation of the bilayer structure can trigger, under some circumstances, the fusion of lipid bilayers [33], and indeed, it was demonstrated that synaptotagmin can lower the energy needed for membrane fusion [34]. Interestingly, several copies of synaptotagmin that are found on a single synaptic vesicle are suggested to be ordered ringwise around the potential fusion site [35] through their interaction with soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors (SNARE) complexes [36]. Hence, upon Ca^{2+} influx and subsequent Ca^{2+} binding by synaptotagmin1, the C2A and C2B domains penetrate the PM, resulting in the local induction of positive membrane curvature under the SNARE complex ring [34]. This causes buckling of the PM within the SNARE ring toward the synaptic vesicle, reducing the distance between the two membranes. Furthermore, the buckled membrane is under curvature stress, which reduces the energy barrier that it has to overcome at the intermediate stage of fusion and, hence, accelerates the fusion reaction [34]. Surprisingly, in a recently published work by Hui and colleagues [37], it was found that while both the C2A and C2B (tested separately) interacted avidly with highly curved membranes, only the C2B and *not* the C2A could promote membrane tubulation. Yet, testing the tubulation ability of the tethered C2A–C2B revealed that interfering with the calcium-binding ability of the C2A inhibited the membrane bending activity of the adjacent C2B [37], suggesting that in the context of the full protein, both C2A and C2B are important for tubulation activity.

We hypothesize that the C2A of DOC2B functions via a similar mechanism. Our working model [7] suggested that DOC2B exerts a facilitating effect on the PM. This model was supported by amperometric spike measurements of DOC2B-overexpressing cells. The data were analyzed and interpreted according to accepted model for the fusion pore [38, 39], suggesting that DOC2B enables a longer dilation time and slower expansion of the fusion pore, which in turn causes more efficient dispersion of catecholamine [7]. We hypothesize that this mechanism of action is based on the ability of the predicted CBLs to move and penetrate the PM. Modeling the movement in the C2A of DOC2B relative to that in rabphilin-3A (Fig. 2c, f) suggests that calcium binding induces conformational changes in DOC2B's C2A domain that can induce penetration of specific amino acids into the PM. Indeed, the amino acids that penetrate the PM in synaptotagmin were partially conserved by DOC2B: The phenylalanine in the C2A was conserved (F222), but the methionine in position 158 was replaced with histidine. In addition, the amino acid adjacent to F222 is arginine (R223), which contributes a positive charge to the CBL1 of DOC2B. The NMR study of rabphilin-3A demonstrated that this area (CBL1) is flexible, having several possible positions (all facing out toward the PM). The existence of arginine (R223) near the flexible phenylalanine (F222) might help to stabilize the CBL1 in comparison to glycine in rabphilin-3A and serine in synaptotagmin, thus promoting more efficient membrane penetration by the C2A of DOC2B. The histidine in position 158 in DOC2B (Fig. 1c, green arrow) is another point variation among the C2As of DOC2B, rabphilin-3A, and synaptotagmin1. Histidine, which is partially positive at natural pH [40] and facing the PM, can also penetrate the PM, and this interaction might be more efficient than that of methionine in position 173 in synaptotagmin1. The relevant amino acids for penetration in the C2B were not conserved—the valine (V304) was replaced with alanine (A298) and the isoleucine (I367) with glycine (G362). Hence, the side chains of the relevant amino acids of the C2B domain of DOC2B are shorter, but their charge is conserved. Based on the above model, we suggest that the CBLs of DOC2B C2A move upon binding two Ca^{2+} ions (Fig. 2f), inserting the relevant amino acids (H158, F222, Fig. 2e) into the PM and creating a hydrogen bond between E219 and R181 that further stabilizes this energetically favorable structure. This mechanism of action might explain DOC2B's efficient promotion of fusion, as demonstrated in the amperometry experiments [7].

Although C2A plays an important role in DOC2B's function, there are some discrepancies in its activity. The C2A of DOC2B binds PS at higher affinity in the presence of calcium than does C2B [16]. However, deletion of C2B abolishes DOC2B translocation in adipocytes while deletion of C2A does not [41]. This finding contradicts

the biochemistry assays [7, 16] and the effects of mutations in DOC2B C2A and C2B. Mutating the third and fourth aspartate motifs of the C2A in DOC2B ($DOC2B^{D218,220N}$) caused constant translocation of the protein to the PM (Fig. 3a, [7, 14]) while similar mutations in the C2B of DOC2B did not change its calcium sensitivity. Examination of the translocation of $DOC2B^{wt}$ and $DOC2B^{D218,220N}$ by Total Internal Reflection Fluorescence Microscopy (TIRF) demonstrated that $DOC2B^{D218,220N}$ maintains some translocation ability (Fig. 3b) and can be dislocated from the PM using the calcium-chelating agent BAPTA-AM [14]. These experiments suggest that the C2A mutation did not completely abolish the protein's dependence on calcium. Hence, in chromaffin and PC12 cells, C2A plays a more direct role in DOC2B's calcium sensitivity.

There are other differences between the different cell types: DOC2B is localized to the membrane in MIN6 beta cells and in 3T3-L1 adipocytes, and no apparent translocation is observed in response to elevation of $[Ca^{2+}]_i$ in this study [42]. Yet in a recent work performed in 3T3-L1 adipocytes, DOC2B showed cytosolic distribution and translocated to the cell membrane only following insulin addition. In addition, the translocation occurred at a slower rate (~5 min [41, 43]) compared to that measured in PC12 cells (~2 s [13]) and chromaffin cells (unpublished data).

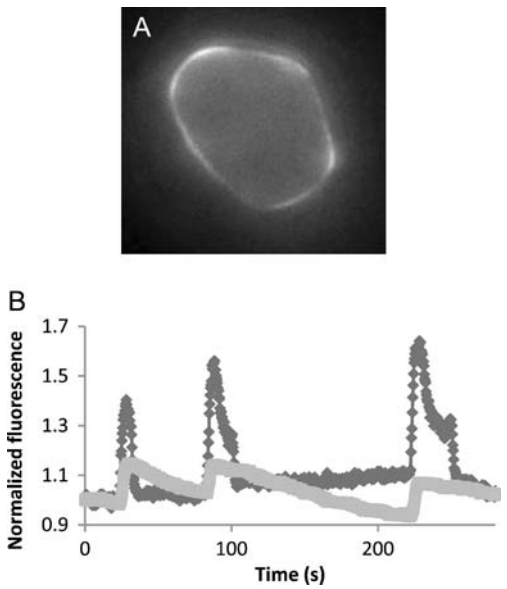

Fig. 3 $DOC2B^{D218,220N}$ localizes to the PM at basal $[Ca^{2+}]_i$. **a** PC12 cell expressing $DOC2B^{D218,220N}$-EGFP at basal $[Ca^{2+}]_i$. $DOC2B^{D218,220N}$-EGFP is found at the PM. **b** Translocation of $DOC2B^{wt}$ and $DOC2B^{D218,220N}$ to the PM following depolarization in PC12 cells. Increase in the fluorescence intensity on the PM (cell's foot) of $DOC2B^{wt}$-EGFP (*dark gray*) or $DOC2B^{D218,220N}$-EGFP (*light gray*) in response to depolarization with 60 mM KCl. Each cell was stimulated three times with three different application lengths (10, 20, and 30 s). Between applications, the cells were washed with external solution

Nevertheless, despite these discrepancies, the different rates of translocation correlate with different phases of secretion in these cells. In adipocytes, the time course of the second phase of insulin secretion is slow and accordingly, so is the translocation of DOC2B. In contrast, in chromaffin cells and in neurons, the rate of vesicle refilling and release is several fold faster and so is DOC2B's translocation. Thus, despite these apparent changes in translocation kinetics, we assume that in all cases DOC2B plays a similar role in maintaining release during repeated or long periods of stimulation. Interestingly, mice lacking DOC2A exhibited normal neurotransmission in the hippocampal CA1–CA3 synapses but abnormal frequency facilitation during prolonged stimulation at 5 Hz [44], suggesting that DOC2A also play a role during repeated stimulation.

The Importance of Sequences Adjacent to the C2 Domain for Calcium Binding

Many C2 core domains contain a Ca^{2+}-binding region with a conserved consensus sequence of acidic residues, but this motif alone does not reflect the large variations in Ca^{2+} and Ca^{2+}-mediated membrane-binding affinities among these domains. The C2A of DOC2B shows high homology with those of rabphilin-3A and synaptotagmin7 (81% and 40%, respectively [14]), but while the C2A of synaptotagmin7 presents high calcium affinity in the presence of phospholipids (1–2 μM with liposomes composed of 25% PS/75% PC [45]), that of rabphilin-3A presents no apparent phospholipid binding (with liposomes of the same composition [46]). Moreover, the C2A of DOC2B shows higher homology to rabphilin-3A C2A than that of DOC2A (81% and 76%, respectively), yet the C2A of DOC2A binds phospholipids in a calcium-dependent manner [14]. A possible explanation may stem from the region upstream of the C2 domain itself. In rabphilin-3A, it is the C2B that shows high phospholipid affinity [46], and recently, it has been found that a number of acidic residues in the linker between C2A and C2B that are *not* part of the C2B core domain of rabphilin-3A interacts with the Ca^{2+}-binding region of this domain [47].

The standard model for the C2 domain–membrane interaction implies that the Ca^{2+} ions function as a bridge between the protein and the phospholipids. According to this model, the phosphate moiety of the phospholipids and the carboxylic moiety of the PS head group complete the coordination spheres of the Ca^{2+} ions. The Ca^{2+}-coordinating water molecules found in the various crystal structures of C2 domains are then displaced by the phospholipids, and no strong specific PS head group–protein interactions are required for the binding [12, 48,

49]. An interesting study by Montaville and colleagues [47] suggested that the standard model for Ca^{2+}-mediated phospholipid binding by the C2 domain is different in the case of the C2B of rabphilin-3A. As already mentioned, a number of acidic residues from the C2A-C2B linker region that are *not* part of the C2B core domain interacts with the Ca^{2+}-binding region of this domain. Because of these interactions, the coordination sphere of the two Ca^{2+} ions is almost completely created by the relevant protein residues of the C2B and the acidic linker that stays bound to the calcium-binding region of the C2B domain upon phospholipid binding [47]. The spacer of DOC2B, like the linker of rabphilin-3A, has several short (up to five amino acids) negatively charged residues that might contribute to Ca^{2+} binding in the C2A of DOC2B. The findings that the spacer—and not the linker—of DOC2A and B exhibits homology with the linker in rabphilin-3A [47], and that the C2A of DOC2B has high affinity for calcium, support the claim that the area upstream of the C2 domain contributes to the domain's calcium affinity. Interestingly, high diversity exists in the spacer region between DOC2A and B, e.g., the DOC2B spacer is 36% longer than that of DOC2A and only 15 amino acids are conserved between the two isoforms in this region. While the C2As of DOC2A and DOC2B show high homology (76%), their calcium affinities differ—that of DOC2A (475 nM) is lower than that of DOC2B (175 nM [14], 225 nM [15]). This can be attributed to the spacer's contribution in DOC2 to calcium/PS binding. In agreement with this, we have data that the C2A domain of DOC2B, by itself, is not sufficient for translocation to the PM upon elevation in $[Ca^{2+}]_i$, strengthening the idea that sequences upstream of the C2 domain contribute to the calcium-dependent membrane binding.

Another difference between DOC2 and rabphilin is the length of their linkers. The linker between the two C2 domains is longer in rabphilin-3A and more glycine-rich than in the DOC2 family, suggesting that in the latter, the range of motion of the two C2 domains is more restricted, especially for DOC2B which has the shortest linker (25 amino acids) in the family. This suggests that the C2 domains of DOC2B are in fixed orientation, facilitating complex formation of C2-Ca^{2+}-PS in the DOC2 family and especially in DOC2B which has the highest affinity for calcium in vivo [14].

Overall, the data presented here strongly support the idea that the C2A of DOC2B plays a vital role in the latter's function, especially in targeting the protein to the PM and in facilitating its effect on vesicle fusion with the PM. More research needs to be performed on the phospholipid-binding ability of the C2B of DOC2B and on the relationship between the tandem C2 domains to achieve a comprehensive understanding of DOC2B function.

DOC2B's Interacting Partners

DOC2B was first described by Sakaguchi and colleagues in 1995 [50], and since its closest isoform, DOC2A, had already been found to participate in exocytosis from PC12 cells [51], Sakaguchi suggested that the second isoform, DOC2B, also plays a role in calcium-dependent exocytosis. In recent years, several reports have been published on the physiological role of DOC2B, suggesting it to be a positive regulator of exocytosis. DOC2B associates with many proteins of the exocytotic machinery that are involved in different steps of the secretory cycle [7, 41–43]. Here, we suggest a possible model for DOC2B's involvement in calcium-triggered exocytosis.

The DOC2 family of proteins interacts with the Munc13 family of proteins primarily via their Mid domain, located within the N-terminal domain of DOC2 (Fig. 1a) [6, 52–55]. Munc13-1 acts as a priming factor in neurons and in chromaffin cells, and overexpression of Munc13-1 and ubMunc13-2 increases the amplitude of the exocytotic response in chromaffin cells [56, 57]. It has also been recently suggested that Munc13-1 has a postpriming function in lowering the energy barrier for vesicle fusion [58]. DOC2B translocates to the PM upon phorbol ester (PE) stimulation only when coexpressed with Munc13 [54], and mutating its Mid domain abolishes this translocation [13]. The interaction between DOC2A and Munc13-1 is enhanced by high K^+ in the presence of Ca^{2+}; however, upon deletion of the C1 domain of Munc13-1, this interaction exists independent of Ca^{2+} [52]. Injection of a synthetic peptide identical to the Mid domain into cholinergic neurons of the superior cervical ganglion inhibited synaptic transmission in an activity-dependent manner [53]. Similarly, injection of the same peptide into the Calyx of Held blocked the potentiating effect of PE administered 5 min after the peptide [59]. Together, these data suggest that the DOC2–Munc13 interaction has an important physiological role when stimulation by PE is involved.

DOC2 translocates to the PM upon elevation of $[Ca^{2+}]_i$. Munc13, on the other hand, does not translocate to the PM upon calcium elevation (unpublished data). We hypothesize that when Munc13 is coexpressed with DOC2, upon elevation of $[Ca^{2+}]_i$ Munc13-1 cotranslocates to the PM following DOC2 (Fig. 4). When the elevation in calcium is brief, Munc13 translocation might be reversible, dislocating back to the cytosol after DOC2 dislocation. However, it is possible that during high-frequency activity, e.g., sustained or intermittent depolarization, the levels of diacylglycerol (DAG) increase [60]. This can cause a more stable interaction of Munc13-1 with the PM via its C1 domain, anchoring Munc13-1 to the PM and enabling its catalytic activity on the fusion step [58]. Thus, according to this hypothesis, the activity of Munc13 at the PM depends on

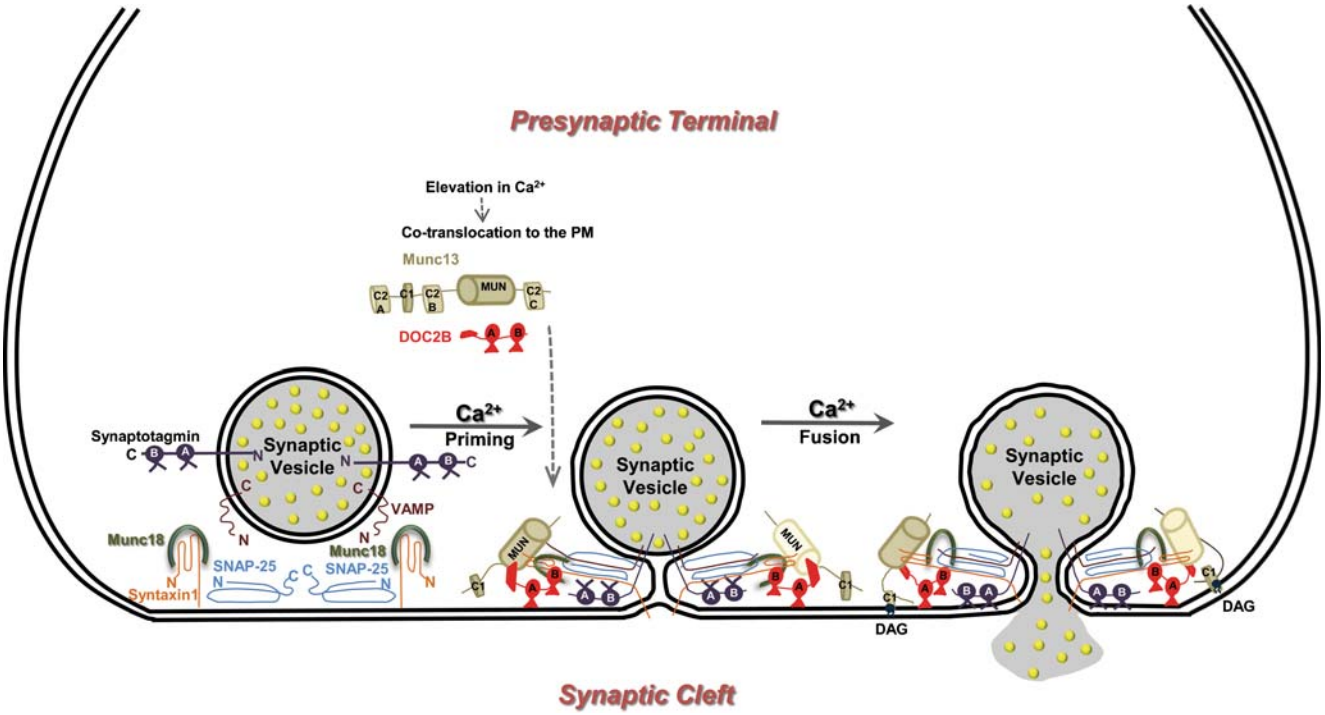

Fig. 4 Schematic model of protein interactions during exocytosis. The scheme depicts the possible protein–protein interactions during the vesicle cycle in the presynaptic terminal. Elevation of calcium to 200–400 nM accelerates vesicle priming, which is associated with the formation of SNARE complexes (syntaxin in *orange*, SNAP-25 in *light blue*, and VAMP in *brown*) and the interaction of synaptotagmins (*purple*) with the assembled SNARE complexes. DOC2B (*red*) and Munc13 (*olive*) co-translocate to the PM and can interact with SNARE complexes. For simplicity only relevant domains of Munc13 (Mun and C1) are illustrated. DOC2B also interacts with Munc18 (*right in khaki*). During stimulation, high calcium allows interaction of the C2 domains of synaptotagmin with the PM, which reduces the energy barrier for fusion and can cause a mechanical perturbation that opens the fusion pore [66]. DOC2B interacts with syntaxin-SNAP25 at the PM via its C2B and its C2A might penetrate into the phospholipids, affecting fusion-pore kinetics

the stimulation frequency and DOC2 translocation, becoming more prominent during periods of high activity. A similar mode of action has been suggested for PKC activity by Oancea and Meyer [61]. Many receptor stimuli induce calcium signals prior to a more persistent increase in DAG concentration. These calcium signals have only a minimal effect on conventional PKC activity in the absence of DAG. However, in the presence of DAG, each calcium spike induces a more pronounced activation cycle of conventional PKC.

In chromaffin cells, elevation of $[Ca^{2+}]_i$ to 300 nM for 30 to 60 s enhances secretion by increasing the number of fusion-competent vesicles, a process that is known as calcium-dependent priming [62]. Overexpression of DOC2B under similar priming conditions, which causes DOC2B translocation to the PM, yielded a twofold increase in the exocytotic response. On the other hand, applying the same stimulation to a cell without priming did not cause an increase in the exocytotic response although the stimulation was continued for 5 s. In contrast, $DOC2B^{D218,220N}$, which is constantly at the PM, enhanced secretion under conditions of no priming. Thus, $DOC2B^{D218,220N}$ exerts a facilitating effect at PM also at low calcium whereas $DOC2B^{wt}$ has to translocate to the PM in order to enhance secretion. These data suggest that DOC2B's action depends on both calcium concentration and the amount of time that the cell maintains an elevated calcium concentration: Calcium is needed to cause DOC2B translocation but has to stay at priming levels (300 nM) for 30 to 60 s to enable DOC2 to achieve its maximal effect. During this period, DOC2B can act directly or via other proteins that cotranslocate to the PM, such as Munc13 (Fig. 4). Support for these suggestions comes from the finding that during repeated stimulation, DOC2B enhances secretion gradually, and its effect becomes larger during later stimulations, suggesting that more than one stimulus is needed to reach its maximal effect [7]. In insulin-secreting cells, DOC2B is suspected of being a regulator of the second phase of insulin secretion (up to 60 s following stimulation) by interacting with syntaxin4 in a Ca^{2+}-dependent manner [41, 43]. The second phase of insulin secretion is slow and prolonged, and this correlates well with DOC2B's relatively slower rate of action at the PM and its ability to support enhanced secretion during repeated stimulations [7]. The

combined data imply that DOC2B requires a certain amount of time in the presence of elevated calcium levels before it can affect secretion. Together with the findings that DOC2B exerts a facilitating effect on the PM, we suggest that DOC2B can both facilitate fusion via the penetration of its C2 domain into the PM and by recruitment of Munc13 to the PM.

Another possible binding partner of DOC2B is Munc18, an important agent of the exocytotic machinery [63, 64]. A direct interaction, regardless of Ca^{2+}, has been identified between DOC2 and Munc18 in several studies [2, 42, 65], while others found an interaction between DOC2 and syntaxin4 [41, 43], or with syntaxin1 in complex with SNAP25 [7]. As DOC2B is highly dependent on calcium [14, 15], these differences might be attributable to the use of different calcium concentrations in these experiments [41, 43]. A possible explanation for its multiple interacting partners might be that the interaction between DOC2B and Munc18 is favored under low Ca^{2+} conditions while increased Ca^{2+} levels facilitate the interaction between DOC2B, Munc13, and the complex of syntaxin-SNAP-25 on the PM to promote exocytosis (Fig. 4).

Many proteins are involved in membrane trafficking in general and exocytosis in particular, and many of them interact with one another. The synaptotagmin family (for review see [66]), rabphilin-3A, DOC2, and synaptotagmin-like protein [67, 68] are all proteins with double C2 domains that have been identified as being involved in membrane trafficking and exocytosis. Is it possible that some of these proteins can substitute for each other? For example, both synaptotagmin1 and DOC2B were found to interact with the SNAREs and with lipids. Is it possible that the similarities in their structures promote analogous interactions? Perhaps the fact that a protein is able to undergo certain in vitro interactions does not mean that these interactions are physiologically relevant. It should also be considered that under various physiological conditions, the levels or the activity of a specific protein can be significantly altered, and this can change the probability for a certain interaction or create a new one. Therefore, perhaps a more integrative, less linear view is needed in this field—and in biology in general.

Methods

Structure Prediction of DOC2B C2A: Two models for DOC2B were built using SCWRL4 [69]. Both were based on rabphilin-3A structures: One was solved with calcium ions (2K3H [17]) and the other without ions (2CHD [18]). The sequence identity between C2A of DOC2B and rabphilin-3A is 80.8%. Since the alignment between the sequences of DOC2B and rabphilin-3A contains no gaps, the sequence of DOC2B was threaded on the structures of rabphilin-3A, and the conformation of the side chains (rotamers) was then modeled using SCWRL4 [69]. For the calcium-binding model, the conformations of amino acids M412, D413, N415, G416, L417, A418, D419, D474, E475, D476, K477, F478, H480, N481, and E482 of the two CBLs were kept as in the template to prevent collapse of the binding pocket, since the modeling process does not include the ions. E475 was not constrained. The calcium-free model made by SCWRL4 was then refined using the fast relax protocol of Rosetta 3.0 [70]. The final model presented here was chosen out of 10,000 decoys based on its lowest score. The models were further assessed using the MolProbity webserver [71, 72] which examines psi and phi angles, Cβ deviations, atom clashes, and rotamers. Twenty structural models for DOC2B with calcium ions were built, based on each of the NMR conformers in the NMR structure of rabphilin-3A (2K3H [17]), in order to check conformations of E219 and R181. We use the model based on the first conformer of rabphilin-3A's NMR structure in Fig. 2d, f for clarity.

Translocation Experiments: In TIRFM PC12 cells (a generous gift from Dr. Nicolas Vitale) were grown in Dulbecco's modified Eagle's medium supplemented with glucose (4,500 mg/l) and L-glutamine (Gibco) and containing 5% fetal bovine serum, 10% horse serum, and 100 U/ml penicillin/streptomycin. For the translocation experiment, cells were plated on glass coverslips, transfected with plasmids encoding fluorescently tagged proteins—*p*-DOC2B^{wt}-EGFP or *p*-DOC2BC2A-mRFP [14] using Lipofectamine™ 2000 (Invitrogen). Imaging was performed 24–32 h posttransfection. Cells were perfused constantly with external solution and excited using a high K^+ solution (70 mM) as described previously [14]. The imaging setup consisted of an Olympus IX-70 inverted microscope with a 60× (TIRF) objective (Olympus), a TILL Photonics TIRF condenser (Gräfelfing, Germany), two solid-state lasers (Laser Quantum, Stockport, UK) emitting at 473 and 532 nm, an Andor Ixon 887 EMCCD camera (Belfast, Northern Ireland), and a dual-view beam-splitting device (Optical Insights, Roper Bioscience, Tuscon, AZ). The equipment was controlled by Metamorph software (Molecular Devices, Downingtown, PA). The analysis was performed using Metamorph software.

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