Doc2b promotes GLUT4 exocytosis by activating the SNARE-mediated fusion reaction in a calcium- and membrane bending–dependent manner

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ABSTRACT The glucose transporter GLUT4 plays a central role in maintaining body glucose homeostasis. On insulin stimulation, GLUT4-containing vesicles fuse with the plasma membrane, relocating GLUT4 from intracellular reservoirs to the cell surface to uptake excess blood glucose. The GLUT4 vesicle fusion reaction requires soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) as the core fusion engine and a group of regulatory proteins. In particular, the soluble C2-domain factor Doc2b plays a key role in GLUT4 vesicle fusion, but its molecular mechanism has been unclear. Here we reconstituted the SNARE-dependent GLUT4 vesicle fusion in a defined proteoliposome fusion system. We observed that Doc2b binds to GLUT4 exocytic SNAREs and potently accelerates the fusion kinetics in the presence of Ca$^{2+}$. The stimulatory activity of Doc2b requires intact Ca$^{2+}$-binding sites on both the C2A and C2B domains. Using electron microscopy, we observed that Doc2b strongly bends the membrane bilayer, and this membrane-bending activity is essential to the stimulatory function of Doc2b in fusion. These results demonstrate that Doc2b promotes GLUT4 exocytosis by accelerating the SNARE-dependent fusion reaction by a Ca$^{2+}$- and membrane bending–dependent mechanism. Of importance, certain features of Doc2b function appear to be distinct from how synaptotagmin-1 promotes synaptic neurotransmitter release, suggesting that exocytic Ca$^{2+}$ sensors may possess divergent mechanisms in regulating vesicle fusion.

INTRODUCTION
Regulated exocytosis is the basis of a wide range of fundamental biological processes, including neurotransmitter release, hormone secretion, and inside–outside distributions of surface transporters and receptors (Schekman and Novick, 2004; Sudhof and Rothman, 2009). One prominent example of regulated exocytosis is the insulin-regulated trafficking of the glucose transporter GLUT4, which plays a central role in maintaining blood glucose homeostasis (Birnbaum, 1989; Charron et al., 1989; James et al., 1989). GLUT4 is normally sequestered in intracellular vesicles in adipocytes and skeletal muscles. In response to elevated levels of blood glucose, insulin binds to cell surface receptors and activates a complex signaling cascade, ultimately leading to the exocytosis of GLUT4-containing vesicles. Once on the cell surface, GLUT4 facilitates the uptake of excess blood glucose into the cell for disposal (Lavan and Lienhard, 1994; Bryant et al., 2002; Watson and Pessin, 2006; Huang and Czech, 2007; Blot and McGraw, 2008; Vassilopoulos et al., 2009; Jewell et al., 2010). Imbalances in GLUT4 exocytosis disrupt body glucose balance and ultimately give rise to insulin resistance and type 2 diabetes (Kewalramani et al., 2010; Hoffman and Elmendorf, 2011).

Although the physiological and medical importance of the GLUT4 exocytic pathway is well established, we are still at the beginning of understanding the underlying molecular mechanisms. GLUT4 exocytosis is mediated by the fusion of GLUT4-containing vesicles with the plasma membrane. Membrane fusion—the merging of two separate lipid bilayers into one—involves substantial lipid
Mechanism of Doc2b in GLUT4 exocytosis

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The fusion reaction is regulated by the Doc2 protein family (Orita et al., 2008). Similar to the well-characterized synaptic Ca^{2+} sensor synaptotagmin-1, Doc2b possesses two homologous Ca^{2+} domains—C2A and C2B. However, Doc2b is a cytoplasmic protein lacking a transmembrane domain (Martens and McMahon, 2008). Doc2b-deficient cells, insulin-triggered GLUT4 exocytosis is abrogated (Fukuda et al., 2009; Ramalingam et al., 2012). However, it remains to be determined how Doc2b regulates the SNARE-dependent GLUT4 vesicle fusion. Doc2 family proteins are also implicated in neurotransmitter release at the chemical synapse (Groffen et al., 2010; Pang et al., 2011; Yao et al., 2011), but attempts to define their functional roles are impeded by the coexistence of other synaptic C2-domain factors that may play redundant/compensatory roles in the fusion reaction (Martens and McMahon, 2008). As such, the functions of Doc2 family proteins in vesicle fusion are still being debated (Groffen et al., 2010; Pang et al., 2011; Yao et al., 2011), providing little insight into the mechanism of Doc2b in GLUT4 exocytosis.

Here we sought to unravel the molecular mechanism of Doc2b by reconstituting it into a defined fusion reaction containing GLUT4 exocytic SNAREs. We observed that Doc2b interacts with GLUT4 exocytic SNAREs and strongly accelerates the fusion kinetics in the presence of Ca^{2+} ions. The stimulatory activity of Doc2b is compatible with multiple v-SNARE isoforms, including VAMP2, VAMP3, and VAMP8, in agreement with the abilities of these v-SNAREs to support GLUT4 exocytosis in vivo (Zhao et al., 2009).

Doc2b also binds to syntaxin-4 monomer, but the Doc2b–syntaxin-4 heterodimer does not block the assembly of the t-SNARE complex. The stimulation of fusion by Doc2b requires intact Ca^{2+}-binding sites on both the C2A and C2B domains, distinct from the activity of synaptotagmin-1 in synaptic vesicle fusion (Bhalla et al., 2005; Stein et al., 2007). Using electron microscopy, we show that Doc2b strongly bends lipid bilayers in the presence of Ca^{2+}. This curvature-inducing activity is critical to the stimulation of fusion by Doc2b. Because synaptotagmin-1 also requires membrane bending to regulate fusion (Martens et al., 2007; Lynch et al., 2008; Hui et al., 2009), these data suggest that membrane curvature induction likely constitutes a general mechanism of exocytic Ca^{2+} sensors.

**RESULTS**

**Doc2b accelerates the SNARE-dependent fusion reaction reconstituted with GLUT4 exocytic SNAREs**

Doc2b can directly bind to membrane bilayers in the presence of Ca^{2+} and phosphatidylserine (PS; Supplemental Figure S7; Groffen et al., 2010; Yao et al., 2011). To examine the association of Doc2b with GLUT4 exocytic SNAREs, we prepared SNARE liposomes using the neutral lipid phosphatidylincholine (PC; Figure 1A). In a...
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cytic SNAREs alone drove a (slow) basal level of membrane fusion of v- and t-SNARE liposomes was monitored through lipid mix chored in separate populations of proteoliposomes (Figure 2A). The into a defined fusion system in which the v- and t-SNAREs were an GLUT4 vesicle fusion. GLUT4 exocytic SNAREs were reconstituted with syntaxin-4 and SNAP-23, and the v-SNARE liposomes contained VAMP2. (B) Fusion of the reconstituted proteoliposomes in the absence or presence of 5 μM Doc2b. The fusion reactions included 1 mM EGTA or CaCl2. Negative controls: 20 μM of the dominant-negative inhibitor VAMP2 CD (cdv2) was added at the beginning of the fusion reactions. (C) Initial rates of the fusion reactions in B. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.

FIGURE 2: Doc2b strongly accelerates the kinetics of the SNARE-dependent membrane fusion. (A) Illustrations of the liposome fusion procedures. The t-SNARE liposomes were reconstituted with syntaxin-4 and SNAP-23, and the v-SNARE liposomes contained VAMP2. (B) Fusion of the reconstituted proteoliposomes with syntaxin-4 and SNAP-23, and the v-SNARE liposomes contained VAMP2. (C) Initial rates of the fusion reaction including 1 mM CaCl2 or EGTA. Negative controls: 20 μM of the dominant-negative inhibitor VAMP2 CD (cdv2) was added at the beginning of the fusion reactions. (C) Initial rates of the fusion reactions in B. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.

The stimulatory activity of Doc2b in the fusion reaction is compatible with multiple v-SNAREs
Whereas VAMP2 constitutes the primary v-SNARE in GLUT4 vesicle fusion, VAMP3 and VAMP8 can serve as alternative/compensatory

FIGURE 3: Dose dependence of Doc2b activity in the SNARE-dependent fusion reaction. Doc2b was added to the reconstituted fusion reaction at the indicated concentrations in the presence of 1 mM CaCl2. Initial fusion rates of the fusion reactions are shown. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.
v-SNAREs (Zhao et al., 2009). Next we examined whether Doc2b could stimulate the fusion reactions in which VAMP2 was substituted with VAMP3 or VAMP8. Proteoliposomes bearing GLUT4 exocytic t-SNAREs (syntaxin-4 and SNAP-23) were directed to fuse with liposomes reconstituted with VAMP2, VAMP3, or VAMP8 (Figure 4A). We found that Doc2b strongly accelerated all of these fusion reactions in the presence of Ca\(^{2+}\) (Figure 4B). Therefore the stimulatory function of Doc2b in the SNARE-dependent fusion reaction is compatible with VAMP2, VAMP3, and VAMP8, in agreement with the abilities of these v-SNAREs to support GLUT4 exocytosis in vivo (Zhao et al., 2009).

The Doc2b–syntaxin-4 heterodimer does not inhibit the assembly of the GLUT4 t-SNARE complex

In the foregoing reconstituted fusion reactions, the t-SNARE complexes were preassembled to examine the membrane-fusing activity of the trans-SNARE fusion engine (Figure 2A). The upstream t-SNARE assembly, however, is a key regulatory step in exocytosis (Jahn and Fasshauer, 2012; Rizo and Sudhof, 2012). Multiple factors have been shown to bind syntaxin monomers and block the assembly of t-SNARE complexes, thereby clamping the fusion reaction at a basal state (Rowland et al., 2011; Jahn and Fasshauer, 2012). Doc2b binds to syntaxin-4 in solution (Fukuda et al., 2009; Ramalingam et al., 2012), but the functional role of this binding is unknown. Here we examined the Doc2b–syntaxin-4 interaction in a membrane environment by reconstituting the syntaxin-4 monomer into proteoliposomes. SNAP-23, the other subunit of the t-SNARE complex, was subsequently added as soluble proteins. In a liposome coflotation assay, we observed that the membrane-anchored syntaxin-4 monomer interacted with Doc2b to form a heterodimer (Supplemental Figure S6). However, Doc2b binding did not block the assembly of syntaxin-4 with SNAP-23 to form the t-SNARE complex (Supplemental Figure S6), indicating that the domain(s) of Doc2b confers the Ca\(^{2+}\) sensitivity, we tested Doc2b mutants bearing substitutions in the conserved Ca\(^{2+}\)-binding sites of the C2 domains. The binding of Ca\(^{2+}\) to C2 domains is coordinated by a series of aspartate residues lining the pockets on one end of the C2 domain (Chapman, 2008; Pang et al., 2011). Although the crystal structure of Doc2b is unavailable, its Ca\(^{2+}\)-binding sites can be modeled based on the structures of the synaptic Ca\(^{2+}\) sensor synaptotagmin-1 (Figure 6, A and B; Shao et al., 1998; Fernandez et al., 2001). A triple substitution D163A/D218A/D220A abolishes the Ca\(^{2+}\) binding to the C2A domain of Doc2b, whereas the D303A/D357A/D359A mutations disrupt the Ca\(^{2+}\)-binding sites on the C2B domain (Figure 6A; Pang et al., 2011). Of importance, these point mutations do not interfere with the folding of the C2 domains based on circular dichroism (CD) measurements (Pang et al., 2011).

Next we reconstituted the Ca\(^{2+}\)-binding–defective Doc2b mutants into the FRET-based, SNARE-dependent fusion reaction to examine their regulatory activities. We found that the stimulation of fusion by Doc2b was abolished when the Ca\(^{2+}\)-binding sites of both C2A and C2B were mutated (Figure 6C). Remarkably, the stimulatory activity of Doc2b was also abrogated when either the C2A or C2B domain was mutated (Figure 6C). Thus Doc2b requires intact Ca\(^{2+}\)-binding sites on both the C2A and C2B domains to accelerate the fusion reaction. Of interest, the synaptic Ca\(^{2+}\) sensor synaptotagmin-1 appears to use only one of its C2 domains to sense Ca\(^{2+}\), whereas Ca\(^{2+}\) binding to the other C2 domain is largely dispensable (Bhalla et al., 2005; Stein et al., 2007). Hence, despite the overall similarities in their domain organization and stimulatory functions, Doc2b and synaptotagmin-1 likely differ in coupling Ca\(^{2+}\) binding to fusion regulation.

The stimulatory function of Doc2b in the SNARE-dependent fusion reaction involves membrane curvature induction

Binding to Ca\(^{2+}\) ions enables the hydrophobic loops of a C2 domain to penetrate into the outer leaflet of a lipid bilayer (Figure 7A;
FIGURE 5: The Doc2b-syntaxin-4 heterodimer does not inhibit the assembly of the GLUT4 t-SNARE complex. (A) Diagram illustrating the experimental procedures for the reconstituted fusion reactions. (B) The binding of Doc2b to syntaxin-4 did not inhibit the SNARE-dependent fusion reaction. Syntaxin-4 liposomes were incubated with or without Doc2b at 4°C for 1 h before SNAP-23 was added. After 2 h at 4°C, VAMP2 liposomes were introduced to initiate fusion. The fusion reactions were carried out in the presence of 1 mM CaCl\(^{2+}\) or EGTA. (C) Initial rates of the indicated fusion reactions in B. Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of indicated fusion reactions in the presence of 1 mM EGTA or CaCl\(^{2+}\) and marked with asterisks. CD spectroscopic analysis demonstrated that these Ca\(^{2+}\) proteins used in the fusion reactions. C2 domains with Ca\(^{2+}\) numbers on the top and asterisks at the bottom. (B) Illustrations of WT and mutant Doc2b C2A and C2B domains that coordinate Ca\(^{2+}\) binding. Mutated residues are indicated with amino acids numbers on the top and asterisks at the bottom. The stimulatory activity of Doc2b in the fusion reaction requires intact Ca\(^{2+}\) binding sites. Mutated residues are indicated with asterisks. CD spectroscopic analysis demonstrated that these Ca\(^{2+}\)-binding mutations do not affect the folding of the C2 domains (Pang et al., 2011). (C) Initial rates of the indicated fusion reactions in the presence of 1 mM EGTA or CaCl\(^{2+}\). Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.

FIGURE 6: The stimulatory activity of Doc2b in the fusion reaction requires intact Ca\(^{2+}\) sites on both the C2A and C2B domains. (A) Sequences showing the aspartic acid residues in the Doc2b C2A and C2B domains that coordinate Ca\(^{2+}\) binding. Mutated residues are indicated with amino acid numbers on the top and asterisks at the bottom. (B) Illustrations of WT and mutant Doc2b proteins used in the fusion reactions. C2 domains with Ca\(^{2+}\)-binding sites mutated are marked with asterisks. WT C2 domains are shown in yellow, and mutant C2 domains are shown in pink and marked with asterisks. CD spectroscopic analysis demonstrated that these Ca\(^{2+}\)-binding mutations do not affect the folding of the C2 domains (Pang et al., 2011). (C) Initial rates of the indicated fusion reactions in the presence of 1 mM EGTA or CaCl\(^{2+}\). Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.

Chapman, 2008; Martens and McMahon, 2008). In synaptic vesicle fusion, this Ca\(^{2+}\)-dependent membrane penetration allows synaptotagmin-1 to locally induce membrane curvature, which is critical to its stimulatory activity in fusion (Martens et al., 2007; Lynch et al., 2008; Hui et al., 2009). However, it remains unclear whether membrane bending constitutes a common mechanism of exocytic Ca\(^{2+}\) sensors. Using electron microscopy (EM), we observed that Doc2b strongly bent lipid membranes in the presence of Ca\(^{2+}\) (Figure 7C), consistent with a previous report (Groffen et al., 2010). To examine the functional role of this membrane-bending activity, we mutated the bilayer-penetrating hydrophobic residues of Doc2b. Structural modeling suggests that the hydrophobic residues N159/F222 (C2A) and N299/I360 (C2B) are located at the tips of the membrane-binding loops that insert into the surface of membrane bilayers (Figure 7A). We mutated these hydrophobic residues into alamines (Figure 7A) and then used EM to test how the mutations affect the membrane-bending activity of Doc2b. We found that the Doc2b mutant bearing mutations in the C2A domain lost the ability to induce membrane curvature (Figure 7C). By contrast, mutation of the C2B domain had little effect on the membrane-bending function of Doc2b (Figure 7C). As expected, mutations of both the C2A and C2B domains abrogated the membrane-bending activity of Doc2b (Figure 7C). These data demonstrate that the hydrophobic residues N159/F222 in C2A are critical to the membrane-bending function of Doc2b. Using a liposome coflotation assay, we found that the membrane-bending-defective Doc2b mutants failed to interact with the membrane bilayer (Supplemental Figure S7). By contrast, none of these mutations affected the association of Doc2b with the GLUT4 exocytic SNAREs (Supplemental Figure S8), indicating that the mutations specifically interfere with the curvature-inducing function of Doc2b. Of importance, CD spectrum measurements demonstrate that mutations of the hydrophobic residues do not affect the overall folding of the Doc2b proteins (Figure 7B).

Next we examined how these Doc2b mutants regulate the SNARE-dependent fusion reaction. We observed that the Doc2b mutant bearing substitutions in the C2A domain failed to accelerate the fusion reaction, whereas the stimulatory activity of the C2B domain mutant was comparable to that of wild-type (WT) Doc2b (Figure 7D). As expected, the Doc2b mutant bearing substitutions in both of the C2 domains was defective in accelerating the fusion reaction.
FIGURE 7: The stimulatory function of Doc2b involves membrane curvature induction. (A) Top, model depicting the binding of Doc2b to the membrane. The two hydrophobic loops (arrows) of the C2 domain insert into the lipid bilayer in the presence of Ca\(^{2+}\) ions (red balls). Modeled from the crystal structure of synaptotagmin-1 C2A domain (PDB: 1BYN) and C2B domain (PDB: 1K5W; Shao et al., 1998; Fernandez et al., 2001). Bottom, sequences of hydrophobic residues in the C2A and C2B domains of Doc2b that are predicted to embed in the lipid bilayer. Mutated residues are indicated with amino acid numbers on the top and asterisks at the bottom. (B) Top, illustrations of WT and mutant Doc2b proteins labeled with M. Bottom, CD spectroscopic analysis of WT and mutant Doc2b proteins. (C) Electron micrographs showing the bending of Folch liposomes by Doc2b in the presence of 1 mM EGTA or CaCl\(_2\). (D) Initial rates of the indicated fusion reactions in the presence of 1 mM EGTA or CaCl\(_2\). Each fusion reaction contained 5 μM t-SNAREs and 1.5 μM v-SNARE. Data are presented as percentage of fluorescence change per 10 min. Error bars, SD.

(Figure 7D). We also mutated the hydrophobic N159/F222 residues of the C2A domain into the bulky tryptophans (Supplemental Figure S9A). We found that the Doc2b tryptophan mutant bound to the membrane bilayer and strongly induced membrane curvature in a Ca\(^{2+}\)-dependent manner (Supplemental Figure S9, B and C). The Doc2b tryptophan mutant strongly accelerated SNAP25-mediated membrane fusion in the presence of Ca\(^{2+}\) (Supplemental Figure S9D). Therefore the stimulatory functions of Doc2b mutants correlate with their abilities to bend lipid bilayers (Figure 7C), indicating that the stimulation of fusion by Doc2b involves membrane curvature induction. Taken together with previous findings of synaptotagmin-1 (Martens et al., 2007; Lynch et al., 2008; Hui et al., 2009), our data suggest that membrane curvature induction likely represents a common mechanism of exocytic Ca\(^{2+}\) sensors in regulating vesicle fusion.

DISCUSSION

Decades of genetic and physiological research have firmly established the essential role of insulin-controlled GLUT4 exocytosis in maintaining blood glucose homeostasis. However, the molecular basis of the GLUT4 exocytic pathway remains poorly understood. It poses significant challenges to delineate complex membrane trafficking pathways that require the dynamic assembly of multiple layers of functional units at membrane–cytosol interfaces. Fusion regulators often operate at similar or overlapping steps of the fusion reaction such that they might exhibit identical loss-of-function phenotypes in vivo, precluding further mechanistic insights (Sudhof, 2004). Traditional biochemical assays (e.g., pull-down and immunoprecipitation), on the other hand, mostly use detergent-solubilized or truncated proteins, which are trapped in dead-end conformations and often behave differently from the native proteins (Jahn, 2004).

We sought to dissect the GLUT4 exocytic pathway from a novel angle by reconstituting GLUT4 vesicle fusion in vitro using purified components. This SNARE-dependent defined fusion system is well suited for addressing the fundamental questions of regulated exocytosis because the protein composition and topology can be precisely controlled. Regulatory factors can be individually added or perturbed without the complications of other molecules naturally present in the cell, allowing their kinetic effects on fusion to be causally established.

In this study, we characterized the soluble Ca\(^{2+}\) sensor Doc2b in GLUT4 exocytosis. Doc2b is critical to insulin-triggered GLUT4 exocytosis, but its molecular mechanism of action has been unclear. When reconstituted into a FRET-based proteoliposome
fusión, GLUT4 exocytosis SNAREs—synaptin-4, SNAP-23, and VAMP2—mede a basal level of membrane fusion. We discovered that Doc2b binds to the GLUT4 exocytic SNAREs and strongly accelerates the fusion kinetics in the presence of Ca\(^{2+}\) ions. Dose-dependence analysis suggests that Doc2b may bind stoichiometrically to GLUT4 exocytic SNAREs during the fusion reaction. The stimulatory activity of Doc2b is compatible with multiple v-SNARE isoforms, including VAMP2, VAMP3, and VAMP8, in agreement with the physiological observations that these v-SNAREs support GLUT4 exocytosis in vivo (Zhuo et al., 2009). Doc2b also binds to syntaxin-4 monomer, but the Doc2b-syntaxin-4 heterodimer does not inhibit t-SNARE assembly or the fusion kinetics, suggesting that Doc2b does not regulate t-SNARE complex assembly. Of interest, the Sec1/Munc18 (SM) protein Munc18c appears to act at similar stages of the SNARE pathway as Doc2b in GLUT4 exocytosis (Jewell et al., 2008; Ramalingam et al., 2012). It is possible that Doc2b binds to the t-SNAREs and induces a local curvature on the target membrane, whereas Munc18c binds to the ternary SNARE complex and promotes the zipping of the trans-SNARE complex. Together Munc18c and Doc2b may form a supracomplex that positively regulates the SNARE-mediated vesicle fusion. It is also possible that Doc2b plays an additional role in fusion by regulating the interaction between Munc18c and syntaxin-4 monomer.

The stimulatory function of Doc2b in the reconstituted SNARE-dependent fusion reaction is strictly dependent on the presence of Ca\(^{2+}\) ions, in agreement with the proposed Ca\(^{2+}\)-sensing role of Doc2b in GLUT4 exocytosis (Fukuda et al., 2009). We discovered that Doc2b uses both the C2A and C2B domains to sense Ca\(^{2+}\). Of interest, the synaptic Ca\(^{2+}\) sensor synaptotagmin-1 requires active Ca\(^{2+}\)-binding sites only in one of its C2 domains, whereas Ca\(^{2+}\) binding to the other C2 domain is largely dispensable (Bhalla et al., 2005; Stein et al., 2007). Thus the C2 domain requirement of Doc2b in GLUT4 exocytosis is distinct from that of synaptotagmin-1 in synaptic vesicle fusion. Another notable difference between Doc2b and synaptotagmin-1 is the topological restriction of their regulatory functions. Like Doc2b, synaptotagmin-1 binds to both SNAREs and membrane bilayers (Chapman, 2008; Martens and McMahon, 2008). However, synaptotagmin-1 is a transmembrane vesicular protein that promotes synaptic vesicle fusion only when localized to vesicles (on the same membrane as the v-SNARE; Mahal et al., 2002; Wang et al., 2011). By contrast, Doc2b is a soluble protein that is recruited to the membrane through binding to SNAREs and phospholipids, intrinsically lacking the topological restriction of synaptotagmin-1. Hence, despite their analogous functions in exocytic Ca\(^{2+}\) sensing, Doc2b and synaptotagmin-1 likely differ in certain features of their regulatory activities.

Doc2 family proteins are also implicated in synaptic neurotransmitter release. It was suggested that Doc2 promotes synaptic vesicle fusion in a Ca\(^{2+}\)-dependent manner (Groffen et al., 2010; Yao et al., 2011). However, in another study, Doc2b was found to regulate synaptic release as a Ca\(^{2+}\)-independent adaptor (Pang et al., 2011). Attempts to solve this discrepancy are impeded by the coexistence of multiple double–C2 domain factors at synaptic terminals, including synaptotagmin-1, 2, and 9, as well as Doc2 proteins (Martens and McMahon, 2008; Walter et al., 2011). By contrast, Doc2b is the only double–C2 domain factor involved in GLUT4 exocytosis, presenting a unique opportunity for dissecting its molecular function in regulated exocytosis. Our findings provide the molecular explanation of the Ca\(^{2+}\)-dependent stimulatory function of Doc2b observed in GLUT4 exocytosis (Fukuda et al., 2009; Ramalingam et al., 2012). It would be interesting to determine whether Doc2b also harbors a second, Ca\(^{2+}\)-independent function in GLUT4 exocytosis.

The final step of the membrane fusion reaction is to merge the two separate lipid bilayers into one (Jahn and Scheller, 2006; Martens and McMahon, 2008). Bilayer merging, however, is opposed by powerful hydrophobic forces, and, for fusion to occur, a high energy barrier must be overcome (Hanson et al., 1997; Martens and McMahon, 2008; van der Bliek, 2009). A major way to overcome this energy barrier is believed to involve the induction of extreme membrane curvature (membrane bending; Chernomordik and Kozlov, 2005; Kozlov et al., 2010; McMahon et al., 2010). Membrane bending results in a curved “dimple” structure that points toward the apposed bilayer. The tip of the dimple is under strong curvature stress, which reduces the energy barrier for membrane fusion and therefore promotes fusion kinetics (Chernomordik and Kozlov, 2005; Graham and Kozlov, 2010). In synaptic vesicle fusion, synaptotagmin-1 has been shown to promote the fusion reaction by bending the lipid bilayer (Martens et al., 2007; Lynch et al., 2008; Hui et al., 2009). However, it is unclear whether this membrane-bending function constitutes a general mechanism for exocytic Ca\(^{2+}\) sensors or represents a specialized function of synaptotagmin-1 in synaptic release. To address this question, it is necessary to dissect a distinct Ca\(^{2+}\) sensor in a nonsynaptic exocytic pathway. Our functional studies of Doc2b indicate that its stimulatory function in the SNARE-dependent vesicle fusion depends on its membrane-bending activity. Doc2b mutants defective in membrane curvature induction can still bind SNAREs but fail to stimulate the fusion kinetics. These data suggest that membrane curvature induction likely represents a common mechanism for the regulatory functions of exocytic Ca\(^{2+}\) sensors in vesicle fusion.

Taken together, these findings establish the molecular mechanism by which Doc2b promotes the SNARE-dependent GLUT4 exocytosis. Furthermore, our findings suggest that exocytic Ca\(^{2+}\) sensors possess both common and specialized mechanisms in regulating vesicle fusion. This study will also serve as a paradigm for dissecting how protein–protein networks mediate and regulate GLUT4 exocytosis in body glucose homeostasis.

**MATERIALS AND METHODS**

**Protein expression and purification**

All recombinant proteins were expressed in *Escherichia coli* and purified by nickel affinity chromatography. GLUT4 exocytic t-SNAREs were composed of an untagged syntaxin-4 subunit and the SNAP-23 protein with an N-terminal hexahistidine tag. The v-SNAREs VAMP3 and VAMP8 were expressed in a similar way as VAMP2 (Shen et al., 2010) and had no extra residues left after the tags were proteolytically removed. SNAREs were stored in a buffer containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4), 400 mM KCl, 1% n-octyl-β-D-glucoside, 10% glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine. Recombinant rat Doc2b (amino acids 125–412) was expressed and purified from *E. coli* in a similar way as described for the synaptic factor Munc18-1 (Shen et al., 2007, 2010). Doc2b mutants were generated by site-directed mutagenesis and expressed following the same procedure as for the wild-type protein.

**Proteoliposome reconstitution**

Proteoliposomes were prepared using an improved reconstitution procedure known to yield homogeneous populations of proteoliposomes that exhibit similar fusion properties as native membranes (Takamori et al., 2006; Holt et al., 2008; Rathore et al., 2010; Shen et al., 2010). All lipids were obtained from Avanti Polar.
Lipids (Alabaster, AL). For t-SNARE reconstitution, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, POPC, POPE, POPS, cholesterol, (N-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine, and N-(Lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine) were mixed at a molar ratio of 60:17:10:10:1:5:1:5. SNARE proteoliposomes were prepared by detergent dilution and isolated on a Nycodenz (Axis-Shield, Oslo, Norway) density gradient flotation (Shen et al., 2010). Complete detergent removal was achieved by overnight dialysis of the samples in Novagen (Gibbstown, NJ) dialysis tubes against the reconstitution buffer (25 mM HEPES, pH 7.4, 100 mM KCl, 10% glycerol, and 1 mM dithiothreitol [DTT]). On SNARE liposomes, the protein:lipid ratio was 1:200 for v-SNAREs and 1:500 for t-SNARE liposomes. Reconstituted liposomes were routinely monitored by electron microscopy with negative staining and dynamic light scattering.

**Liposome fusion assay**

Fusion reactions and data analysis were performed as previously described (Shen et al., 2010). A standard fusion reaction contained 45 μl of unlabeled t-SNARE liposomes and 5 μl of labeled v-SNARE liposomes and was conducted in a 96-well Nunc plate (Nunc/Thermo Fisher Scientific, Rochester, NY) at 37°C. The fusion reaction was carried out in a reaction buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10% glycerol, and 1 mM dithiothreitol [DTT]). On SNARE liposomes, the protein:lipid ratio was 1:200 for v-SNAREs and 1:500 for t-SNARE liposomes. Reconstituted liposomes were routinely monitored by electron microscopy with negative staining and dynamic light scattering.

**Liposome cofloation assay**

Binding of Doc2b to liposomes was performed using a cofloation assay essentially in the same manner as previously described (Shen et al., 2007). Doc2b was incubated with liposomes at 4°C with gentle agitation in the presence of 1 mM ethylene glycol tetraacetic acid or CaCl2. After 1 h, an equal volume of 80% Nycodenz (wt/vol) in reconstitution buffer was added and transferred to 5 × 41 mm centrifuge tubes. The liposomes were overlaid with 20 μl of 35 and 30% Nycodenz and then with 20 μl of reconstitution buffer on top. The gradients were centrifuged for 4 h at 52,000 rpm in a Beckman SW55 rotor (Beckman Coulter, Brea, CA). Samples were collected from the 0/30% Nycodenz interface (2 × 20 μl) and analyzed by SDS-PAGE.

**Circular dichroism spectroscopy**

CD spectra were measured using a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a 1-mm quartz cell. The readings were made at 0.1-nm intervals, and each data point represented the average of six scans at a speed of 50 nm/min over the wavelength range 200–260 nm. The data were converted into mean-residue-weighted molar ellipticity using the equation

\[
[\theta]_{MRW} = 1000C/\text{m}, \quad \theta = \text{the measured ellipticity (mdeg), } n = \text{the number of residues, and } l = \text{the path length (cm)}.
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**Electron microscopy**

EM imaging of liposomes was carried out at the Boulder Laboratory for 3-D Electron Microscopy of Cells (Boulder, CO). Brain lipid extract (Folch fraction I; Sigma-Aldrich, St. Louis, MO) was dried and resuspended at 1 mg/ml in HEPES-buffered saline (50 mM HEPES, pH 7.4, and 100 mM NaCl). The resuspended lipid bilayers were then sonicated for 10 min to generate Folch liposomes. Folch liposomes (0.3 mg/ml) were incubated with WT or mutant Doc2b proteins (10 μM) in the presence of 1 mM EDTA or CaCl2 for 10 h at room temperature. Subsequently the samples were stained with 2% uranyl acetate and observed under a Philips CM100 transmission electron microscope (Philips, Amsterdam, Netherlands).

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Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Smith CA, Bennett ME, Brandie FM, Aran V, Verma A, McNew JA, Bryant NJ, Gould GW (2008). Complete detergent removal was achieved by overnight dialysis of the samples in Novagen (Gibbstown, NJ) dialysis tubes against the reconstitution buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10% glycerol, and 1 mM dithiothreitol [DTT]). On SNARE liposomes, the protein:lipid ratio was 1:200 for v-SNAREs and 1:500 for t-SNARE liposomes. Reconstituted liposomes were routinely monitored by electron microscopy with negative staining and dynamic light scattering.

**Liposome fusion assay**

Fusion reactions and data analysis were performed as previously described (Shen et al., 2010). A standard fusion reaction contained 45 μl of unlabeled t-SNARE liposomes and 5 μl of labeled v-SNARE liposomes and was conducted in a 96-well Nunc plate (Nunc/Thermo Fisher Scientific, Rochester, NY) at 37°C. The fusion reaction was carried out in a reaction buffer (25 mM HEPES, pH 7.4, 50 mM KCl, 10% glycerol, and 1 mM dithiothreitol [DTT]). Fusion was followed by measuring the increase in NBD fluorescence at 538 nm (excitation 460 nm) every 2 min in a BioTek Synergy HT microplate reader (BioTek, Winooski, VT). At the end of the reaction, 10 μl of 2.5% dodecylmaltoside was added to the liposomes. Fusion data were presented as the percentage of maximum fluorescence change. To assess the regulatory activity of Doc2b, we incubated v- and t-SNARE liposomes with or without 5 μM Doc2b at 37°C. The maximum fusion rate within the first 10 min of liposome fusion was used to represent the initial rate of a fusion reaction. In fusion reactions with decreases in initial fluorescence (due to temperature change), the phase of fluorescence decrease was omitted from the calculation. Full accounting of statistical significance was included for each figure based on at least three independent experiments.

**Liposome cofloation assay**

Binding of Doc2b to liposomes was performed using a cofloation assay essentially in the same manner as previously described (Shen et al., 2007). Doc2b was incubated with liposomes at 4°C with gentle agitation in the presence of 1 mM ethylene glycol tetraacetic acid or CaCl2. After 1 h, an equal volume of 80% Nycodenz (wt/vol) in reconstitution buffer was added and transferred to 5 × 41 mm centrifuge tubes. The liposomes were overlaid with 200 μl each of 35 and 30% Nycodenz and then with 20 μl of reconstitution buffer on the top. The gradients were centrifuged for 4 h at 52,000 rpm in a Beckman SW55 rotor (Beckman Coulter, Brea, CA). Samples were collected from the 0/30% Nycodenz interface (2 × 20 μl) and analyzed by SDS-PAGE.

**Circular dichroism spectroscopy**

CD spectra were measured using a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a 1-mm quartz cell. The readings were made at 0.1-nm intervals, and each data point represented the average of six scans at a speed of 50 nm/min over the wavelength range 200–260 nm. The data were converted into mean-residue-weighted molar ellipticity using the equation

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[\theta]_{MRW} = 1000C/\text{m}, \quad \theta = \text{the measured ellipticity (mdeg), } n = \text{the number of residues, and } l = \text{the path length (cm)}.
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