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Synaptotagmin-1 Docks Secretory Vesicles to Syntaxin-1/SNAP-25 Acceptor Complexes

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SUMMARY

Docking, the initial association of secretory vesicles with the plasma membrane, precedes formation of the SNARE complex, which drives membrane fusion. For many years, the molecular identity of the docked state, and especially the vesicular docking protein, has been unknown, as has the link to SNARE complex assembly. Here, using adrenal chromaffin cells, we identify the vesicular docking partner as synaptotagmin-1, the calcium sensor for exocytosis, and SNAP-25 as an essential plasma membrane docking factor, which, together with the previously known docking factors Munc18-1 and syntaxin, form the minimal docking machinery. Moreover, we show that the requirement for Munc18-1 in docking, but not fusion, can be overcome by stabilizing syntaxin/SNAP-25 acceptor complexes. These findings, together with cross-rescue, double-knockout, and electrophysiological data, lead us to propose that vesicles dock when synaptotagmin-1 binds to syntaxin/SNAP-25 acceptor complexes, whereas Munc18-1 is required for the downstream association of synaptobrevin to form fusogenic SNARE complexes.

INTRODUCTION

Calcium-dependent exocytosis of synaptic or secretory vesicles can be elicited within fractions of a millisecond upon calcium influx into the releasing cell. In order to achieve such exquisite temporal precision, secretory vesicles undergo a number of maturation steps before calcium influx, leaving them in a semistable so-called "release-ready" or primed state, where membrane fusion can be fast. In electron micrographs of neurosecretory cells, many secretory vesicles are found docked at the target membrane; however, their number typically exceeds the number of release-ready vesicles. Docking thus appears to be an intermediate maturation state of a vesicle, immediately preceding the step at which vesicles become release ready. Whereas a picture of the release-ready vesicle is emerging, the molecular mechanism of docking and its connection to the priming and fusion reaction remains unresolved (for a review, see Verhage and Sørensen, 2008).

Two proteins have been firmly implicated in docking, Munc18-1 (Voets et al., 2001b) and syntaxin-1 (de Wit et al., 2006). Deficiency of either of these proteins produced robust docking phenotypes, and in agreement with several other findings (Hammarlund et al., 2007; Weimer et al., 2003), syntaxin-1 and Munc18-1 are now widely accepted as docking factors (Verhage and Sørensen, 2008). In addition to their role in docking, syntaxin-1 and Munc18-1 also have essential functions downstream of docking. Syntaxin-1 forms, together with synaptosome-associated protein of 25 kDa (SNAP-25) and the vesicle-associated membrane protein-2 (VAMP-2)/synaptobrevin-2, the core SNARE (soluble N-ethylmaleimide-sensitive factor [NSF]-attachment protein receptor) complex (Jahn and Scheller, 2006; Rizo and Rosenmund, 2008). The formation of this complex between vesicle and plasma membrane appears to underlie the priming reaction, and final C-terminal assembly coincides with fusion triggering (Sørensen et al., 2006). In addition to syntaxin-1 and Munc18-1, several other proteins have been implicated in docking in several types of secretory cells, such as rab3 and rab27, rabphilin3A, granuphilin, and exophilin4/Slp2a, and function mutations in several priming genes in *C. elegans* (*RIM/unc-10*, *(M)unc-13,* or *CAPS/unc-31*) produce a strong reduction of vesicles with a "contact patch" (for a review, see Verhage and Sørensen, 2008).

It is known that Munc18-1 interacts with neuronal SNARE proteins in two distinct modes: i.e., with isolated syntaxin-1 alone in a "closed" conformation and with assembled SNARE

complexes containing syntaxin-1 in an ''open'' conformation (Toonen and Verhage, 2007). Munc18-1 binding to the assembled SNARE complex involves an interaction with the N-terminal H(abc) domain of syntaxin-1 and the four-helical bundle of the assembled SNARE complex (Dulubova et al., 2007). Recently, it was shown that Munc18-1 binding to assembled neuronal SNARE complexes enhanced membrane fusion in an in vitro liposome assay (Shen et al., 2007), whereas in the calyx of Held synapse, Munc18-1 binding to the N-terminal of syntaxin-1 is essential for exocytic membrane fusion (Khvotchev et al., 2007).

Prior to binding of synaptobrevin-2 to syntaxin-1/SNAP-25 at the target membrane, syntaxin-1 is believed to form a 1:1 heterodimer with SNAP-25 as an intermediate step (Fasshauer and Margittai, 2004; Zilly et al., 2006). Stabilization of these ''acceptor'' 1:1 syntaxin-1/SNAP-25 complexes is important for the acceleration of fusion in vitro (Pobbati et al., 2006). Recent studies (Zilly et al., 2006) suggest that Munc18-1 controls the assembly of syntaxin-1/SNAP-25 heterodimers and may even promote the formation of this ''acceptor'' complex relative to a proposed nonproductive syntaxin-1/SNAP-25 2:1 complex (''off pathway'' [Fasshauer et al., 1997; Xiao et al., 2001; Zilly et al., 2006]); however, whether this plays a role for exocytosis in vivo is unknown. Hence, syntaxin-1 and Munc18-1 probably operate at the receiving end of the docking process, but it is unclear how and which vesicular partner(s) and other proteins are involved.

Docking of secretory vesicles to the target membrane must involve vesicular proteins. While the proteins present on synaptic vesicles have been systematically identified (Jahn and Scheller, 2006), none of these has been assigned as a docking factor. Biochemical evidence suggest that at least two vesicle proteins bind to established docking factors on the plasma membrane and can therefore be considered candidate docking factors: synaptobrevin-2 and synaptotagmin-1 (Chieregatti et al., 2002; Rickman et al., 2004; Schiavo et al., 1997; Söllner et al., 1993). We recently confirmed that the *synaptobrevin-2* null mutation does not produce docking phenotypes similar to *munc18-1* or *syntaxin-1* null (Gerber et al., 2008), consistent with earlier findings (Borisovska et al., 2005). Therefore, synaptotagmin-1 seems to be the prime candidate for a vesicular docking protein, but evidence for this hypothesis is lacking.

In the present study, we addressed the involvement of the syntaxin-1/SNAP-25 acceptor complex and binding of vesicular synaptotagmin-1 to this complex using (mutant) mouse embryonic chromaffin cells as a model. Mouse embryonic chromaffin cells are a preferred model to study docking, because vesicles are sparse and around 40%–50% of them found docked to the plasma membrane, making docking phenotypes more evident than in other systems. We have analyzed null mutants for the genes encoding these four proteins and studied the effects of rescue and cross-rescue using electron microscopy and cell physiology. We identify two docking factors, synaptotagmin-1 and SNAP-25, and show that stabilizing syntaxin-1/SNAP-25 acceptor complexes rescue the docking defect in *munc18-1* null mutants. Together with published data, this allows us to synthesize a minimal docking model.

RESULTS

SNAP-25 Overexpression Rescues the Docking Phenotype in Munc18-1-Deficient Cells

Recent in vitro studies (Zilly et al., 2006) suggest that Munc18-1 promotes the formation of the syntaxin-1/SNAP-25 ''acceptor'' complex relative to a proposed nonproductive syntaxin-1/SNAP-25 2:1 complex (''off-pathway'' [Fasshauer et al., 1997; Xiao et al., 2001; Zilly et al., 2006]). We tested the possibility that in the absence of Munc18-1 too few acceptor SNARE complexes (1:1) exist in the cell to dock vesicles by overexpressing SNAP-25 on a *munc18-1* null background, thereby favoring the formation of 1:1 complexes by law of mass action (Pobbati et al., 2006). Strikingly, SNAP-25 overexpression fully restored docking in the absence of Munc18-1 (Figures 1A, 1C, and 1D), without affecting cell morphology and total vesicle number (Figure S1 and Table S1 available online). Vesicle fusion was assayed with a combination of flash photorelease of caged-calcium to trigger fusion and membrane capacitance and amperometric measurements to monitor exocytosis (Figure 1E). SNAP-25 overexpression did not restore vesicle fusion (Figures 1E and 1F), neither during the burst phase (within 1 s of releasing calcium), nor during the sustained phase (between 1 and 5 s after calcium release), consistent with previous findings that Munc18-1 has an additional role downstream of docking (Gulyás-Kovács et al., 2007).

The cellular level of syntaxin-1 is reduced to about 50% in *munc18-1* null chromaffin cells, which, however, cannot explain the docking defect in the absence of Munc18-1 (Gulyás-Kovács et al., 2007). Interestingly, quantification of plasma membrane syntaxin-1-levels after SNAP-25 overexpression revealed a partial recovery (Figure S2), suggesting that the expressed SNAP-25 is in fact binding and stabilizing syntaxin-1. As an additional control, we overexpressed another syntaxin-1 binding protein, Munc13-1. Overexpression of Munc13-1 was previously shown to enhance secretion in chromaffin cells (Ashery et al., 2000), and Munc13-1 is required for docking in the nematode *C. elegans* neurons (Hammarlund et al., 2007). However, Munc13-1 overexpression failed to rescue the docking phenotype in *munc18-1* null cells (Figures 1C and 1D). Hence, cellular factors that interact with syntaxin-1 and promote secretion are not sufficient to rescue the *munc18-1* null phenotype.

A Synaptobrevin Fragment Rescues the Docking Phenotype in *munc18-1*-Deficient Cells

To stabilize syntaxin-1/SNAP-25 acceptor complexes in a different way, we expressed a C-terminal 49–96 fragment of synaptobrevin-2 (SybCT) in *munc18-1* null cells. This fragment, which is displaced by full-length synaptobrevin, blocks formation of proposed nonproductive 2:1 syntaxin-1/SNAP-25 complexes, thereby accelerating fusion in liposome-fusion experiments in vitro (Pobbati et al., 2006). In addition, C-terminal synaptobrevin fragments also help to structure the membrane-proximal portion of acceptor complexes and prevent association of the N-terminal domain of syntaxin (Melia et al., 2002). Indeed, the SybCT fragment, like SNAP-25 overexpression, rescued the docking phenotype in *munc18-1* null cells (Figures 2B–2D and S1). As a control, an N-terminal 1–70 peptide (SybNT), which

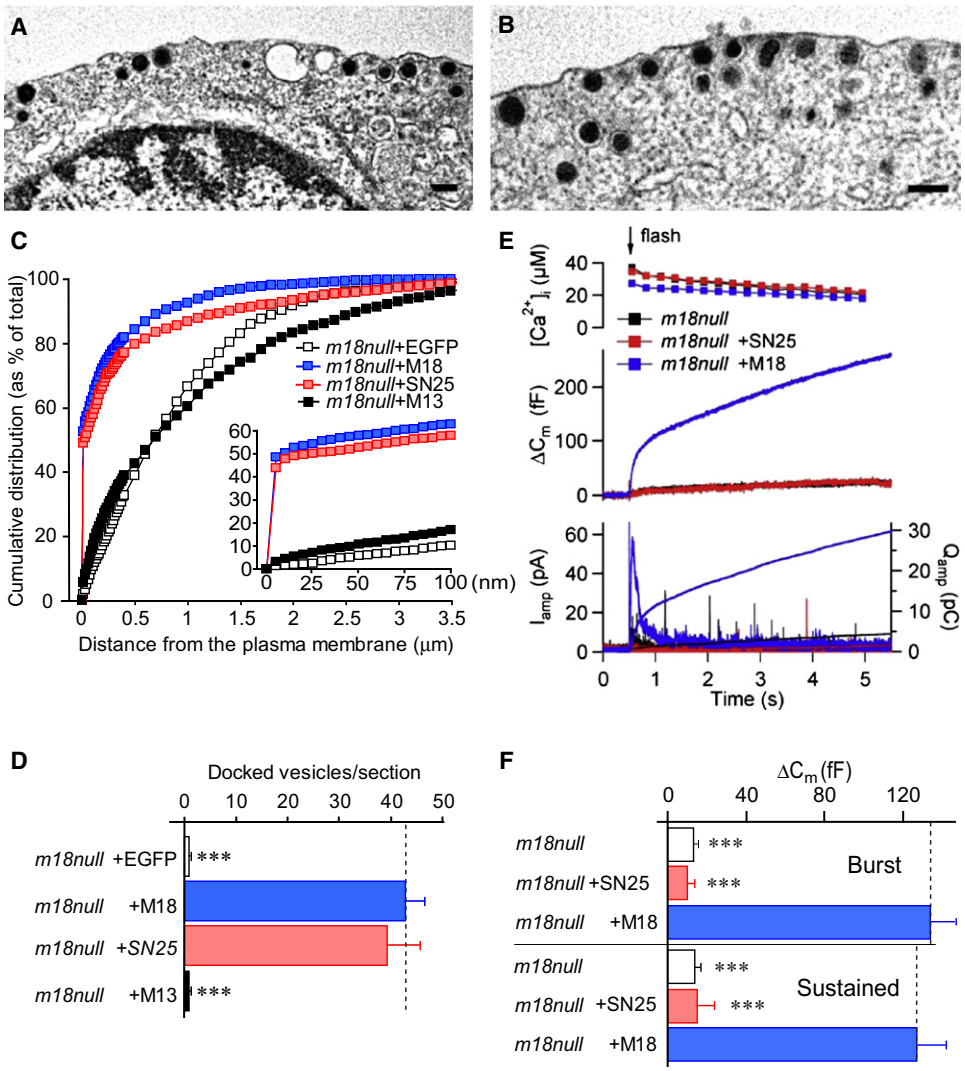

Figure 1. SNAP-25 Rescues Docking in *munc18-1* Null Chromaffin Cells

(A and B) Electron micrographs from cultured *munc18-1* null chromaffin cells expressing SNAP-25 (A) or Munc18-1 (B). The scale bar represents 200 nm.

(C) Normalized cumulative distribution of vesicles as a function of distance from the plasma membrane. The inset shows cumulative vesicle distribution in the submembrane region within 0–100 nm.

(D) Number of docked vesicles per section. In (C) and (D), Munc13-1 expression was used as a control. See Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are shown as means ± SEM; ***p < 0.001 by Student's t test compared to controls: *munc18-1* null + Munc18-1. SN25, SNAP-25; M18, Munc18-1; M13, Munc13-1; EGFP, enhanced green fluorescent protein.

(E and F) Release of primed secretory vesicles in response to rapid Ca^{2+} uncaging triggered by UV flash (E) and quantification (F) of burst (0–1 s) and sustained (1–5 s) phases of *munc18-1* null chromaffin cells expressing SNAP-25 or Munc18-1. See Table S2 for number of cells (n) and animals (N); data are shown as means ± SEM; for both parameters; indicated is the result of Mann-Whitney tests comparing to the rescue situation (***p < 0.001).

does not stabilize acceptor complexes or accelerate fusion (Pobbati et al., 2006), did not (Figures 2A, 2C, 2D, and S1). Similar to SNAP-25 overexpression, SybCT did not rescue secretion (Figures 2E and 2F), again consistent with previous findings that Munc18-1 has a postdocking function (Gulyás-Kovács et al., 2007; Shen et al., 2007). Neither SybCT nor SybNT affected secretion in wild-type cells (Figure S3). Together, these experiments suggest that Munc18-1 promotes docking by promoting the formation, stability, or function of syntaxin-1/SNAP-25 acceptor complexes.

Snap-25 Null Mutant Cells Have a Docking Phenotype

The involvement of syntaxin-1/SNAP-25 acceptor complexes in docking suggests a direct requirement for SNAP-25 in docking, similar to syntaxin-1. We reanalyzed docking in *Snap-25* null cells. *Snap-25*-deficient cells exhibited a strong docking phenotype (Figures 3B, 3E, 3F, 4E, and S4), which was, however, slightly less severe than in Munc18-1- or syntaxin-1-deficient cells (de Wit et al., 2006; Voets et al., 2001b). These data are not consistent with previous observations (Sørensen et al., 2003), which could be explained by differences in culture

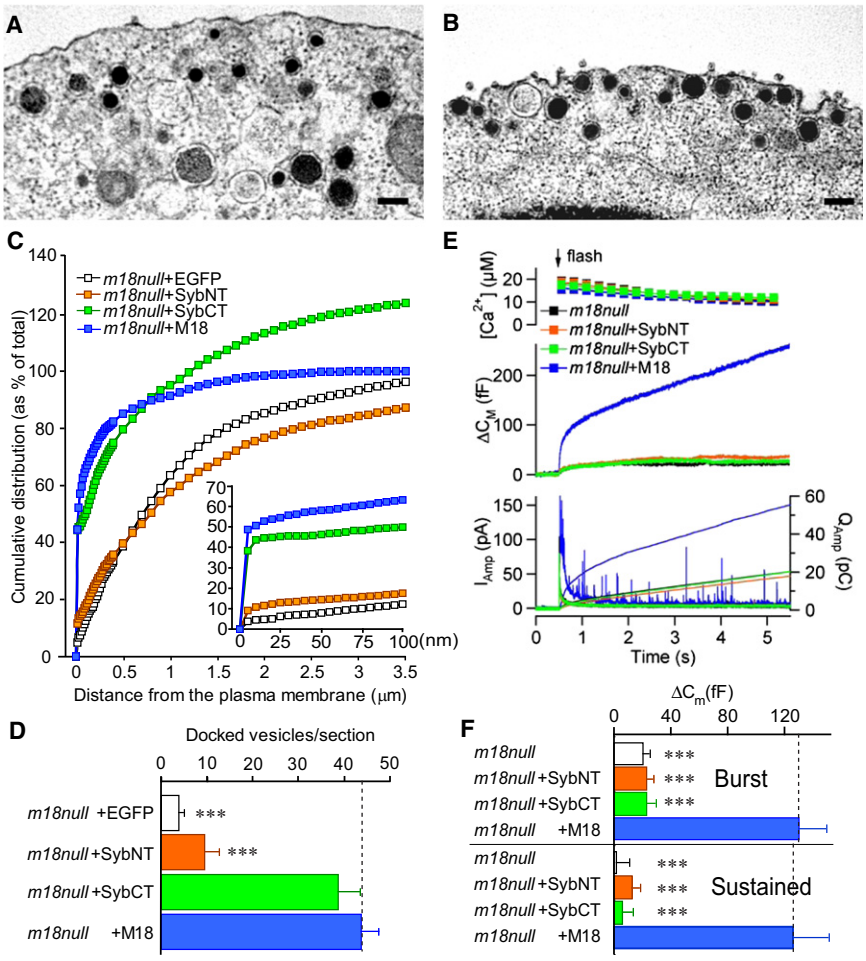

Figure 2. C-Terminal Synaptobrevin Fragment Restores Docking in the Absence of Munc18-1

(A and B) Electron micrographs from primary cultured *munc18-1* null chromaffin cells expressing either the 1–70 N-terminal (SybNT; A) or 49–96 C-terminal (SybCT; B) fragment of synaptobrevin. The scale bar represents 200 nm.

(C) Normalized cumulative distribution of vesicles as a function of distance from the plasma membrane. Inset shows cumulative vesicle distribution in the submembrane region within 0–100 nm. SybCT overexpression leads to a significantly higher total number of vesicles (see Table S1). It is conceivable that this manipulation promotes endocytosis or vesicle biogenesis, which would be consistent with previous findings (Salem et al., 1998), or inhibits fusion of intracellular organelles. Previous manipulations with synaptobrevin function have also produced changes in vesicle number (Broadie et al., 1995; Gerber et al., 2008). Because SybCT have a larger total vesicle pool, all curves were normalized to controls (*munc18-1* null + Munc18-1). Hence, instead of normalizing to the number of vesicles in each group, we normalized to one group. In this way, the curve "overshoots" at larger distances from the membrane. Normalizing of each curve separately would obscure this difference.

(D) Number of docked vesicles per section. See Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are shown as means ± SEM; ***p < 0.001 by Student's t test compared to control: *munc18-1* null + Munc18-1. SybNT and CT, N-terminal fragment 1–70 and C-terminal fragment 49–96 of synaptobrevin-2, respectively.

(E and F) Membrane capacitance responses (E) and quantification (F) of burst (0–1 s) and sustained (1–5 s) phases of SybNT and SybCT expressing *munc18-1* null chromaffin cells. See Table S2 for number of cells (n) and animals (N); data are shown as means ± SEM; ***p < 0.001 by the Mann-Whitney test compared to controls (see above).

conditions, cell treatment, or fixation methods, but cannot be explained by differences in either the definition or measurement of docked vesicles since *Snap-25* null and wild-type chromaffin cells show docking differences after using the same criteria as previously used (see Figure S4F). To circumvent the culturing procedure altogether, we also analyzed docking in intact adrenal glands and confirmed the docking phenotype (Figures 4 and S4). In addition, we analyzed docking in intact adrenal glands that were rapidly frozen under high pressure to circumvent chemical fixation artifacts. We observed the same docking difference between *Snap-25* null and wild-type chromaffin cells after rapid freezing compared to chemically fixed intact adrenal glands, as well as cultured chromaffin cells (Figures 4 and S4). Overexpression of SNAP-25 on the *Snap-25* null background rescued the phenotype (Figures 3C, 3E, 3F, and S1), whereas no increase of docking was observed after SNAP-25 overexpression in wild-type cells (Figures 3E and 3F). Strikingly, in contrast to SNAP-25 overexpression in *munc18-1* null cells, Munc18-1 overexpression in *Snap-25* null cells did not rescue the docking phenotype (Figures 3D, 3E, 3F, and S1). These observations demonstrate that SNAP-25 is essential for docking and that Munc18-1 cannot promote docking in its absence.

The total number of vesicles was significantly different between control and *Snap-25* null adrenals, but not between cultured cells of both genotypes after chemical fixation. Figure 4F indicates that this difference probably arises from a reduced number in the control rather than an increase in the mutant adrenals. Probably, the slow penetration of fixative leads to a loss of vesicles by exocytosis, but only if exocytosis is not impaired.

Vesicular Synaptotagmin-1 Provides a Link between Vesicles and Docking Complexes at the Target

Next we attempted to identify the vesicular component to dock vesicles to syntaxin-1/SNAP-25 acceptor complexes. Two candidates, which bind these complexes, have been identified:

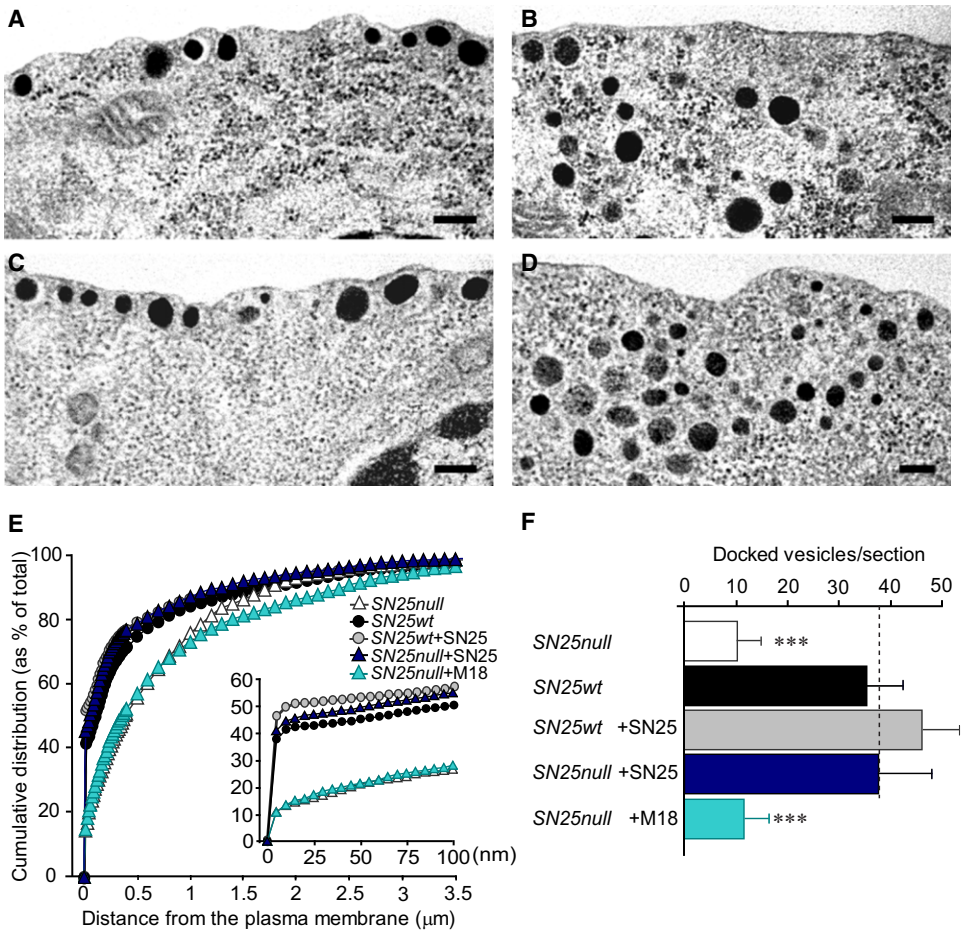

Figure 3. Munc18-1 Fails to Rescue Docking in *Snap-25* Null Chromaffin Cells

(A–D) Electron micrographs from cultured wild-type (A) and *Snap-25* null chromaffin cells either untransfected (B) or expressing either SNAP-25 (C) or Munc18-1 (D). The scale bar represents 200 nm.

(E) Normalized cumulative distribution of vesicles as a function of distance from the plasma membrane. Inset shows cumulative vesicle distribution in the submembrane region within 0–100 nm.

(F) Number of docked vesicles per section. See Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are shown as means ± SEM; ***$p < 0.001$ by Student's t test compared to controls: *Snap-25* null + SNAP-25 or wild-type littermates. SN25, SNAP-25; M18, Munc18-1.

synaptobrevin-2 and synaptotagmin-1 (Rickman et al., 2004; Schiavo et al., 1997; Söllner et al., 1993). We recently confirmed that the *synaptobrevin-2* null mutation does not produce docking phenotypes similar to *munc18-1* or *syntaxin-1* null (Gerber et al., 2008), consistent with earlier findings (Borisovska et al., 2005), leaving synaptotagmin-1 as a prime candidate. Vesicle secretion is heavily impaired in *synaptotagmin-1* null chromaffin cells (Voets et al., 2001a), but docking has not been assessed. We observed a strong docking defect in *synaptotagmin-1* null cells (Figures 5B, 5E, S1, and S5), similar to *Snap-25* null cells. The expression level of SNAP-25, as well as Rab3, Rab27, and their effector proteins Rabphilin and Granuphilin, which are also implicated in the docking step (Fukuda, 2006), were unchanged in *synaptotagmin-1* null chromaffin cells (Figure S6).

To further explore the possibility that synaptotagmin-1 provides the link between vesicles and syntaxin-1/SNAP-25 acceptor complexes, we performed four additional sets of experiments. First, we tested whether SNAP-25 overexpression still rescued the docking phenotype in cells deficient for both Munc18-1 and synaptotagmin-1. Unlike overexpression of either Munc18-1 or SNAP-25 in *munc18-1* single-null cells, overexpression of these proteins in *munc18-1/synaptotagmin-1* double-null cells no longer rescued the docking phenotype (Figures 6A, 6C, S1, and S5). This indicates that synaptotagmin-1 is required for syntaxin-1/SNAP-25-dependent docking and also excludes the possibility that SNAP-25, synaptotagmin-1, and Munc18-1 promote docking via separate (parallel) pathways.

Second, we expressed soluble tandem C2 domains (C2AB) of synaptotagmin-1 in wild-type cells, which competes with endogenous synaptotagmin-1 for binding to the syntaxin-1/SNAP-25 acceptor (Rickman et al., 2006). Indeed, expression of soluble C2AB produced a phenocopy of the *synaptotagmin-1* null (Figures 5A, 5E, S1, and S5). In contrast, expression of a soluble C2AB domain bearing a mutation that was previously reported

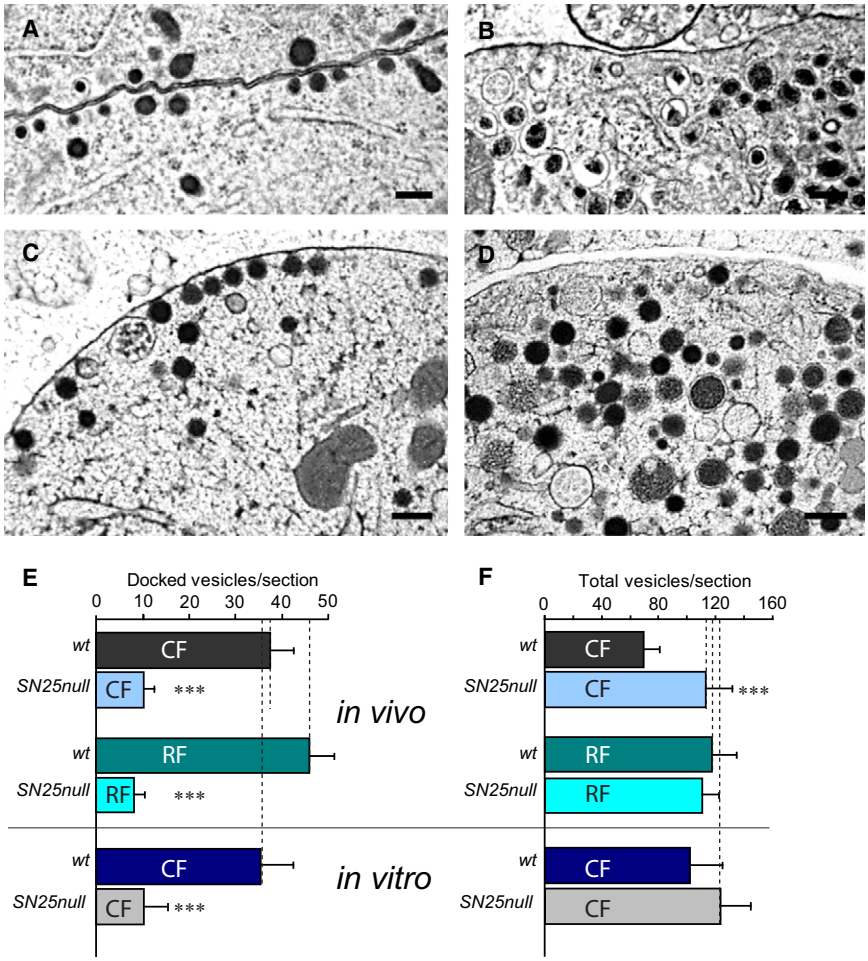

Figure 4. Docking Defect in *Snap-25* Null Mouse Chromaffin Cells In Vitro and In Vivo

(A–D) Representative electron micrographs from SNAP-25 wild-type (A and C) and *Snap-25* null littermate (B and D) chromaffin cells in an intact medulla of the adrenal gland after chemical fixation (A and B) or after high-pressure rapid freezing (C and D). The scale bar represents 200 nm.

(E) Number of docked vesicles per section. The docking phenotype in the *Snap-25* null cells is slightly less severe than in the *munc18-1* null cells, probably because SNAP-25 homologs are expressed in chromaffin cells (SNAP-23, SNAP-49) and may partially rescue the phenotype (see Sørensen et al., 2003).

(F) Total number of vesicles per section.

(E and F) Analysis from in vivo (upper four bins) and in vitro (lower two bins) preparations; see Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are shown as means ± SEM; ***p < 0.001 by Student's t test compared to the wild-type. *wt*, wild-type; SN25, SNAP-25; CF, chemical fixation; RF, high-pressure rapid freezing.

to have a reduced affinity for SNAP-25 (Y311N [Rickman et al., 2006]) did not affect docking (Figures 5A, 5E, S1, and S5), indicating that binding of synaptotagmin to SNAP-25 is indeed relevant for docking. Parallel electrophysiological analyses revealed a reduced sustained component of release in C2AB expressing cells, but no effect in cells that express C2AB$^{[Y311N]}$ (Figures 5C and 5F). The reduction in the sustained release component is consistent with a role for synaptotagmin-1 binding to SNAP-25 in vesicle recruitment. This secretion phenotype is distinct from the phenotype found in *synaptotagmin-1* null cells (Voets et al., 2001a) in that it still displays a fast component, which is probably caused by the presence of endogenous synaptotagmin-1.

Third, to resolve the possible confounding effect of endogenous synaptotagmin-1 and to confirm its specificity, we analyzed docking and secretion in *synaptotagmin-1* null cells rescued with either full-length, wild-type synaptotagmin-1 or the synaptotagmin-1 mutant with the reported reduced SNAP-25 affinity (Y311N). Both proteins were expressed >10-fold above the endogenous expression level (Figure S6). Whereas full-length synaptotagmin-1 rescued both docking and secretion as expected, the Y311N mutant failed (Figures 5B, 5D, 5E, 5F, and S5). This differential effect between the wild-type and mutant versions of synaptotagmin-1 further support the conclusion that a SNAP25-synaptotagmin interaction is a central element in vesicle docking. In addition, we observed that SNAP-25 overexpression was not able to rescue docking in *synaptotagmin-1* null cells (Figures 5B, 5E, and S5), indicating that the SNAP-25 docking acceptor is not functional in the absence of synaptotagmin-1 and also implying that other proteins, such as Slp-4 or synaptobrevin-2, are not sufficient to dock vesicles to the SNAP-25 acceptor complex.

Fourth, we addressed the question whether overexpression of SNAP-25 mutations that reduce binding to synaptotagmin-1 rescue the *munc18-1* null docking phenotype equally well as wild-type SNAP-25 (Figure 1). At least two binding epitopes for synaptotagmin-1 have been identified in SNAP-25: AA 51–55 and 179–193 (Lynch et al., 2007; Rickman et al., 2006; Zhang et al., 2002). However, these regions are also involved in other central aspects of SNAP-25 function (such as SNARE complex formation), which complicates interpretation of mutagenesis experiments to address the relevance of SNAP-25-synaptotagmin interaction for docking. Indeed, proper interactions between SNAP-25 and syntaxin-1 are probably also required for docking (see above). Therefore, we tested SNAP-25 mutations all along the SNARE bundle, including a N-terminal and a middle mutation in the interaction layers (M32A/V153A and L50A/I171A [Sørensen et al., 2006]), the 51–55 region (D51K/E52K/E55K [Lynch et al., 2007; Rickman et al., 2006; Zhang et al., 2002]), and a deletion of the C-terminal end (SNAP-25$^{[\Delta 26]}$), which correspond to cleavage by Botulinum Toxin E and which include the second proposed synaptotagmin-1 binding epitope (179–193 [Zhang et al., 2002]; Figure 6D). All four mutations produced some

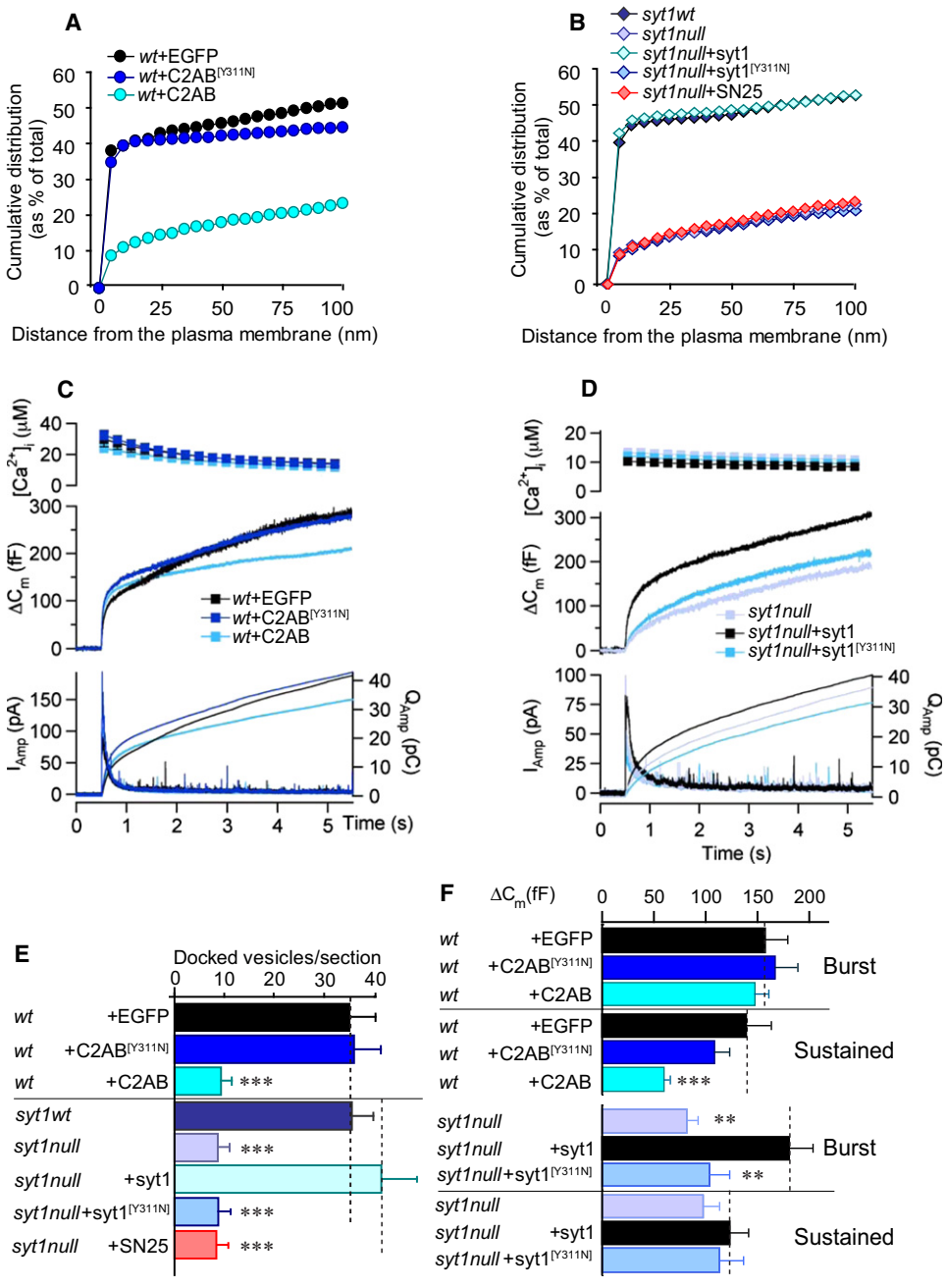

Figure 5. Synaptotagmin-1 Binding to SNAP-25 Is Essential for Docking

(A and B) Normalized cumulative distribution of vesicles in the 0–100 nm submembrane region.

(E) Number of docked vesicles per section. See Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are shown as means ± SEM; ***p < 0.001, **p < 0.01 by Student's t test compared to controls: wild-type + EGFP or wild-type synaptotagmin-1 littermates. *wt*, wild-type; C2AB and C2AB[Y311N], soluble tandem C2 domains of synaptotagmin-1 with or without Y311N mutation; syt1, full-length synaptotagmin-1; syt1[Y311N], synaptotagmin-1 bearing the Y311N mutation; SN25, SNAP-25; EGFP, enhanced green fluorescent protein.

(C, D, and F) Membrane capacitance responses (C and D) and quantification (F) of burst (0–1 s) and sustained (1–5 s) phases of wild-type cells expressing soluble C2AB or C2AB[Y311N] (C and F), and *synaptotagmin-1* null cells expressing either full-length synaptotagmin-1 or synaptotagmin-1 bearing the Y311N mutation (D and F). See Table S2 for number of cells (n) and animals (N); data are shown as means ± SEM; ***p < 0.001 by the Mann-Whitney test compared to control wild-type + EGFP; **p < 0.01 by the Mann-Whitney test compared to *synaptotagmin-1* null rescued with full-length synaptotagmin-1.

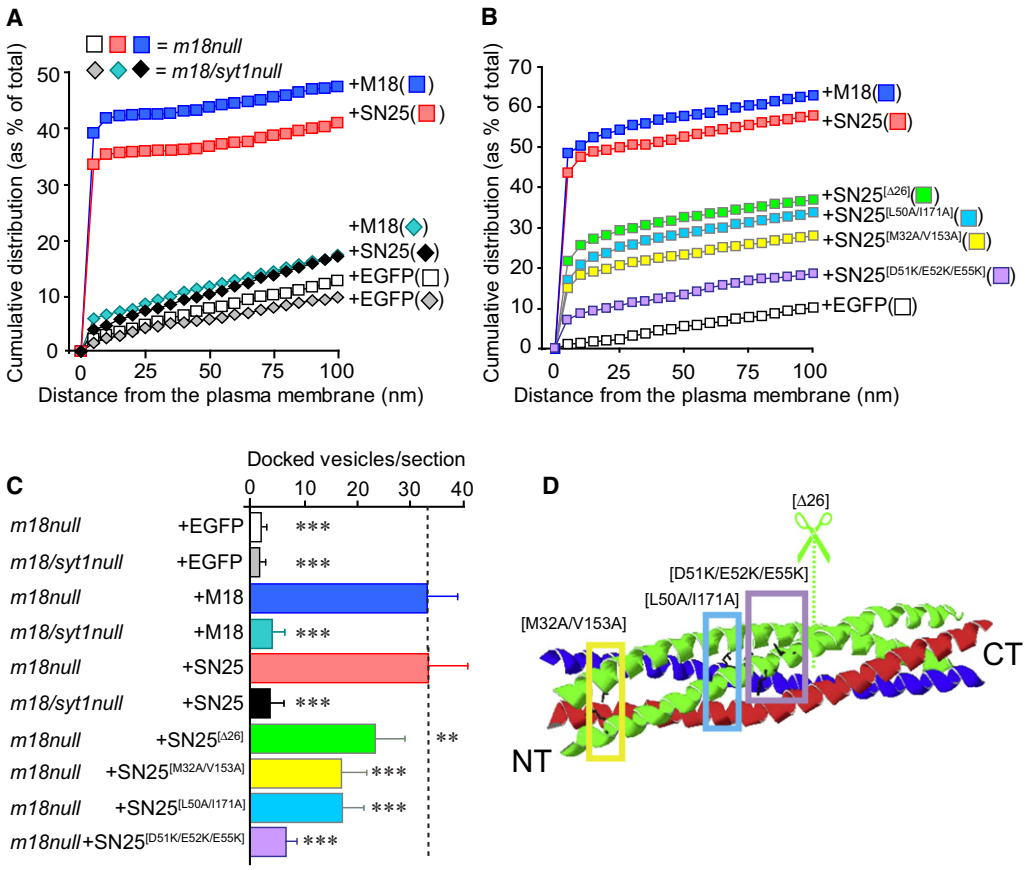

Figure 6. SNAP-25 Binding to Synaptotagmin-1 Is Required for Docking

(A and B) Normalized cumulative distribution of vesicles in the 0–100 nm submembrane region.

(C) Number of docked vesicles per section. See Table S1 for number of cells (n) and animals (N) and total number of vesicles; data are means ± SEM; ***$p < 0.001$, **$p < 0.01$ by Student's t test compared to *munc18-1* null + Munc18-1 or *munc18-1* null + SNAP-25. SN25, SNAP-25; $SN25^{[L50A/I171A]}$, SNAP-25 with alanine substitutions in layer-1; $SN25^{[M32A/V153A]}$, SNAP-25 with alanine substitutions in layer-6; $SN25^{[\Delta 26]}$, Botulinum Toxin E truncated SNAP-25; $SN25^{[D51K/E52K/E55K]}$, SNAP-25 with lysine substitutions in D51, E52, and E55; EGFP, enhanced green fluorescent protein.

(D). Graphical representation of the SNAP-25 mutations that were used in our study. Colored boxes indicate the position of the lysine substitutions in D51, E52, and E55 (magenta), as well as the alanine substitutions in layer-1 (L50A/I71A; lighter blue) and layer-6 (M32A/V153A; yellow), whereas the position of the Botulinum Toxin E truncation is marked by scissors. Blue, synaptobrevin; red, syntaxin; green, SNAP-25. NT, N terminus sites; CT, C terminus sites.

rescue of the docking phenotype, but rescue was in all cases significantly impaired compared to wild-type SNAP-25 (Figures 6B, 6C, S5, and S7). This indicates that all parts of the SNARE bundle directly or indirectly contribute to docking.

Together, these four sets of experiments indicate that binding of vesicular synaptotagmin-1 to SNAP-25, probably as part of assembled syntaxin-1/SNAP-25 acceptor complexes, docks secretory vesicles. Importantly, the experiments with synaptotagmin Y311N and with SNAP-25 mutations, which have been shown to impair secretion in chromaffin cells (Sørensen et al., 2006), show that in the presence of Munc18-1 there is a correlation between mutations that impair secretion and those that impair docking. This is not the case in the absence of Munc18-1, where docking can be fully rescued by stabilizing the 1:1 SNAP-25:syntaxin acceptor complex, while secretion remains abolished (above). Thus, Munc18-1 plays an essential postdocking role (Gulyás-Kovács et al., 2007; Shen et al., 2007).

DISCUSSION

Our current data identify two genes, *Snap-25* and *synaptotagmin-1*, that, together with two previously characterized genes, *munc18-1* and *syntaxin-1*, are required for docking of secretory vesicles. We addressed the involvement of the syntaxin-1/SNAP-25 acceptor complex and found that two conditions that favor the formation of syntaxin-1/SNAP-25 acceptor complexes rescue the docking defects in *munc18-1* null mutants: SNAP-25 overexpression and expression of truncated synaptobrevin. Furthermore, null mutations for SNAP-25 and the vesicular protein synaptotagmin-1 abolish docking, and SNAP-25 no longer rescues docking in *synaptotagmin-1/munc18-1* double-null mutants. By using synaptotagmin-1 and SNAP-25 mutations that affect their interaction, we confirmed that both proteins act in concert for correct anchoring of secretory vesicles to fusion sites. Moreover, the rescue of docking, but not fusion, after expression of SNAP-25 or the synaptobrevin-2 C-terminal

fragment on the *munc18-1* null background indicates that Munc18-1 is not an essential constituent of the docking complex itself, but plays an essential downstream role. Together, the null mutation and (cross-) rescue experiments indicate that the corresponding four proteins work together to dock vesicles and at the same time suggest that Munc18-1 plays a unique, orchestrating role. While docking is established between syntaxin-1/SNAP-25 acceptor complexes at the target membrane and synaptotagmin-1 on the vesicle membrane, Munc18-1 promotes the formation or stability of the correct acceptor SNARE complexes.

Munc18-1 can interact with both "closed" and "open" syntaxin-1 (Toonen and Verhage, 2007), but it is unclear which binding mode is essential to perform its function in docking. Munc18-1 binding to "open" syntaxin-1 involves an interaction with the N-terminal H(abc) domain of syntaxin-1 and the four-helical bundle of the assembled SNARE complex (Dulubova et al., 1999, 2007; Khvotchev et al., 2007). We have previously shown that N-terminal interaction is not sufficient for docking, since a docking phenotype similar to *syntaxin-1* and *munc18-1* null was observed in chromaffin cells from knockin mice that express a mutant syntaxin-1 that only allows N-terminal interaction (Gerber et al., 2008). In addition, when we expressed the well-characterized D34N/M38V double mutant of Munc18-1 that is known to perturb the interaction with "closed" syntaxin (Naren et al., 1997; Schütz et al., 2005), we observed that docking was not restored in *munc18-1* null chromaffin cells (Gulyás-Kovács et al., 2007). Other studies have shown that Munc18-1 binding to "open" syntaxin is essential to execute fusion (Burkhardt et al., 2008; Dulubova et al., 2007; Khvotchev et al., 2007). In our present study, we have managed to experimentally separate docking and fusion phenotypes in *munc18-1* null chromaffin cells. Our observations that SNAP-25 and SybCT overexpression, which both increase the number of syntaxin-1/SNAP-25 dimers, restore docking implies that Munc18-1 promotes the existence/stability of intermediate syntaxin-1/SNAP-25 dimers at the target membrane and therefore probably binds to these intermediate complexes. This increased number of acceptor complexes is not sufficient to restore fusion in the absence of Munc18-1, which firmly establishes a postdocking role for Munc18-1 in SNARE-dependent fusion, as also indicated before (Gulyás-Kovács et al., 2007). Currently, it is unclear whether Munc18-1's function downstream of docking requires either binding to intermediate syntaxin-1/SNAP-25 dimers alone or also binding to assembled SNARE complexes (containing synaptobrevin-2) to promote fusion as shown previously in vitro (Shen et al., 2007). In addition, our experiments with synaptotagmin-1 and SNAP-25 mutations, which have been shown to impair secretion (Sørensen et al., 2006), show that in the presence of Munc18-1 a correlation exists between mutations that impair secretion and those that impair docking. This is not the case in the absence of Munc18-1, emphasizing its postdocking role in SNARE-dependent fusion.

This study identifies synaptotagmin-1 as a vesicular docking factor that binds to the assembled docking acceptor discussed above and has the capacity to anchor vesicles to the target membrane. This docking role of synaptotagmin-1 is consistent with previous findings in invertebrate synapses (Jorgensen et al., 1995; Loewen et al., 2006; Reist et al., 1998), which, however, have not been specifically interpreted in terms of docking because of additional phenotypes in these synapses: large effects on undocked vesicle populations near the active zone, which has been related to the increased mini rate observed in these mutant synapses (Reist et al., 1998), impaired recycling (Jorgensen et al., 1995), and/or impaired recruitment (Loewen et al., 2006). Interestingly, a mutation used in the latter study is in an area of the molecule that was later identified to interact with SNAP-25 (Rickman et al., 2006).

The docking role of synaptotagmin-1 proposed here does not conflict with its well-established role in fusion (Chapman, 2008; Fernandez-Chacon et al., 2001; Martens et al., 2007). However, while its role in fusion is strictly Ca^{2+} dependent (Chapman, 2008; Fernandez-Chacon et al., 2001; Martens et al., 2007), its role in docking is probably Ca^{2+} independent, since resting chromaffin cells have a strong docking phenotype in the absence of synaptotagmin-1 and its Ca^{2+} affinity is insufficient to be activated by resting Ca^{2+} levels in the cytosol. This is in line with a Ca^{2+}-independent, upstream role previously suggested in rescue experiments in fly neuromuscular junction (Loewen et al., 2006). It is tempting to speculate that on top of this principally Ca^{2+}-independent docking role, synaptotagmins may also contribute to the well-known but incompletely understood Ca^{2+}-dependent acceleration of vesicle recruitment/docking/priming (for a review, see Verhage and Sørensen, 2008).

Secretory systems typically express multiple synaptotagmins. In chromaffin cells, synaptotagmin-7 can partially compensate for the loss of synaptotagmin-1 (Schonn et al., 2008), but the secretion phenotype of the *synaptotagmin-1* null cells is still drastic (Voets et al., 2001a). In analogy, the docking phenotype in *synaptotagmin-1* null cells is also drastic, but still slightly less severe than the *munc18-1* null phenotype. This may be explained by a partial compensation by other synaptotagmins. The presence of multiple synaptotagmins, with different Ca^{2+} sensitivities and our new evidence that they are not only involved in fusion (and endocytosis), but also in docking, may require reinterpretation of previous studies on these proteins. Most studies assess upstream processes by measuring the final one (fusion) and thereby sample a composite measure of the combined effects of experimental manipulations on all upstream steps. For these combined effects to be dissected, new methodologies may be required to directly assess these upstream steps and to go beyond what current secretion assays have revealed about the complexity of the secretory pathway.

In vertebrate synapses, docking phenotypes for Munc18-1, syntaxin-1, SNAP-25, and synaptotagmin-1 have not been described or are at least less evident (for a review, see Verhage and Sørensen, 2008). It is possible that these proteins are dispensable for synaptic vesicle docking and that distinct mechanisms dock vesicles in synapses. However, it seems more likely that docking principles are conserved among secretory systems. This idea is strongly supported by the fact that docking phenotypes have been observed in invertebrate synapses upon mutations in three of the four genes (Hammarlund et al., 2007; Jorgensen et al., 1995; Reist et al., 1998; Weimer and Richmond, 2005). However, these phenotypes are generally subtle and sometimes require advanced methodology and new docking definitions to become evident (Hammarlund et al., 2007; Weimer and

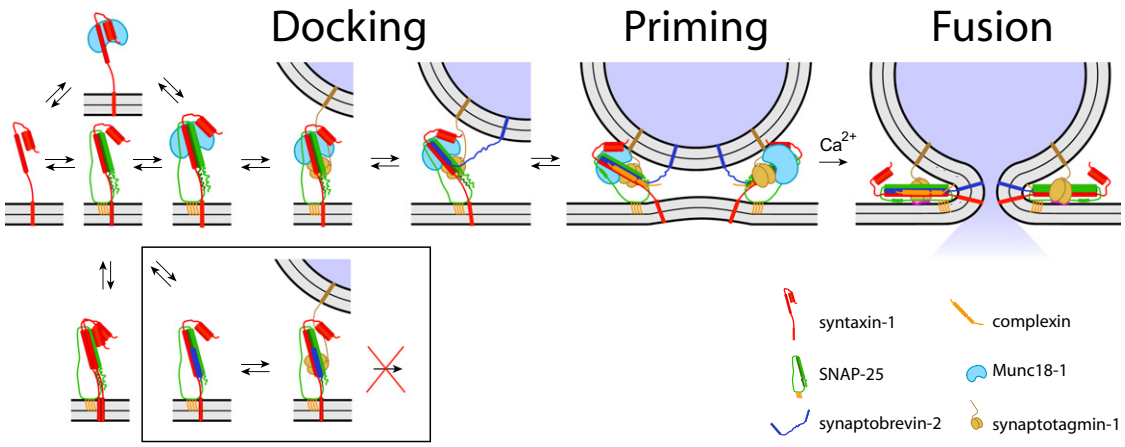

Figure 7. Working Model for the Exocytotic Pathway from Docking to Fusion

Model depicts the subsequent steps in the pathway, starting with several initial protein complexes consisting of syntaxin-1 (red), SNAP-25 (green), and/or Munc18-1 (light blue), until the final fusion, which also involves synaptobrevin-2/VAMP-2 (dark blue) and synaptotagmin-1 (yellow). The bottom row indicates nonproductive protein complexes ("off pathways"), which do not lead to fusion, either by integrating two syntaxins and one SNAP-25 into a single complex (2:1 complex; bottom left) or by formation of trimeric SNARE complexes in *cis*, with or without synaptotagmin-1 associated (bottom right). See the main text for a detailed description of the proposed steps in the pathway.

Richmond, 2005). In the case of synaptotagmin, invertebrate phenotypes are robust (Jorgensen et al., 1995; Reist et al., 1998), but additional phenotypes were observed (see above) that prevented a specific interpretation in terms of docking. It is likely that docking phenotypes are less evident in vertebrate synapses either because of redundancy arising from the expression of multiple isoforms for some of the docking genes identified here or because structurally unrelated proteins that are not expressed in chromaffin cells restrict undocking of synaptic vesicles even when essential docking factors are not expressed. Finally, it is plausible that undocking and docking phenotypes are simply not as evident in the densely packed nerve terminal.

With the currently identified four genes for docking and the link to SNARE complex assembly, a consistent (minimal) working model for the exocytotic pathway from the initial docking step until the final fusion reaction can now be synthesized for the first time (Figure 7), proposing the following four steps: First, Munc18-1 binds the closed conformation of syntaxin-1. Munc18-1 interacts with two epitopes in syntaxin-1, the Habc domain, and the N-terminal domain (Dulubova et al., 2007; Dulubova et al., 1999; Khvotchev et al., 2007). Second, SNAP-25 binds the syntaxin-1/Munc18-1 heterodimer (Burkhardt et al., 2008; Zilly et al., 2006). Third, secretory vesicles reach the target membrane area and associate via synaptotagmin-1 to this trimeric syntaxin-1/Munc18-1/SNAP-25 complex, which effectuates docking. This binding requires the C2B domain of synaptotagmin-1 (Bhalla et al., 2006; Dai et al., 2007; Gaffaney et al., 2008; Lynch et al., 2007; Mackler et al., 2002; Rickman et al., 2006; Tang et al., 2006; Xue et al., 2008), and recent studies suggest that Munc18-1's function here is to further help stabilize the syntaxin-1/SNAP-25 (1:1) acceptor complex for subsequent binding of synaptobrevin-2 (Weninger et al., 2008). In addition, since only vesicles docked in the presence of Munc18-1 are able to fuse, Munc18-1 might help restrict fusion to specific sites on the plasma membrane (Medine et al., 2007). By attaching the vesicle to the plasma membrane, the calcium sensor for exocytosis—synaptotagmin-1—has the additional function of localizing vesicles close to calcium channels, as originally proposed by Neher and Penner more than 14 years ago (Neher and Penner, 1994). Fourth, synaptobrevin-2 then binds to the synaptotagmin-1/syntaxin-1/Munc18-1/SNAP-25 complex and the four helical SNARE bundle forms, which subsequently allows complexins to associate with the four helical SNARE bundle, and ultimately the vesicle fuses upon Ca^{2+} entry. It has been proposed that synaptobrevin-2 replaces Munc18-1 (Zilly et al., 2006), but, given the proposed fusion-promoting actions of Munc18-1 while associated to SNARE complexes (Shen et al., 2007), Munc18-1 may also continue to associate with the ternary SNARE complex until fusion is triggered.

EXPERIMENTAL PROCEDURES

Cell Culture, Expression Constructs, and Transfection

Snap-25 (Sørensen et al., 2003), *synaptotagmin-1* (Geppert et al., 1994), *munc18-1* (Verhage et al., 2000), and *munc18-1/synaptotagmin-1* null animals were obtained by crossing of heterozygotes and recovered by Cesarean section at embryonic day 17 (E17) or E19. Chromaffin cells from null and wild-type littermates were cultured as described (Sørensen et al., 2003). Acute expression of heterologous genes was induced with Semliki Forest Virus (SFV). SNAP-25 variants (SNAP-25 or SNAP-25 mutants SNAP-25$^{[\Delta 9]}$, SNAP-25$^{[\Delta 26]}$, SNAP-25$^{[L50A/I171A]}$, SNAP-25$^{[M32A/V153A]}$ [Sørensen et al., 2006], and SNAP-25$^{[D51K/E52K/E55K]}$ [Lynch et al., 2007; Rickman et al., 2006; Zhang et al., 2002]), Munc18-1 (Toonen et al., 2006), N-terminal fragment 1–70 (SybNT), and C-terminal fragment 49–96 (SybCT) of synaptobrevin-2 (Pobbati et al., 2006), or soluble C2 domains of synaptotagmin-1 (C2AB) (Rickman et al., 2006) and full-length synaptotagmin-1 (syt1) constructs with or without the Y311N mutation, as well as Munc13-1 (Ashery et al., 2000), were expressed from a bicistronic message containing a poliovirus internal ribosomal entry site (PV-IRES) and EGFP. In some experiments, EGFP alone was used as a control. Mutations were introduced by standard methods. All constructs were verified by DNA sequencing. For mouse chromaffin cells, 4–6 hr was allowed for expressing the proteins after infection with virus.

Microscopical Methods

Immunofluorescence of Membrane Sheets

Six hours after infection of *munc18-1* null chromaffin cells with SNAP-25 variants, plasma membrane sheets were generated, fixed, washed, and blocked as described previously (Sørensen et al., 2006).

Immunocytochemistry

Embryonic wild-type chromaffin cells were cultured on poly-L-lysine coated coverslips. After infection with full-length synaptotagmin-1, constructs with or without the Y311N mutation and noninfected controls were fixed, washed, and blocked, and fluorescence quantification was performed by synaptotagmin immunostaining.

Electron Microscopy of Adrenal Glands and Cultured Chromaffin Cells

Adrenal glands were removed from control and *Snap-25* null littermates at embryonic day 18 and fixed in 2.5% glutaraldehyde in cacodylate buffer 2 hr at room temperature and further processed as previously (Gerber et al., 2008; Voets et al., 2001b). For the omission of chemical fixation, adrenal glands were rapidly frozen and cryosubstituted in Epon.

In addition, chromaffin cells from *Snap-25*, *synaptotagmin-1*, *munc18-1*, or *munc18-1/synaptotagmin-1* (double) null, littermate controls (E18), and wild-type mice were plated on rat tail type 1 collagen-coated coverslips (Cellocate, Eppendorf, Germany) and infected (DIV2, 2 days in vitro) with SFV constructs. Cells were fixed for 45 min at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), washed, embedded, and analyzed as before (Toonen et al., 2006). Analysis of secretory vesicle distribution was done blinded for the genotype of the animal. Docked vesicles were without any measurable distance between granule and plasma membrane.

For more details of microscopical methods, see the Supplemental Experimental Procedures.

Electrophysiological Analyses

Whole-cell patch clamp, membrane capacitance measurements, amperometry, ratiometric intracellular calcium $[Ca^{2+}]_i$ measurements, and flash photolysis of caged Ca^{2+} were performed as described previously (Sørensen et al., 2006).

Electrophoresis and Immunoblotting

Adrenal glands were collected at E18 from *synaptotagmin-1* null and wild-type littermates and homogenized in SDS-PAGE buffer. For immunodetection of SNAP-25, Rab27, Rab3A-D, Rabphilin3A, and Slp4/Granuphilin four adrenals per lane were loaded on an 11% gel. Proteins were transferred to PVDF membranes, detected with alkaline phosphatase-conjugated secondary antibodies, and enhanced by chemifluorescence. Blots were scanned on a Fuji Imager. For antibody details, see the Supplemental Experimental Procedures.

Statistics

All statistical analyses were performed with the Student's t test, one/two-way ANOVA test, Tukey test, or Mann-Whitney test as indicated. Numbers included in the various bar diagrams indicate mean ± SEM.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00906-4.

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