The SNAP-25 Linker as an Adaptation Toward Fast Exocytosis

Gábor Nagy,**†‡ Ira Milosevic,**‡§ Ralf Mohrmann,* Katrin Wiederhold,|| Alexander M. Walter,* and Jakob B. Sørensen*

*Department of Cell Biology, Yale University, School of Medicine, ‡Present addresses: †National Centre for Stereotactic Radiosurgery, Royal Hallamshire Hospital, Sheffield S10 2JF, United Kingdom; §Department of Cell Biology, Yale University, School of Medicine, New Haven, 06510 CT.

**Molecular Mechanism of Exocytosis, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany; ||Department of Neurobiology, Max Planck Institute for Biophysical Chemistry, 37077 Göttingen, Germany

Submitted December 6, 2007; Revised May 23, 2008; Accepted June 18, 2008
Monitoring Editor: Thomas F. J. Martin

The assembly of four soluble N-ethylmaleimide-sensitive factor attachment protein receptor domains into a complex is essential for membrane fusion. In most cases, the four SNARE-domains are encoded by separate membrane-targeted proteins. However, in the exocytotic pathway, two SNARE-domains are present in one protein, connected by a flexible linker. The significance of this arrangement is unknown. We characterized the role of the linker in SNAP-25, a neuronal SNARE, by using overexpression techniques in synaptosomal-associated protein of 25 kDa (SNAP-25) null mouse chromaffin cells and fast electrophysiological techniques. We confirm that the palmitoylated linker-cysteines are important for membrane association. A SNAP-25 mutant without cysteines supported exocytosis, but the fusion rate was slowed down and the fusion pore duration prolonged. Using chimeric proteins between SNAP-25 and its ubiquitous homologue SNAP-23, we show that the cysteine-containing part of the linkers is interchangeable. However, a stretch of 10 hydrophobic and charged amino acids in the C-terminal half of the SNAP-25 linker is required for fast exocytosis and in its absence the calcium dependence of exocytosis is shifted toward higher concentrations. The SNAP-25 linker therefore might have evolved as an adaptation toward calcium triggering and a high rate of execution of the fusion process, those features that distinguish exocytosis from other membrane fusion pathways.

INTRODUCTION

The soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are central to vesicular trafficking (Fasshauer, 2003; Jahn and Scheller, 2006; Rizo et al., 2006) and characterized by a stretch of 60–70 amino acids, known as the SNARE motif (Terrian and White, 1997; Weimbs et al., 1997). The assembly of four SNARE domains into a coiled-coil bundle is a general mechanism in fusion of intracellular membrane compartments. The bundle is held together by layers of hydrophobic interaction, with the exception of the middle layer (layer “0”), which is formed by an arginine and three glutamines (Sutton et al., 1998). The SNARE domains can be subdivided into four homologous groups, named after their contribution to the zero layer: R, Qa, Qb, and Qc (Fasshauer et al., 1998b). SNARE assembly requires the participation of one domain from each group, resulting in a certain degree of specificity (McNew et al., 2000; Scales et al., 2000a).

In most cases, the four SNARE-domains are encoded by separate membrane-targeted proteins, but the SNAREs driving the fusion of vesicles with the plasma membrane (exocytosis) are special in that three proteins provide the four domains (Weimbs et al., 1998; Fukuda et al., 2000). One of the SNAREs in this pathway, exemplified by the best-known isoform synaptosomal-associated protein of 25 kDa (SNAP-25), seems to have been created by fusion of the Qb and Qc SNAREs. This arrangement necessitates a flexible linker, which runs back along the complex from the C-terminal end of the first (Qb) SNARE-domain and connects to the N-terminal end of the second SNARE-domain (Qc). This antiparallel linker is a special feature of exocytotic SNARE complexes in eukaryotic organisms from yeast to human.

The only function so far ascribed to the linker domain is the membrane-targeting of SNAP-25 and SNAP-23 through the palmitoylation of four to five cysteine residues in the N-terminal end of the linker (Gonzalo et al., 1999; Loranger and Linder, 2002). However, other members of the family (SNAP-29, SNAP-46, Sec9, and SPO20) lack linker cysteines. It remains controversial whether palmitoylation is needed for the function of SNAP-25 in exocytosis, or only for membrane targeting, and it is unknown whether the linker plays any other, more active role in determining the special features that distinguish exocytosis from other membrane fusion reactions: calcium-triggering and a high rate of execution of the fusion process.

Ca2+-triggered exocytosis from Snap-25 null mouse chromaffin cells is nearly abolished, but it can be rescued by viral expression of SNAP-25 isoforms (Sorensen et al., 2003b).
Here, we used this approach and fast electrophysiological techniques to address the role of the SNAP-25 linker domain in exocytosis. By mutating linker-cysteines in SNAP-25 and substituting the SNAP-23 linker for its SNAP-25 counterpart, we verify the function of cysteines in membrane targeting, and we show that the cysteine-containing part of the SNAP-25 and SNAP-23 linker is interchangeable. However, a short 10-amino acid stretch at the C-terminal end of the SNAP-25 linker is necessary for fast calcium triggering of exocytosis. These data establish the SNAP-25 linker as an integral part of the membrane fusion machinary, and they suggest that the arrangement of the Qa and Qb motifs in one protein together with a linker is an adaptation toward fast exocytosis.

MATERIALS AND METHODS

Cell Culture and Expression Constructs

Snap-25 null animals and control litters were obtained by crossing heterozygotes and recovered by Cesarean section at embryonic days 17–19. Primary culture of mouse embryonic chromaffin cells was prepared and cultured as described previously (Sorensen et al., 2003b). SNAP-25a cysteine mutants and SNAP-25a/SNAP-23 chimeras were produced by polymerase chain reaction (PCR), and all constructs were verified by DNA sequencing. Semiliki Forest virus particles expressing mutant/chimeras together with enhanced green fluorescent protein from a bicistronic message were generated as described (Ashery et al., 1999). For bacterial expression of the SNAP-25a and SNAP-23 linker domain constructs, corresponding DNAs were cloned into a pET28a vector via the Ndel/EcoRI cleavage sites.

Immunofluorescence on Plasma Membrane Sheets

Plasma membrane sheets were generated 6 h after viral infection of mouse embryonic chromaffin cells and subsequently fixed, washed, and blocked as described previously (Nagy et al., 2005). The membrane sheets were incubated with the primary antibodies (mouse anti-SNAP-25, dilution 1:100; rabbit anti-syntaxin 1, dilution 1:100; Synaptic Systems, Göttingen, Germany) for 3 h and subsequently with cyanine (Cy3-) and Cy5-coupled secondary antibodies (goat anti-rabbit/anti-mouse horseradish peroxidase-conjugated immunoglobulin G, 1:2000; Jackson ImmunoResearch Laboratories), the membranes were washed three times and incubated in ECL Western blotting detection reagent (Amersham Hyperfilm-ECL; GE Healthcare Bio-Sciences, Uppsala, Sweden). After incubation with secondary antibodies (goat anti-rabbit/anti-mouse horseradish peroxidase-conjugated immunoglobulin G, 1:2000; Jackson ImmunoResearch Laboratories), the membranes were washed three times and incubated in ECL Western blotting detection reagent (SuperSignal, West Pico; Pierce Chemical, Rockford, IL). Chemiluminescence was detected by a digital gel documentation system; quantification was performed by densitometry using ImageJ software (National Institutes of Health, Bethesda, Maryland). The expression level was corrected for the infection efficiency, which was estimated as the ratio of green fluorescent protein (GFP)-expressing cells to the total cell number.

RESULTS

Palmitoylation of the SNAP-25 Linker

We asked whether the palmitoylation of linker cysteines in SNAP-25 is necessary for exocytosis triggering per se, or merely plays a role for membrane targeting. To that end, we expressed SNAP-25a mutants, where one or all four linker cysteines were replaced by serines (denoted C/S mutants) expressed SNAP-25a mutants, where one or all four linker cysteines were replaced by serines (denoted C/S mutants) and SNAP-25a cysteine mutants expressing mutant/chimera together with enhanced green fluorescent protein from a bicistronic message were generated as described (Fasshauer et al., 1998a). For expression of SNAP-25a linker peptide (NKLKSSDAYKKAWGNYDQGVVIASQPARVDERMAICSBGIRYNTN) and SNAP-23 linker peptide (NRTKIFESCKNYKTWWGDGGDNSPSNVVSKOIPRTNGQPCQ-QOTTGAASGGYKRIKTNDARE) the fragments were introduced into the pET28a plasmid between the Ndel and the EcoRI sites. Protein expression was induced in Escherichia coli BL21(DE3) by adding 0.8 mM isopropyl-β-D-thiogalactopyranoside to a bacterial culture grown in Luria-Bertani medium to OD600 = 0.8. The bacteria were pelleted and resuspended in extraction buffer (500 mM NaCl, 50 mM Tris, pH 7.4, and 8 mM imidazole). Cells were sonicated in the presence of 6 M urea and subsequently incubated with nickel-nitrilotriacetic acid. After 1 h at 8°C, the cell lysates were washed with extraction buffer, and the recombinant protein was eluted with 400 mM imidazole (plus half of the extraction buffer). The His-tag was cleaved off by thrombin during overnight dialysis in 20 mM Tris, 50 mM NaCl, and 1 mM dithiothreitol, pH 7.4. After purification by ion exchange chromatography on an Akta system (GE Healthcare Bio-Sciences) using Mono-S column (GE Healthcare Bio-Sciences), all peptides were pure as judged by SDS- polyacrylamide gel electrophoresis (PAGE) analysis. Protein concentration was determined by absorption at 280 nm, and peptides were stored at −20°C. SDS-PAGE was carried out as described previously (Schagger et al., 1988). For testing SNARE-complex assembly, equimolar amounts of the SNAP-25a mutants were incubated for one hour at room temperature. When testing for SDS resistance, samples were solubilized in SDS sample buffer (not boiled) before analysis on a 12% polyacrylamide gel.

Circular Dichroism (CD) Spectroscopy Measurements

CD measurements were performed using a Jasco model J-720 instrument (Applied Photophysics, Leatherhead, United Kingdom) by using quartz cuvettes with 1-mm pathlength (Helma, Mülheim, Germany). All experiments were carried out in 20 mM sodium phosphate buffer, pH 7.4, in the presence of 100 mM NaCl at 25°C. Liposomes (65% brain phosphatidylcholine, 30% brain phosphatidylserine, 5% phosphatidylinositol-4,5-bisphosphate were mixed in chloroform and dried, Avanti Polar Lipids, Alabaster, AL) were prepared in 20 mM Tris buffer with 100 mM NaCl, pH 7.4, by the SMART system (GE Healthcare Bio-Sciences).

Palmitoylation of the SNAP-25 Linker

We asked whether the palmitoylation of linker cysteines in SNAP-25 is necessary for exocytosis triggering per se, or merely plays a role for membrane targeting. To that end, we expressed SNAP-25a mutants, where one or all four linker cysteines were replaced by serines (denoted C/S mutants) using the Semiliki Forest Virus expression system in chromaffin cells isolated from Snap-25 null mice. We first investigated the targeting of SNAP-25a C/S mutants to the plasma membrane by generating plasma membrane sheets from expressing chromaffin cells. Punctate SNAP-25a immunostaining of all C/S mutants was found (Figure 1A). However, quantitatively all C/S mutants showed significant reductions in the staining compared with overexpressed WT SNAP-25a (Figure 1B). For quantification of the overall expression level we used Western blotting of bovine chromaffin cells, because cultures from a single mouse embryo do not yield sufficient protein level for quantification. It showed that all single cysteine mutants were expressed at similar level as wild-type (WT) SNAP-25a (Figure 1C; WT SNAP-25a

Molecular Biology of the Cell
Dual Role of SNAP-25 Linker Domain

Figure 1. The linker cysteines function in membrane targeting of SNAP-25. (A) Plasma membrane sheets were generated from Snap25 null chromaffin cells expressing SNAP-25a cysteine mutants (serine substitutions) and immunostained for SNAP-25. The SNAP-25 staining was punctate for all single cysteine mutants and WT SNAP-25a. The quadruple SNAP-25a 4xC/S mutant was present in only a few puncta on the plasma membrane sheets, indicating defective expression of the quadruple SNAPP-25a 4xC/S mutant on exocytosis. Even though the amount of this mutant on the plasma membrane was strongly impaired, the mutant still reached a plasma membrane level of 40% of endogenous expression levels, comparable with the level in SNAP-25 heterozygotes (~50%), in which no secretory defect was identified (Sorensen et al., 2003b) (Supplemental Figure 1). This presented an opportunity for testing whether the palmitoylation plays a role for exocytosis per se, in addition to membrane targeting. Even this mutant resulted in significantly reduced release from the two pools were similar to the WT situation (Figure 2, A and B). The observed increase in RRP size in the presence of C/S-1 or C/S-4 was not statistically significant. Also the sustained rate of release was unperturbed. Therefore, the abolition of single cysteines in the SNAP-25a linker region does not impair the ability of SNAP-25a to mediate fast Ca\(^{2+}\)-triggered exocytosis.

We further tested the effect of the SNAP-25a 4xC/S mutant on exocytosis. Even though the amount of this mutant on the plasma membrane was strongly impaired, the mutant still reached a plasma membrane level of 40% of endogenous expression levels, comparable with the level in SNAP-25 heterozygotes (~50%), in which no secretory defect was identified (Sorensen et al., 2003b) (Supplemental Figure 1). This presented an opportunity for testing whether the palmitoylation plays a role for exocytosis per se, in addition to membrane targeting. Even this mutant resulted in significantly reduced release from the two pools were similar to the WT situation (Figure 2, A and B). The observed increase in RRP size in the presence of C/S-1 or C/S-4 was not statistically significant. Also the sustained rate of release was unperturbed. Therefore, the abolition of single cysteines in the SNAP-25a linker region does not impair the ability of SNAP-25a to mediate fast Ca\(^{2+}\)-triggered exocytosis.

We further tested the effect of the SNAP-25a 4xC/S mutant on exocytosis. Even though the amount of this mutant on the plasma membrane was strongly impaired, the mutant still reached a plasma membrane level of 40% of endogenous expression levels, comparable with the level in SNAP-25 heterozygotes (~50%), in which no secretory defect was identified (Sorensen et al., 2003b) (Supplemental Figure 1). This presented an opportunity for testing whether the palmitoylation plays a role for exocytosis per se, in addition to membrane targeting. Even this mutant resulted in significantly reduced release from the two pools were similar to the WT situation (Figure 2, A and B). The observed increase in RRP size in the presence of C/S-1 or C/S-4 was not statistically significant. Also the sustained rate of release was unperturbed. Therefore, the abolition of single cysteines in the SNAP-25a linker region does not impair the ability of SNAP-25a to mediate fast Ca\(^{2+}\)-triggered exocytosis.
mediate phenotype between the Snap-25 knockout and the wild-type situation caused by the lower expression level, but that the 4xC/S mutant causes slower secretion even on the level of individual fusion events. In addition, the 4xC/S mutation resulted in a mild and less significant increase in the charge of each spike, i.e., the quantal size.

Overall, our data show that the association of the SNARE complex with the plasma membrane through SNAP-25 palmitoylation is important for optimizing the speed of exocytosis triggering and the duration of the fusion pore.

The SNAP-25 Linker Domain Speeds Up Exocytosis Triggering

We reported previously that the expression of the ubiquitous isoform SNAP-23 in Snap-25 null chromaffin cells supports some rescue of calcium-dependent exocytosis, but without a burst component (Sorensen et al., 2003b). Here, we first repeated this experiment with similar results, and then applied stronger stimulation into the Snap-25a linker mildly reduced the level of exocytosis (red traces, n = 47 cells expressing SNAP-25a 4xC/S; black traces, n = 41 control cells; gray traces show secretion from the Snap-25a C/S-1 for comparison). The amplitudes (mean ± SEM) of the fast and slow burst were significantly decreased and the fast fusion time constant was significantly increased in cells overexpressing SNAP-25a 4xC/S. In addition, the secretory delay (time between photorelease of Ca$^{2+}$ and start of capacitance increase) was significantly increased. *p < 0.05 and **p < 0.01 (Mann–Whitney test).

Table 1. Single fusion event characteristics as measured by amperometry

<table>
<thead>
<tr>
<th></th>
<th>No. of cells</th>
<th>No. of spikes</th>
<th>Amplitude (pA)</th>
<th>Q$^{1/3}$ (pC)$^{1/3}$</th>
<th>Half width (ms)</th>
<th>Rise time (ms)</th>
<th>Foot duration (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP-25a in −/−</td>
<td>15</td>
<td>569</td>
<td>21.7 ± 1.7</td>
<td>0.53 ± 0.01</td>
<td>4.4 ± 0.6</td>
<td>0.63 ± 0.08</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>4xC/S in −/−</td>
<td>13</td>
<td>562</td>
<td>27.8 ± 2.8</td>
<td>0.58 ± 0.02*</td>
<td>4.6 ± 1.0</td>
<td>0.73 ± 0.15</td>
<td>4.2 ± 0.8*</td>
</tr>
<tr>
<td>SN25L23 in −/−</td>
<td>9</td>
<td>561</td>
<td>21.6 ± 1.7</td>
<td>0.57 ± 0.02</td>
<td>4.6 ± 0.7</td>
<td>0.57 ± 0.04</td>
<td>2.4 ± 0.8</td>
</tr>
</tbody>
</table>

The table gives mean ± SEM of the cell median for each parameter. The foot duration of the quadrupel cysteine mutation was significantly longer (p = 0.017) and the charge of the spike slightly larger (p = 0.040) than in cells rescued with wild-type SNAP-25a.

* p < 0.05 (Mann–Whitney test comparing to SNAP-25a rescue).
Overexpression of a chimeric protein (SN25aL23), where the SNAP-25 SNARe domains from SNAP-25 were joined by the SNAP-23 linker. The crossovers of the chimera were the lysine-83(SNAP-25)/lysine-78(SNAP-23), and the glutamate-148(SNAP-25)/glutamate-153(SNAP-23) (also see Figures 4 and 5). The chimera (denoted SN25aL23) was overexpressed at similar levels as SNAP-25 WT protein (Figure 1C). When overexpressed in Snap-25 null chromaffin cells, SN25aL23 did not fully restore secretion (Figure 3), but it led to a slowdown and a reduction in exocytosis within the first second of stimulation (Figure 3A). This partial rescue already indicates that the linker plays another role than just membrane targeting (see also below). We investigated whether stronger stimulation might recover exocytosis driven by the chimera. We increased [Ca\textsuperscript{2+}] to 60–80 μM to speed up exocytosis without eliciting capacitance increases uncorrelated to amperometric charge, which typically happens at >100 μM in an all-or-none manner (Xu et al., 1998). Indeed, we found that at 60–80 μM calcium, the burst of secretion within the first 0.5 s was largely restored, whether evaluated by capacitance, or by amperometric measurements (Figure 3B). The agreement between the two types of measurements shows that these recordings were not severely contaminated with fusing vesicles not containing catecholamines.

To understand the consequences for exocytosis of replacing the SNAP-25 linker with its SNAP-23 counterpart, we cells stimulated to ~20 μM [Ca\textsuperscript{2+}]. Black trace, a Snap-25 null cell rescued by SNAP-25a WT expression and displaying large secretion (compare with A); and red trace, example of rescue with SN25L23. Shown are also the kinetic fits to the traces (blue and red smooth lines) and the fitted kinetic parameters. For comparison under conditions of similar amplitudes, the SN25L23 trace is also shown scaled to WT amplitude at 1 s after the flash (red dotted trace). (D) Example traces from two cells stimulated to ~60 μM [Ca\textsuperscript{2+}]. Note expanded time scale. Color-coding as in C. The SN25L23 cell displays an exocytotic burst that could be fitted with a sum of two exponentials, with time constants 90.7 and 946 ms, respectively. The chimera (denoted SN25aL23) was overexpressed at similar levels as SNAP-25 WT protein (Figure 1C). When overexpressed in Snap-25 null chromaffin cells, SN25aL23 did not fully restore secretion (Figure 3), but it led to a slowdown and a reduction in exocytosis within the first second of stimulation (Figure 3A). This partial rescue already indicates that the linker plays another role than just membrane targeting (see also below). We investigated whether stronger stimulation might recover exocytosis driven by the chimera. We increased [Ca\textsuperscript{2+}] to 60–80 μM to speed up exocytosis without eliciting capacitance increases uncorrelated to amperometric charge, which typically happens at >100 μM in an all-or-none manner (Xu et al., 1998). Indeed, we found that at 60–80 μM calcium, the burst of secretion within the first 0.5 s was largely restored, whether evaluated by capacitance, or by amperometric measurements (Figure 3B). The agreement between the two types of measurements shows that these recordings were not severely contaminated with fusing vesicles not containing catecholamines.

To understand the consequences for exocytosis of replacing the SNAP-25 linker with its SNAP-23 counterpart, we conditions. The high-calcium stimulations only elicited slightly more release of catecholamines than stimulation to 20–30 μM, and the secretion followed a similar time course as during flashes to 20–30 μM calcium (Supplemental Figure 2, A and B). Thus, Snap-25 null cells do not support fast LDCV release even when expressing SNAP-23 and stimulated to [Ca\textsuperscript{2+}] > 100 μM.

Next, we asked whether there are any functional differences between the SNAP-23 and SNAP-25 linkers, which both act to anchor the protein in the plasma membrane. We therefore constructed chimeric proteins where the two SNARe domains from SNAP-25 were joined by the SNAP-23 linker. The crossovers of the chimera were the lysine-83(SNAP-25)/lysine-78(SNAP-23), and the glutamate-148(SNAP-25)/glutamate-153(SNAP-23) (also see Figures 4 and 5). The chimera (denoted SN25aL23) was overexpressed at similar levels as SNAP-25 WT protein (Figure 1C). When overexpressed in Snap-25 null chromaffin cells, SN25aL23 did not fully restore secretion (Figure 3), but it led to a slowdown and a reduction in exocytosis within the first second of stimulation (Figure 3A). This partial rescue already indicates that the linker plays another role than just membrane targeting (see also below). We investigated whether stronger stimulation might recover exocytosis driven by the chimera. We increased [Ca\textsuperscript{2+}] to 60–80 μM to speed up exocytosis without eliciting capacitance increases uncorrelated to amperometric charge, which typically happens at >100 μM in an all-or-none manner (Xu et al., 1998). Indeed, we found that at 60–80 μM calcium, the burst of secretion within the first 0.5 s was largely restored, whether evaluated by capacitance, or by amperometric measurements (Figure 3B). The agreement between the two types of measurements shows that these recordings were not severely contaminated with fusing vesicles not containing catecholamines.

To understand the consequences for exocytosis of replacing the SNAP-25 linker with its SNAP-23 counterpart, we}

---

**Figure 3.** Overexpression of a chimeric protein (SN25aL23), where the SNAP-25 SNARe domains were fused to the SNAP-23 linker in Snap-25 null chromaffin cells slow down exocytosis. (A) Exocytosis was stimulated and measured as described in the legend to Figure 2. The SN25aL23 chimera resulted in less exocytosis during the first 0.5 s after flash stimulation to 15–20 μM Ca\textsuperscript{2+}, as measured by both membrane capacitance increase and amperometry (SN25aL23, red trace is mean of 34 cells; SNAP-25a, black trace is mean of 35 cells). (B) Exocytosis within the first 0.5 s after the flash was largely rescued when stimulating to higher Ca\textsuperscript{2+} concentrations (60–80 μM), whereas sustained secretion was now impaired (SN25aL23, n = 26 cells; SNAP-25a, n = 26 cells). (C) Example traces from two
performed a detailed kinetic analysis of the capacitance traces. This was done by fitting a sum of exponential functions to individual capacitance traces to identify the size of the releasable vesicle pools, RRP and SRP, together with their fusion kinetics (Figure 3, C and D). It was immediately clear that in SN25aL23-expressing cells the fusion kinetics was dramatically slowed down. At $[\text{Ca}^{2+}]_i < 35 \mu$M (Figure 3, A, C, and E), only a single, slow phase of exocytosis was discernible. In addition, the delay between the $[\text{Ca}^{2+}]_i$, increase and the start of the capacitance trace was much longer than in cells expressing SNAP-25 WT (Figure 3C). The fastest resolvable time constant of release was increased by a factor $\approx 20$ (Figure 3E, bottom left), whereas the secretory delay was threefold longer in the SN25aL23-expressing cells (Figure 3E, bottom right). At higher postflash $[\text{Ca}^{2+}]_i$ (Figure 3, B, D, and F), both WT and mutant cells displayed faster kinetics, as indicated by faster time constants and shorter secretory delays (Figure 3, D and F). In SN25aL23-expressing cells, two exocytotic phases were now distinguishable (Figure 3D). Strikingly, when considering cells with a calcifying cells, two exocytotic phases were now distinguishable (Figure 3D). The fast- and slow time constant of release and the secretory delay were longer in the chimera, indicating slower exocytosis triggering with the SNAP-23 linker. In addition to the fast burst component, a second slow burst could not be identified. The graphical representation in Figure 3, G and H, shows that with increasing $[\text{Ca}^{2+}]_i$, the rate constant of fast release was speeded up, whereas the delays became shorter, as described previously (Voets, 2000). This calcium-dependence of secretion was shifted toward much higher concentrations in the chimeric construct containing the SNAP-23 linker. In addition to the effect on exocytosis triggering, at high $[\text{Ca}^{2+}]_i$, it became clear that the sustained rate of secretion was reduced in the chimeric construct (Figure 3, B and F, top right). This parameter has often been associated with the rate of refilling the releasable vesicle pools.

Recording of single resolved amperometric spikes did not reveal significant differences between SNAP-25a and SNAP25aL23 (Table 1), in line with the lack of significant differences following SNAP-25a and SNAP-23 overexpression (Sorensen et al., 2003b). This shows that a slow-down of exocytosis triggering (found both in 4C/S mutant and in the SNAP25aL23 chimera) in some cases might correlate with a change in fusion pore duration; in other cases not. Thus, exocytosis triggering and fusion pore expansion is driven by partly different—but probably overlapping—processes.

Overall, the replacement of the SNAP-23 for the SNAP-25 linker causes a displacement of the calcium dependence of exocytosis toward much higher concentrations without changing the releasable vesicle pool sizes, and it also seems to affect slower phases of exocytosis that assay vesicle priming (see Discussion).
SNAP-25, nevertheless displayed the linker phenotype (Supplemental Figure 3). Conversely, the chimera where the N-terminal part—and therefore the linker cysteines—was from SNAP-23, but the rest of the linker from SNAP-25 (SN23aL23Δ10-2) exhibited full rescue and fast secretion indistinguishable from SNAP-25a (Figure 6B).

Overall, these data show that the membrane-anchoring part of the linker containing the palmitoylated cysteines is interchangeable between SNAP-25 and SNAP-23 without implications for expression level, membrane targeting or in vitro SNARE complex formation, whereas the C-terminal part of linker surprisingly is necessary for fast exocytosis triggering.

A Short Amino Acid Stretch in the C-Terminal Half of the SNAP-25 Linker Speeds Up Exocytosis

To further narrow down the decisive part of the linker, we continued testing the chimeras displayed in Figure 5. In brief, we found that all mutants depicted in red displayed the linker phenotype, i.e., secretion like SN25aL23, whereas mutants in green had a SNAP-25a-like secretory phenotype and mutants in blue showed an intermediate phenotype. The minimum region that gave an unperturbed SNAP-25-like phenotype was a 10-amino acid stretch encompassing the positions 120–129 in SNAP-25. Chimeras including this stretch were expressed and targeted to the membrane at wild-type levels (Figures 1C and 4B) and restored secretion to normal amplitude and kinetics (Figure 7 and Supplemental Figure 4A), regardless of whether the construct included the extra seven amino acids present in SNAP-23 N-terminal of this domain. However, a chimera containing the extra three amino acids (GAA) present in SNAP-23 immediately C-terminal of the 10-amino acid stretch from SNAP-25 displayed an intermediate phenotype (Supplemental Figure 4B), showing that the domain needs to be fused directly to the remaining part of the linker, which is largely conserved between SNAP-25 and SNAP-23.

Intermediate Phenotypes Identify the Critical Properties of the SNAP-25 Linker

The critical stretch in the SNAP-25 linker consists of the amino acid sequence (in single-letter code) VVDEREQMAI, which does not to our knowledge correspond to any known consensus sequences for protein–protein interaction domains. The domain starts with two and ends with three hydrophobic amino acids, which are separated by three charged and one hydrophilic residue. In the next set of experiments, we tried to determine which properties of this domain are required for fast triggering.
A chimeric protein where the two initial valines were substituted for the SNAP-23 residues isoleucine and threonine (SN25aL23Δ10-6) resulted in a construct with an intermediate phenotype between the linker phenotype and SNAP-25a (Figure 8A). With this construct, the normal amplitude of the exocytotic burst was recognizable, but the triggering rate was still somewhat decreased and also the sustained component of release was depressed. Therefore, the N-terminal hydrophobicity of the SNAP-25 domain is necessary for full rescue. Another chimera (SN25aL23Δ10/10-4) was created by substituting the last three amino acids of the stretch (MAI) with the uncharged SNAP-23 residues (QTT). This construct displayed the linker phenotype (Figure 8B); thus, the C-terminal part of the domain is absolutely necessary for fast exocytosis triggering.

Interestingly, a chimera where only the methionine-127 from SNAP-25 was substituted for the glutamine residue in the SNAP-23 linker (SN25aL23Δ10/8) sufficed to speed the secretion up compared with the linker phenotype, even though not quite as much as when all three C-terminal amino acids MAI were from SNAP-25 (SN25aL23Δ10/7, Figure 9, A and B). The latter construct restored the size of the

Figure 5. Overview of SNAP-25a/SNAP-23 chimeric constructs. SNAP-25 is depicted in green, SNAP-23 in orange. The sequences of the SNAP-25a and SNAP-23 linker domains are shown aligned, the color of the bar above indicates identical (red) and variant (blue) positions. The ends of the first and second SNARE domain are shown as hatched green boxes attaching to the linker domains. Gaps in the sequences are shown with dots. The names of the chimeras are shown to the left of the sequences, color-coded to show the secretory phenotype. Chimeras in green showed a secretory phenotype indistinguishable from SNAP-25a, red chimeras showed the linker phenotype, i.e., incomplete rescue and lower rate of exocytosis triggering at intermediate [Ca²⁺], which was partly overcome at higher concentrations, blue chimeras displayed an intermediate phenotype between the linker phenotype and SNAP-25a.

Figure 6. The second half of the SNAP-25 linker is necessary for fast exocytosis. (A) Secretion in the SN25aL23Δ10 chimera, in which the extra 10 amino acids in the SNAP-23 linker were deleted (see Figure 5). Exocytosis in the SN25aL23Δ10 chimera was depressed during stimulations to 20–30 μM [Ca²⁺] (light blue traces, n = 19 cells), but it is largely rescued as assayed by amperometry during flashes to higher [Ca²⁺] (blue traces, n = 20 cells). Note that the “overrescue” of the capacitance increase during stimulation to >100 μM is due to the fusion of vesicles that contain no catecholamines (see text for explanation). Therefore, only the amperometric trace contains information about the release of catecholamine-containing vesicles during high-[Ca²⁺] stimulation. For comparison is shown the rescue with WT SNAP-25a in Snap25a−/− (black traces). (B) A chimera where the second half of the linker is from SNAP-25 (refer to Figure 5; SN25aL23Δ10-2) rescues secretion when expressed in Snap-25a−/− (green traces, n = 22 cells) similar to WT SNAP-25a (black traces, n = 22 cells).
burst, but still left time constants of triggering and the delay slowed down by a factor 2–4. Also the sustained component of release was depressed. This is interesting, because in this construct the proline-127 from SNAP-23 was present, and the central charges (DERE) in SNAP-25 were missing. Furthermore, the two chimeras where the last three amino acids (MAI) were from SNAP-25 (SN25aL23/H900410-6 and SN25aL23/H900410-7) displayed indistinguishable phenotypes (compare Figure 8A and Figure 9A), even though the former construct contained the middle charged stretch (DEREQ) from SNAP-25, whereas the latter had the SNAP-23 sequence (NGQQQ). Thus, both the C-terminal and N-terminal hydrophobicity of this stretch and especially methionine-127 is important for fast exocytosis triggering, whereas the middle stretch seems less important, because even a proline in this area does not impair secretion.

To investigate whether mutations in the domain from amino acid 120–129 changes exocytosis in the context of the full-length SNAP-25 protein, we generated the single point-mutation SNAP-25 M127Q (Figure 5). This single mutation sufficed to significantly increase the time constant of fast release and the secretory delay (Figure 9, C and D). In addition, the sustained component was reduced, even though this was not quite significant when testing with a Mann–Whitney test (Figure 9D). Thus, a mutation in the full-length SNAP-25a protein reproduces the main features of similar mutations in the context of the SNAP-23 linker.

Altogether, we conclude that the hydrophobicity of the N- and C-terminal ends of this linker domain is of critical importance for fast triggering, and the domain cannot be further subdivided without losing functionality. Therefore, the 10 amino acids in the SNAP-25 linker from position 120–129 display the features of a single protein domain acting in fast exocytosis triggering.

**DISCUSSION**

A linker connecting the Qb and Qc SNARE domains is a unique feature of the exocytotic fusion pathway; however, no active role for the linker in exocytosis had been identified. Here, we show that the linker is intimately involved in fast Ca\(^{2+}\) triggering. Thus, this part of the SNARE complex warrants further attention.
We analyzed the role of the palmitoylated cysteines in a null background, by expression in Snap-25 knockout chromaffin cells. Our data confirm the function of the cysteines in targeting SNAP-25 to the plasma membrane (Hess et al., 1992; Veit et al., 1996; Lane and Liu, 1997; Gonzalo et al., 1999; Vogel and Roche, 1999; Gonelle-Gispert et al., 2000; Koticha et al., 2002; Loranger and Linder, 2002; Kammer et al., 2003), in which it acts in exocytosis by binding to the Q-SNARE partner syntaxin-1. Removing single cysteines reduced the amount of SNAP-25 on the plasma membrane to <50%, but it did not impair secretion, as previously shown in insulin-secreting cells (Gonelle-Gispert et al., 2000). A SNAP-25 without cysteines was expressed at lower levels than WT protein, but still induced significant rescue. The resulting secretion was slowed down and the duration of the fusion pore increased. Thus, the cysteines are important for the speed of exocytosis. The observed rescue agree with the finding by several groups that nonpalmitoylated SNAP-25 can support membrane fusion in vitro assays (Scales et al., 2000b; Schuette et al., 2004) or after infusion into BoNT/E-treated PC12 cells (Scales et al., 2000b). The slow-down of secretion found here using high time-resolution methods would not have been noticeable in those intrinsically slow assays.

It has been suggested that SNAP-25 and SNAP-23 are differentially targeted to membrane rafts, due to the presence of an extra cysteine in SNAP-23 (Salaun et al., 2005a,b). Others have failed to find SNAREs in lipid rafts (Lang et al., 2001), but they identified cholesterol-dependent SNARE clusters in plasma membrane sheets. We showed that the secretory phenotype of a chimeric SNAP-25a construct where the N-terminal half of the linker—including all cysteines—was from SNAP-23 did not deviate from SNAP-25a in our assay. Thus the difference in secretory phenotype between SNAP-25 and SNAP-23, which was found after overexpression (Sorensen et al., 2003b), cannot be due to a different arrangement of cysteines. Likewise, we found that the cysteine clusters of the two SNAP-25 splice variants are functionally equivalent (Nagy et al., 2005). Nevertheless, it remains possible that at lower expression levels differential targeting of SNAP-25 and SNAP-23 could be functionally important.

Using chimeric proteins we identified a 10-amino acid stretch in the C-terminal half of the SNAP-25 linker—which is necessary for fast exocytosis triggering. This stretch begins immediately C-terminal of the minimal domain required for palmitoylation of SNAP-25 in vivo (Gonzalo et al., 1999) and did not affect expression level or membrane targeting. It consists of hydrophobic and charged amino acids, whereas the stretch in SNAP-23 is hydrophilic, but uncharged. Testing of chimera underlined the importance of the hydrophobicity for functionality. This domain is conserved in SNAP-25 from zebrafish to human (Figure 10A), whereas in the fly and worm one or two of the initial hydrophobic amino acids are present together with the important methionine and one-three charges in the middle (Figure 10A). An interesting exception is formed by the sea urchin, where the critical methionine is replaced by a cysteine. Cysteines are quite hydrophobic, even when not palmitoylated. Limited structural studies performed using CD spectroscopy showed...
The most remarkable effect of replacing the SNAP-25 linker with its SNAP-23 counterpart is a displacement of the calcium dependence of fast exocytosis triggering toward higher concentrations (Figure 3, G and H). This displacement also seems to affect vesicles fusing from the SRP (Figure 3, D and F). The ability of a SNAP-23 domain to displace the calcium dependence of fast exocytosis triggering toward higher values is surprising, because it was shown previously that SNAP-23 induce vesicle priming and fusion from the SRP. Simulations of the model in Figure 10B by using a SNAP-25 orthologue based on a recent classification (Kloeper et al., 2007). The 10 amino acids important for exocytosis triggering are marked by a shaded box. (B) Sequential model for exocytosis in the chromaffin cells. Vesicles become release-competent through the priming reaction (note that this reaction is assumed reversible, even though it for simplicity is shown with a single arrow). Primed vesicles can belong to the SRP, or the RRP. The RRP vesicles fuse ~10 times faster than SRP vesicles. that both are unstructured in solution (Supplemental Figure 5), in agreement with previous data (Margittai et al., 2001). Also, our electrophysiological data indicate the lack of secondary structure, since replacing the middle part of the 10-amino acid stretch with residues from SNAP-23, which involved the insertion of a proline, did not further exacerbate the intermediate phenotype found upon replacement of the first two valines. Finally, the stretch is flanked to both sides by helix-breakers (prolines, glycines), making it unlikely that this domain would change the structure of the SNARE-domains through a cis-action. The most remarkable effect of replacing the SNAP-25 linker with its SNAP-23 counterpart is a displacement of the calcium dependence of fast exocytosis triggering toward higher concentrations (Figure 3, G and H). This displacement also seems to affect vesicles fusing from the SRP (Figure 3, D and F). The ability of a SNAP-23 domain to displace the calcium dependence toward higher values is surprising, because it was shown previously that SNAP-23 induce vesicle docking and fusion in N2A cells at low, resting, calcium levels (Chieregatti et al., 2004). However, Chieregatti et al. (2004) also showed that synaptotagmin-7 suppresses SNAP-23–induced basal release. Synaptotagmin-7 is expressed in and modulates secretion from chromaffin cells (Schonn et al., 2008). Thus, the ability of SNAP-23 to stimulate exocytosis under resting conditions depends on the expression of auxiliary proteins and/or the cell type. In addition, we found that the rate of sustained release at high $[\text{Ca}^{2+}]$, was slowed down by the SNAP-23 linker (Figure 3, B and F). In a sequential model of exocytosis (Figure 10B), the priming rate can be measured from the sustained component, but only if the fusion rate is kept much higher than the priming rate during the experiment. In the SN25L23 chimera, this assumption breaks down because of the dramatic slowdown of fusion triggering, which might have caused newly recruited vesicles to become "trapped" in the SRP. Simulations of the model in Figure 10B by using triggering rates estimated from Figure 3 showed that about half of the decrease in sustained component might be explained by the decrease in fusion triggering (data not shown), whereas the other half might represent a real depression of the vesicle priming reaction.

The displacement of the intracellular calcium dependence of exocytosis triggering is a very specific phenotype. Indeed, it is striking that the only molecular manipulations that are known—or are likely based on published literature—to change the intracellular calcium-dependence of exocytosis triggering are mutations in synaptotagmins and SNAP-25 (Sorensen et al., 2003a; Wang et al., 2003; Chieregatti et al., 2004; Rhee et al., 2005; Wang et al., 2005; Nagy et al., 2006; Pang et al., 2006; Sorensen et al., 2006). In contrast, manipulations of proteins involved in exocytosis more often lead to changes in pool sizes and/or recruitment in the absence of a change in triggering: examples include tomosyn (Yizhar et al., 2004), Munc13 (Ashery et al., 2000), Munc18 (Voets et al., 2001; Gulyas-Kovacs et al., 2007), CAPS1 (Speidel et al., 2005), SV2 (Xu and Bajjalieh, 2001), Snapin (Tian et al., 2005), synaptotobrevin (Borisovsky et al., 2005), and α-SNAP/NSF (Xu et al., 1999). Our present work extends the limited number of molecular manipulations that change the intracellular calcium dependence of exocytosis triggering to include the SNAP-25 linker. There is ample evidence that synaptotagmin-1 binds to the SNARE complex or to individual SNAREs through interactions with one or both C2-domains (Bennett et al., 1992; Sollner et al., 1993; Chapman et al., 1995; Schiavo et al., 1997; Zhang et al., 2002; Rickman and Davletov, 2003; Shin et al., 2003; Bai et al., 2004; Bhalla et al., 2006; Pang et al., 2006; Lynch et al., 2007). It is possible that the SNAP-25 linker participates in synaptotagmin binding in situ. Another possibility, with would agree with the importance of the hydrophobic residues, is that the SNAP-25 linker affects interactions with the membrane that take place simultaneously with $\text{Ca}^{2+}$ binding to synaptotagmin-1. Finally, the linker might affect stages of SNARE complex assembly, which could change both vesicle priming and fusion reactions (Sorensen et al., 2006), possibly by stabilizing an intermediate conformation of the SNARE complex (An and Almers, 2004). The picture emerging is that SNAREs, synaptotagmins, and lipids form an integrated fusion machine (Bhalla et al., 2006; Pang et al., 2006; Dai et al., 2007), whose calcium dependence is set by the properties of the entire assembly. The SNAP-25 linker must be seen as an integral part of this machine and it is tempting to speculate that the fusion of the Qb and Qc-SNARE motifs might have evolved as an adaptation toward calcium triggering of exocytosis.

ACKNOWLEDGMENTS

We thank Dirk Reuter and Ina Herfort for expert technical assistance, and Ralf B. Nehring for advice during the construction of the mutants. We are deeply indebted to Erwin Neher for ongoing discussions and support. We thank Dirk Fasshauer, Reinhard Jahn, and Josep Rizo for comments on an earlier version of the manuscript. This work was supported by Deutsche Forschungsgemeinschaft grant SFB523-B16 (to J.B.S.).

REFERENCES


Figure 10. (A) Alignment of the critical linker domain in some SNAP-25 orthologues based on a recent classification (Kloeper et al., 2007). The 10 amino acids important for exocytosis triggering are marked by a shaded box. (B) Sequential model for exocytosis in the chromaffin cells. Vesicles become release-competent through the priming reaction (note that this reaction is assumed reversible, even though it for simplicity is shown with a single arrow). Primed vesicles can belong to the SRP, or the RRP. The RRP vesicles fuse ~10 times faster than SRP vesicles.


