Cysteine residues of SNAP-25 are required for SNARE disassembly and exocytosis, but not for membrane targeting

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The release of neurotransmitter at a synapse occurs via the regulated fusion of synaptic vesicles with the plasma membrane. The fusion of the two lipid bilayers is mediated by a protein complex that includes the plasma membrane target soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptors (t-SNAREs), syntaxin 1A and synaptosome-associated protein of 25 kDa (SNAP-25), and the vesicle SNARE (v-SNARE), vesicle-associated membrane protein (VAMP). Whereas syntaxin 1A and VAMP are tethered to the membrane by a C-terminal transmembrane domain, SNAP-25 has been suggested to be anchored to the membrane via four palmitoylated cysteine residues. We demonstrate that the cysteine residues of SNAP-25 are not required for membrane localization when syntaxin 1A is present. Analysis of the 7 S and 20 S

complexes formed by mutants that lack cysteine residues demonstrates that the cysteines are required for efficient SNARE complex dissociation. Furthermore, these mutants are unable to support exocytosis, as demonstrated by a PC12 cell secretion assay. We hypothesize that syntaxin 1A serves to direct newly synthesized SNAP-25 through the Golgi transport pathway to the axons and synapses, and that palmitoylation of cysteine residues is not required for targeting, but to optimize interactions required for SNARE complex dissociation.

Key words: *N*-ethylmaleimide-sensitive fusion protein, palmitoylation, syntaxin, vesicle-associated membrane protein, vesicle fusion.

INTRODUCTION

Synaptosome-associated protein of 25 kDa (SNAP-25) is a core component of the synaptic soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein (SNAP) receptor (SNARE) complex. This complex is formed together with the vesicle SNARE (v-SNARE), vesicle-associated membrane protein (VAMP)/synaptobrevin, and the other target SNARE (t-SNARE), syntaxin 1 [1–3]. According to the SNARE hypothesis, cognate v- and t-SNARE partners co-ordinate the targeting and docking of transport vesicles to cellular organelles [4]. Recently this hypothesis has been challenged by the demonstration that v- and t-SNAREs are highly promiscuous in their binding partners [5], although more selective interactions can be detected *in vivo* [6]. Also, recent studies have attributed the catalysis [7,8] and the Ca²⁺-sensitivity of the membrane fusion event between vesicle and plasma membrane to the SNARE complex [9], suggesting that the SNAREs may directly mediate fusion and are not restricted to targeting fusion events to intracellular locations.

Many ultrastructural studies demonstrate that syntaxin 1A and SNAP-25 distinctly localize to axonal membranes, being enriched in presynaptic terminals, whereas they seem to be excluded from dendritic and somal plasma membranes [10–12].

Despite the fact that syntaxin 1A is a type II transmembrane protein, its mode of targeting has not been an issue of dispute. It is assumed to follow a similar route to VAMP, also a type II membrane protein, which is inserted into the endoplasmic reticulum and is then transported via the Golgi [13]. In contrast, the targeting of SNAP-25, which does not possess a transmembrane domain, has been the subject of much controversy.

The central domain of SNAP-25, encoded by either exon 5a or 5b, depending on the splice alternative, contains four cysteine residues [14]. Numerous studies have shown that one or more of these cysteine residues is fatty acylated via the thioester linkage of palmitate in cultured cells and *in vivo* [15–17]. It has been proposed that the change in splice variant, which leads ultimately to the repositioning of one of the cysteine residues and an overall change in charge, could contribute to differential targeting of the SNAP-25 isoforms, a and b [16,18]. Based on mutational studies that eliminate cysteine residues, palmitoylation has been argued to be a prerequisite for membrane localization [16,19]. However, it is hard to imagine how simple palmitoylation of SNAP-25 could lead to its distinct subcellular localization in axons and nerve terminals. Alternatively, it could be hypothesized that a membrane receptor or chaperone is required not only to pull SNAP-25 to the membrane, but to also direct it into the axonal trafficking pathway. Indeed, recent studies have demonstrated

Abbreviations used: BHK cells, baby hamster kidney cells; BoNT/E, botulinum neurotoxin serotype E; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GH, growth hormone; GST, glutathione S-transferase; HA, haemagglutinin; NSF, *N*-ethylmaleimide-sensitive fusion protein; PBST, PBS containing 0.1% Triton X-100; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25 kDa; SNARE, SNAP receptor; t-SNARE, target SNARE; v-SNARE, vesicle SNARE; syntaxin ΔTM, mutant of syntaxin 1A lacking a transmembrane domain; VAMP, vesicle-associated membrane protein.

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that chemical deacylation does not remove SNAP-25 from the membrane [20]. Additional support for this hypothesis has come from studies which have suggested that a receptor is necessary for correct targeting of SNAP-25 to the membrane and that trafficking from the Golgi to the plasma membrane is required for palmitoylation [17,20,21].

In the present study we demonstrate that a dose-dependent decrease in membrane association due to the removal of cysteine residues can be entirely overcome by the presence of syntaxin 1A. More importantly, our data clearly show that the central cysteine residues are required for efficient exocytosis, due to their involvement in SNARE complex dissociation. Our results, therefore, shed light on the mechanism of targeting of palmitoylated hydrophilic proteins to distinct cellular membranes and on the potential role of palmitoylation for the function of SNARE proteins in membrane fusion.

EXPERIMENTAL

Cell lines and vectors

DH5 α *Escherichia coli* cells (Gibco BRL, Gaithersburg, MD, U.S.A.) were used for molecular cloning. The PC12 and COS7 cell lines were obtained from the A.T.C.C. (Manassas, VA, U.S.A.), and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 50 units/ml penicillin (Gibco BRL) and 50 μ g/ml streptomycin (Gibco BRL). Baby hamster kidney cells (BHK cells) were provided by Dr Angela Wandinger-Ness (Department of Pathology, University of New Mexico, Albuquerque, NM, U.S.A.), and were maintained in Glasgow minimum essential medium (Gibco BRL) containing 10% (v/v) FBS, 50 units/ml penicillin and 50 μ g/ml streptomycin. The plasmid vector HA/pCDNA (where HA corresponds to haemagglutinin) was prepared as described in Washbourne et al. [22]. The plasmid encoding the BoNT/E-His₆ light chain construct (where BoNT/E corresponds to botulinum neurotoxin serotype E) was a gift from the late Dr Heiner Niemann.

Mutagenesis

The individual Cys $\rightarrow$ Gly mutations (see 1, 2 and 3 C $\rightarrow$ G in Figure 2C) were made by PCR mutagenesis using the forward primer 5'-CACCCTACCATGGCCGAGG-3' and a reverse degenerate 50mer spanning the cysteine codons and the *Hind*III restriction site. The first base of each cysteine codon was given a 50% possibility of being either an A or a C, to code for either Cys or Gly respectively. The PCR products were cut with *Nco*I and *Hind*III, purified and inserted into the 5' end of the SNAP-25 cDNA at the *Hind*III site. Clones were screened by sequencing. The mutants Cys $\rightarrow$ Ser and Cys $\rightarrow$ Gly in which all four cysteine residues were substituted with serine or glycine residues (see 1 + 2 + 3 + 4 in Figure 2C) were made using the mutagenic primers 5'-GATTTAAGCTTGTTACGGGACTCACACTAAGCCCGCTGAATTTTCCTAG-3' and 5'-GATTTAAGCTTGTTACCGGGACCCACACCAAGCCCGCCGAATTTTCCTAG-3' respectively and the same forward primer and analogous procedure. These were subsequently inserted into HA/pCDNA. The BoNT/E insensitive point mutant Em2 was made as described in Washbourne et al. [22]. The mutants Cys $\rightarrow$ Ser/Em2 and Cys $\rightarrow$ Gly/Em2 were prepared by subcloning the SNAP-25 *Nde*I fragment 5' to the Em2 mutation within Em2/HA/pCDNA. All mutagenesis and subcloning steps were checked by sequencing. Oligonucleotide synthesis and DNA sequencing were performed

at the Protein and Nucleic Acid Core Facility, The Scripps Research Institute, La Jolla, CA, U.S.A. and the Center for Genetics in Medicine, University of New Mexico, Albuquerque, NM, U.S.A.

Transfection and membrane association

Prior to transfection, COS7 cells were grown overnight at a density of 0.25×10^6 cells/well in six-well plates. pCDNA3, syntaxin 1A/pCDNA3 and SNAP-25 cysteine substitution/pCDNA3 constructs (0.875 μ g) were combined with 3.8 μ g of pBluescript and 15 μ l of LIPOFECTAMINE[™] reagent (Gibco BRL) in a total volume of 300 μ l of Optimem (Gibco BRL) and incubated at 24 °C for 30 min. The LIPOFECTAMINE[™]/DNA mixture was then diluted 5-fold with Optimem and used to replace the medium of the COS7 cells. After incubating the cells for 6 h at 37 °C, 1 ml of DMEM containing 20% FBS was added. The medium was completely changed after a further 12 h. After 54 h the cells were trypsinized, collected, washed in PBS and lysed by Dounce homogenization in hypotonic buffer [10 mM Tris/HCl (pH 7.4), 5 mM EDTA, 5 mM dithiothreitol, 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin and 2.5 mM PMSF]. After pelleting nuclei at 3000 *g*, cytoplasmic and membrane components were separated by centrifugation at 100 000 *g* for 30 min. Equal amounts of protein were then separated by SDS/PAGE, transferred on to nitrocellulose membranes and probed with monoclonal antibodies raised against SNAP-25 (SMI-81; Sternberger Monoclonals, Lutherville, MD, U.S.A.) and ¹²⁵I-labelled secondary antibodies. The amount of SNAP-25 in each fraction was measured on a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and compared in order to obtain a relative measure of SNAP-25 association with membrane components.

Immunofluorescence

Prior to transfection, BHK cells were plated at a density of 5×10^4 cells/well in 24-well plates on polylysine-coated coverslips and left for 18 h. Cells were transfected with LIPOFECTAMINE[™] as described above for COS7 cells and left for 36 h. The coverslips were washed twice with PBS, then fixed with warm 4% (w/v) paraformaldehyde/4% (w/v) sucrose and incubated at 37 °C for 20 min. After permeabilizing with 0.2% Triton X-100, the coverslips were incubated with antibodies at appropriate dilutions in PBS containing 1% (w/v) BSA for 1 h at 24 °C. After washing with PBS the coverslips were incubated with both anti-(mouse IgG)-Cy3 and anti-(rabbit IgG)-FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) for 30 min. After washing six times, the coverslips were mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Slides were viewed using an Olympus BX40 fluorescence microscope with a SPOT digital camera.

Purification of recombinant proteins

Recombinant BoNT/E-His₆ light chain expression and purification was achieved as described by Washbourne et al. [22]. Expression and purification of recombinant NSF-His₆ and α -SNAP-His₆ was adapted from the method of Whiteheart et al. [23]. Briefly, the supernatant from sonicated bacteria was loaded on to a Ni²⁺-nitrilotriacetate column (Qiagen, Valencia, CA, U.S.A.). Imidazole-eluted fractions were subsequently visualized in a Coomassie Brilliant Blue-stained 12% (w/v) polyacrylamide gel and the best fractions were pooled and loaded on to a PD10

column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) to remove the imidazole. Recombinant VAMP, SNAP-25 and syntaxin were prepared from glutathione S-transferase (GST) fusion constructs (pGEX4T3; Amersham Pharmacia Biotech) as described previously [24]. GST–syntaxin 1A was eluted from the resin using 50 mM GSH in PBS containing 0.1 % Triton X-100 (PBST). GSH was then eliminated by passing through a PD10 column. VAMP and SNAP-25 were cleaved from the GST bound to the resin by incubating with thrombin. The supernatant was collected, and released protein was further eluted with PBST. Purity was determined by Coomassie Brilliant Blue staining of SDS/PAGE gels and aliquots were stored at -80°C .

Enzymic synthesis of [^3H]palmitoyl-CoA

[9,10- ^3H]Palmitic acid (60 Ci/mmol) was purchased from DuPont (New England Nuclear, Boston, MA, U.S.A.). [^3H]Palmitoyl-CoA was enzymically synthesized from [^3H]palmitic acid (588 Ci/mol), purified by reversed-phase chromatography on a C_{18} sep-pack cartridge and stored at -20°C in 5 mM sodium acetate buffer (pH 5.2) [25]. Spectral and TLC analyses revealed that it was 80–90 % pure.

Thermostability of SNARE protein interactions

GST–syntaxin 1A and thrombin-cleaved recombinant SNAP-25b were incubated together with GSH–agarose overnight and then thoroughly washed with PBST. Subsequently the resin was incubated with 1 nM [^3H]palmitoyl-CoA for 2 h at 37°C . After washing, the resin was aliquoted and heated to temperatures ranging between 52 and 74°C (with 2° increments). The resin was immediately separated from the supernatant by centrifugation through a SPIN-X tube filter (Costar, Corning, NY, U.S.A.), and both the supernatant and the pellet were subjected to SDS/PAGE. After Coomassie Brilliant Blue staining and scanning, the gels were equilibrated in Amplify (Amersham Pharmacia Biotech), dried and exposed to Hyperfilm ECL[®] (Amersham Pharmacia Biotech). As we only found approx. 6.5 % efficiency in palmitate labelling of SNAP-25 (one palmitate per SNAP-25), we assumed that the Coomassie Brilliant Blue-stained protein represented unpalmitoylated protein, whereas the autoradiography data represented palmitoylated SNAP-25.

Immunoprecipitation of SDS-resistant complexes

COS7 cells were transfected with LIPOFECTAMINE[™] as described above. Immunoprecipitation of SDS-resistant complexes was performed as described previously [22]. Briefly, transfected cells were harvested in lysis buffer [20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1 % (v/v) Triton X-100, 0.5 % deoxycholate, 0.05 % SDS and 1 mM PMSF] and immunoprecipitated using Protein G–Sepharose beads (Amersham Pharmacia Biotech) to which an anti-syntaxin antibody, HPC-1 (Sigma, St Louis, MO, U.S.A.), had been conjugated. The beads were washed with lysis buffer and the bound proteins were eluted at 50°C in 60 μl of Laemmli sample buffer containing 1 % (w/v) SDS and no reducing agent. The samples were split into two, and one aliquot was boiled for 10 min. Samples were electrophoresed, transferred on to nitrocellulose, probed with antibodies raised against VAMP (MAB-333; Chemicon, Temecula, CA, U.S.A.), syntaxin and SNAP-25 and then visualized using horseradish peroxidase-conjugated secondary antibodies and a STORM PhosphorImager.

Gradient fractionation of the 20 S complex

COS7 cells were grown overnight at a density of 10^6 cells per plate in 10 cm culture dishes, and were transfected with VAMP2/pCDNA3, syntaxin 1A/pCDNA3 and either SNAP-25b/HA/pCDNA or Cys-negative mutants (SNAP-25 Cys $\rightarrow$ Ser/HA/pCDNA or SNAP-25 Cys $\rightarrow$ Gly/HA/pCDNA) as described above. After 48 h the cells were harvested with trypsin and lysed in 1 ml of lysis buffer. After pelleting insoluble matter, the lysate was split into two and incubated with 0.5 mM ATP, either 2 mM MgCl_2 or 2 mM EDTA, 20 μg of NSF-His₆ and 9 μg of α -SNAP-His₆ in a final volume of 600 μl for 30 min at 4°C . These samples were then loaded on to 10–35 % glycerol gradients and centrifuged as described in Sollner et al. [26]. The gradients were fractionated and the aliquots were precipitated with acetone. The fractions were then subjected to gel electrophoresis and Western blotting, and were subsequently probed with anti-syntaxin 1 antibodies and horseradish peroxidase-conjugated secondary antibodies, followed by detection with ECL[®] and a PhosphorImager.

Transfection of PC12 cells and assay of growth hormone (GH) release

Transfection of PC12 cells and quantification of GH release was carried out as described by Washbourne et al. [22]. Briefly, PC12 cells were transfected with GH plasmid pXGH5 and either control pcDNA3 or the toxin-resistant Em2 constructs using LIPOFECTAMINE[™]. Cells were permeabilized after 72 h with 20 μM digitonin, incubated with or without 14 nM BoNT/E-His₆ light chain and then challenged by the addition of buffer containing either no Ca^{2+} or 10 μM Ca^{2+} . Buffer samples and the cells were processed and assayed for human GH levels using an ELISA kit according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, U.S.A.). In all experiments the amount of human GH release is expressed as a percentage of total cellular content of human GH.

RESULTS

Transmembrane domain of syntaxin 1A determines the cellular localization of SNAP-25

SNAP-25 is a membrane-associated protein of axons and synaptic terminals. As it possesses no transmembrane domain, membrane association may be mediated by palmitoyl residues covalently bound to the cysteine residues within the central portion of the protein [15,19]. Recent indirect evidence [20] based on chemical deacylation suggests, however, that SNAP-25 could be maintained at the plasma membrane through protein–protein interactions. In neurons and neuroendocrine cells SNAP-25 is tightly associated with its t-SNARE partner, syntaxin 1A, a type II membrane protein, whose association with the lipid bilayer is mediated through a C-terminal hydrophobic transmembrane domain [1]. Recent evidence that SNAP-25 is in fact driven to the membrane by its association with syntaxin utilized a deletion mutant in which the entire cysteine cluster, spanning 12 amino acids, was deleted [27]. To address the targeting effect of syntaxin and the relative contribution of each cysteine residue in relation to syntaxin we used two methods. We first substituted all cysteine residues to either serine or glycine, and examined their intracellular localization with and without either wild-type syntaxin 1A or a mutant of syntaxin 1A lacking a transmembrane domain (syntaxin ΔTM) in transfected BHK cells. Since a recent report

Figure 1 Subcellular localization of SNAP-25 is dependent on the transmembrane domain of syntaxin 1A

SNAP-25 follows syntaxin localization when co-transfected into non-neuronal cells. Wild-type SNAP-25 (SNAP-25 wt) and SNAP-25 Cys → Ser (C>S) were transfected alone (a and b) or with either wild-type syntaxin 1A (syntaxin wt; d, d', e and e') or syntaxin Δ TM (g, g', h and h') into BHK cells. The two panels on the left show syntaxin wt and syntaxin Δ TM (c and f) staining in the absence of SNAP-25. SNAP-25 was stained using a rabbit antibody raised against the C-terminus and anti-(rabbit IgG)-FITC, and syntaxin was stained using monoclonal antibody HPC-1 and anti-(mouse IgG)-Cy3. Slides were viewed using an Olympus BX40 fluorescence microscope and images were captured with a SPOT digital camera. Scale bar = 3 μ m.

described that syntaxin is retained in the Golgi in neurosecretion-incompetent cells [28], this phenomenon functions as an assay to investigate whether syntaxin is able to direct SNAP-25 localization. We also examined every possible combination of cysteine residue substitutions for their ability to direct SNAP-25 to the membrane with and without syntaxin.

We performed immunofluorescence on the transfected BHK cells and stained for syntaxin 1A and SNAP-25. From Figure 1 it is evident that wild-type SNAP-25 and the mutant of SNAP-25 in which the cysteines had been mutated to serines localized differentially. Whereas wild-type SNAP-25 accumulated at the plasma membrane (Figure 1a), expression of the Cys-negative mutant (Cys → Ser) led to a diffuse distribution of protein, indicative of cytosolic localization (Figure 1b). The expression of the Cys-negative Cys → Gly mutant gave the same subcellular localization as the Cys → Ser mutant (results not shown). Wild-type syntaxin 1A transfected into BHK cells showed, as expected, an internal membrane localization, which co-localized with Golgi markers, such as 58K and GM130 (results not shown). Expression of syntaxin did not seem to lead to Golgi disassembly in these cells, in contrast with what has been described for syntaxin expression in PC12-27 and NRK cells [28]. The coexpression of syntaxin 1A with SNAP-25 constructs, however, greatly changed the subcellular distribution of SNAP-25 immunofluorescence. Syntaxin 1A and SNAP-25 clearly co-localized, regardless of whether syntaxin 1A possessed a transmembrane domain or not. In the case of wild-type syntaxin 1A, both SNAP-25 and syntaxin

1A take on a perinuclear membrane localization (Figures 1d, 1d', 1e and 1e'), clearly resembling the distribution of syntaxin staining when syntaxin 1A is expressed alone (Figure 1c). Deletion of the transmembrane domain resulted in the diffuse localization of syntaxin throughout the cytosol (Figure 1f), and this localization was followed by the co-transfected SNAP-25, with or without cysteine residues (Figures 1g, 1g', 1h and 1h').

The cells expressing wild-type syntaxin 1A consistently showed a more compact morphology, which was presumably not simply due to overexpression, since cells overexpressing syntaxin Δ TM or Cys-negative SNAP-25 constructs showed a normal flattened appearance similar to that of parental BHK cells (results not shown). Interestingly, cells expressing wild-type SNAP-25 appeared to have an intermediate morphology.

In order to evaluate the individual contribution of each cysteine residue to membrane association, site-directed mutants were generated to create substitutions of all possible combinations of cysteine residues to glycine (Figure 2A). Transfection of these SNAP-25 constructs into COS7 cells and subsequent cell fractionation clearly demonstrated a dose-dependent decrease in membrane association due to the absolute number of cysteine residues present in the molecule (Figures 2B and 2C). Substitution of one cysteine residue reduced the association with the membrane fraction from 55.7 to 18.5%. The substitution of any two cysteines resulted in a further drop to approx. 6.5% and this level remained the same for further substitutions. These data are in agreement with previous studies, which were also performed in

Figure 2 Membrane association of SNAP-25

(A) The four cysteine residues of SNAP-25b (25B) were replaced with glycine residues either individually or in combination. The conservation of the cysteine motif is apparent from the alignment with SNAP-25a (25A) and the non-neuronal counterpart, SNAP-23 (23). (B) The membrane association of SNAP-25 in transfected COS7 cells is shown as a function of the number of replaced cysteine residues. In the presence of syntaxin (white bars) membrane association (assoc.) remained unchanged. Results are presented as the means $\pm$ S.E.M. for the different substitution combinations shown in (C). (C) Membrane association of SNAP-25 shown individually for each possible Cys $\rightarrow$ Gly (C>G) substitution. Data are from a single experiment except for wild-type (wt) and 1+2+3+4, where the results are presented as means $\pm$ S.E.M. ($n = 3$).

transfected non-neuronal cells [16,19]. In contrast, when the cells were co-transfected with a 2-fold molar excess of plasmid expressing syntaxin 1A, the membrane association of either wild-type or cysteine-substituted versions of SNAP-25 remained largely complete, amounting to 62.6 % of total protein, regardless of the number of cysteine residues present in the molecule (white bars in Figures 2B and 2C). The membrane association of coexpressed SNAP-25 and syntaxin was confirmed by subcellular fractionation by sucrose gradient centrifugation. Whereas wild-type SNAP-25 and syntaxin co-sedimented to a lower part of the gradient, largely co-localizing with the 58K Golgi marker [29], Cys-negative SNAP-25 and syntaxin Δ TM, either individually or coexpressed, sedimented in the top fractions, indicating a soluble cytoplasmic localization. Consistent with the results shown in Figure 2, coexpression of full-length syntaxin led to the majority of Cys-negative SNAP-25 co-sedimenting in a lower portion of the gradient, demonstrating syntaxin-mediated membrane association (results not shown).

Presence of cysteine residues facilitates SNARE complex disassembly

In order to establish whether the cysteine residues and their palmitoylation function within the SNARE complex, rather than simply mediating membrane targeting, both Cys $\rightarrow$ Ser and Cys $\rightarrow$ Gly substitution mutants of SNAP-25 were assayed for their ability to form binary and ternary complexes. We considered that the presence or lack of cysteine residues and the presence of

palmitoylation might affect the integral stability of the protein-protein interactions. Analysis of the thermostability of the binary interaction of bacterially expressed syntaxin 1A with SNAP-25 showed no difference in the stability of syntaxin-SNAP-25 dimers formed with either the Cys $\rightarrow$ Ser mutant (Figure 3A) or palmitoylated SNAP-25 (Figure 3B). The melting temperatures were 62.8, 62.5 and 62.6 $^{\circ}$ C for the wild-type, Cys $\rightarrow$ Ser and [3 H]palmitoylated proteins respectively.

We then determined whether the Cys-negative mutants were able to form the core complex together with syntaxin and VAMP in transfected cells. The core complex of SNAP-25, VAMP and syntaxin is resistant to solubilization with SDS, and can only be dissociated in SDS by boiling [30]. Consequently, we transfected wild-type and Cys-negative mutants of SNAP-25 into COS7 cells together with VAMP2 and syntaxin 1A. Complexes were immunoprecipitated from detergent extracts of these cells using anti-syntaxin antibodies and the eluates were incubated at 50 and 95 $^{\circ}$ C [22]. In this assay, the formation of an SDS-resistant heat-stable complex is inferred by the resistance of the complex to dissociation at 50 $^{\circ}$ C compared with complete dissociation at 95 $^{\circ}$ C [22]. While the complex itself is only poorly distinguished under these gel conditions and with these antibodies, the relative proportion of the SNARE proteins resolved as separately migrating proteins after dissociating (95 $^{\circ}$ C) treatment compared with that obtained after non-dissociating (50 $^{\circ}$ C) treatment serves to monitor the retention of these SNARE components in the SNARE core complex. For example, in Figure 3(C) it can be seen that when only VAMP 2 and syntaxin 1A were transfected,

Figure 3 No influence of cysteine residues or palmitoylation on binary and ternary SNARE interactions

(A) Wild-type SNAP-25b (wt) or SNAP-25 Cys → Ser (C>S) was incubated with syntaxin1a-GST and GSH-agarose at the indicated temperatures, and the supernatants were separated from the resin and analysed on Coomassie Brilliant Blue-stained gels. SNAP-25 levels in the supernatant were compared with the total and expressed as a percentage. Results are presented as means $\pm$ S.E.M. ($n = 3$). C, °C. (B) SNAP-25b was bound to syntaxin1a-GST and GSH-agarose and subsequently palmitoylated with [3 H]palmitoyl-CoA. The resin was then incubated at the indicated temperatures and the supernatants were separated from the resin and analysed on Coomassie Brilliant Blue-stained gels and by autoradiography. Wild-type SNAP-25 (wt) levels and palmitoylated SNAP-25 (3H-Pal) levels within the same samples were compared with the total, and expressed as a percentage. Results are presented as means $\pm$ S.E.M. ($n = 3$). C, °C. (C) Syntaxin 1A (S) and VAMP2 (V) were transfected with and without SNAP-25 or SNAP-25 Cys → Ser (C>S) into COS7 cells. Syntaxin immunoprecipitates were incubated either at 50 or 95 °C, then subjected to SDS/PAGE and blotted for syntaxin, VAMP and SNAP-25.

VAMP resolved as a separately migrating protein regardless of the incubation temperature, consistent with the inability of these two SNARE components to form an SDS-resistant complex without SNAP-25 [30]. However, transfection of SNAP-25 or Cys → Ser SNAP-25 caused VAMP 2 and syntaxin 1A to be retained in the SDS-resistant complex, since after incubation at 95 °C, a larger proportion of these SNAREs were dissociated and resolved as separately migrating proteins. As both wild-type SNAP-25 and the Cys → Ser mutant showed a heat-labile complex we concluded that the cysteine residues are not critical for assembly of SDS-resistant complexes.

In order to probe the functionality of the SDS-resistant complex, we determined whether NSF and α -SNAP were able to bind to and dissociate the core complex, thereby transiently forming the 20 S complex [31]. In order to accomplish this, detergent extracts of transfected cells were incubated with recombinant NSF and α -SNAP in the presence of ATP and EDTA to prevent ATP hydrolysis and promote 20 S complex formation. In contrast, to allow NSF-mediated dissociation of the 20 S complex, detergent extracts were incubated with NSF

and α -SNAP in the presence of ATP and $MgCl_2$. The extracts were subsequently fractionated on glycerol gradients [26]. From the gradient profiles shown in Figures 4(A) and 4(C), and the representative blots in Figure 4(E) (left-hand panel), it can be seen that wild-type syntaxin and SNAP-25 migrated to fractions 5–8 and also 9–12 in the presence of EDTA, corresponding to sedimentation coefficients of 7 S and 20 S respectively. In the presence of hydrolysable ATP (i.e. with $MgCl_2$) these complexes were disrupted and syntaxin was only found in the upper portion of the gradient (fractions 1–4), corresponding to the monomeric form. Although clearly dissociated from the more rapidly sedimenting complex, a large proportion of the wild-type SNAP-25 sedimented to fraction 4 of the gradient (Figure 4C), consistent with the formation of multimeric forms probably associated through disulphide bonds between the cysteine residues of wild-type SNAP-25 molecules. The Cys → Ser mutant of SNAP-25 was also effectively incorporated into the higher molecular mass complexes and was more efficient in sequestering syntaxin 1A into this complex as seen by the reduction of syntaxin in fractions 1–4 (Figures 4B and 4E, right-hand panel). In contrast with the complex with wild-type SNAP-25, this 7 S core complex was not disrupted by NSF in the presence of $MgCl_2$ (Figures 4B, 4D and 4E). Similar results were obtained with complexes formed with the Cys → Gly SNAP-25 mutant and also using $MgATP$ and $MgATP[S]$ to promote and disable the dissociating activity of NSF, confirming that substitution of cysteine residues leads to an inappropriately stable complex (results not shown). Considering the result that palmitoylation does not affect the strong interaction between SNAP-25 and syntaxin, the inability to dissociate the Cys-negative SNAP-25-containing 7 S complexes could reflect the need for palmitoylation in order to compromise the stability of the ternary complex and facilitate dissociation by NSF (see the Discussion section).

Cys-negative SNAP-25 does not promote exocytosis in transfected PC12 cells

In order to probe whether the abolition of SNARE complex dissociation by mutating the cysteine residues of SNAP-25 also disrupts secretion, we used a recently developed assay for monitoring Ca^{2+} -dependent exocytosis from PC12 cells [22]. This assay is based on the cotransfection of a human growth hormone (hGH) construct with a BoNT/E-insensitive mutant of SNAP-25. Intoxication of the transfected cells with BoNT/E functionally 'knocks out' endogenous SNAP-25 by cleaving 26-amino-acid residues from its C-terminus [32,33]. Subsequent challenge with Ca^{2+} determines the ability of the transfected mutant to sustain secretion. We combined the BoNT/E-insensitive mutation, Em2, with the cysteine substitution mutations, giving rise to Cys → Ser/Em2 and Cys → Gly/Em2. These, when tested in the PC12 human GH-secretion assay system, did not affect normal Ca^{2+} -dependent release levels in the absence of neurotoxin (Figure 5A). Expression levels of all mutants were comparable as assayed by Western blotting with an anti-HA tag antibody (results not shown). In the presence of toxin, the Em2 mutant demonstrated a restoration of Ca^{2+} -dependent secretion almost to levels of secretion in the absence of toxin, as previously described [22]. The Cys → Ser/Em2 and Cys → Gly/Em2 mutants were not, however, able to restore exocytosis in BoNT/E intoxicated cells (Figure 5B). These constructs were unable to significantly raise human GH release above that of intoxicated cells transfected with empty vector (pCDNA3). Interestingly, in the absence of neurotoxin, the Cys-negative constructs did not appear to affect the level of human GH secretion. Since the majority of cells expressing human GH can be expected to be

Figure 4 Lack of mutant SNARE complex dissociation by NSF

The cysteine residues of SNAP-25 are required for SNARE complex dissociation by NSF and α -SNAP. Lysates from COS7 cells transfected with syntaxin 1a, VAMP2 and wild-type SNAP-25b (wt; **A** and **C**) or SNAP-25 Cys $\rightarrow$ Ser (C>S; **B** and **D**) were incubated with α -SNAP, NSF, ATP and either EDTA ($\circ$) or $MgCl_2$ ($\bullet$) for 30 min prior to glycerol gradient centrifugation. The amount of syntaxin (**A** and **B**) and SNAP-25 (**C** and **D**) detected on blots of the gradient fractions is expressed as a percentage of the total syntaxin, and results are presented as means $\pm$ S.E.M. ($n = 3$). (**E**) Representative blots of the fractions from the glycerol gradients. The electrophoretic mobilities of syntaxin and SNAP-25 are indicated by solid and open arrows respectively. Standards, not shown, were centrifuged in analogous gradients with ovalbumin (44 kDa) and γ -globulin (150 kDa) sedimenting in fractions 3–4 and 7–9 respectively.

coexpressing the SNAP-25 constructs, this suggests that while the Cys-negative mutants cannot function in this assay, they do not exert a dominant-negative effect on the existing SNARE-mediated neuroexocytosis.

DISCUSSION

SNAP-25 is a necessary component of the protein machinery that mediates the fusion of neurotransmitter-containing vesicles at synapses [1,32,33]. It has been well documented that this neuronal protein is found specifically at the plasma membrane or on transport vesicles in axons and synaptic terminals, not in dendrites or at the cell body plasma membrane [10,11,34]. SNAP-25 does not possess a transmembrane domain as do its partners, syntaxin 1A and VAMP. In contrast, SNAP-25 contains a central domain with four cysteine residues, which have been shown to be palmitoylated [15,19]. The role of the palmitoylation of proteins is, however, unclear. Palmitoylation has been argued to be necessary for the membrane association of numerous

hydrophilic proteins, such as growth-associated protein ('GAP')-43, p21^{ras} and G-protein α subunits [35–37]. Palmitoylation of the cysteine quartet of SNAP-25 occurs *in vitro* and *in vivo* and, indeed, appears to be sufficient for membrane association in transfected cells [16,19]. In general, palmitoylation occurs at membranes, but it is not clear as to whether it occurs spontaneously *in vivo* or by catalysis of a non-identified palmitoyl acyltransferase [38,39]. In the case of SNAP-25, palmitoylation *in vitro* has been demonstrated to occur without the requirement for an enzyme [40]. However, regardless of the mechanism driving palmitoylation, it is not clear how general membrane anchoring of SNAP-25 by palmitoylation alone could provide sufficient targeting information to localize SNAP-25 selectively to axons and synaptic terminals. We therefore hypothesized that SNAP-25 would need to be targeted by some other mechanism to axons and synaptic terminals.

In the present study, we have addressed this issue by establishing that mutant versions of SNAP-25 unable to be palmitoylated at any possible combination of cysteine residues

Figure 5 Cysteine residues of SNAP-25 are required for exocytosis

The lack of cysteine residues in SNAP-25 hampers the restoration of exocytosis, but does not exert a dominant-negative effect. PC12 cells transfected with SNAP-25 constructs and a GH construct were permeabilized and incubated in the absence (A) or presence (B) of 100 nM BoNT/E prior to raising the Ca^{2+} concentration. GH levels were measured and expressed as secretion over low Ca^{2+} background levels compared with total GH. Results are presented as means $\pm$ S.E.M. (A, $n = 3$; B, $n = 8$).

are still efficiently targeted to membranes when in the presence of syntaxin 1A. This suggests that the high affinity association with syntaxin 1A is sufficient to keep non-palmitoylated SNAP-25 at membranes, a finding that corroborates recent evidence in PC12 cells [27]. Whereas palmitoylation presumably leads to membrane insertion, binding of SNAP-25 to syntaxin in the absence of palmitate leads to membrane association, which may be aided by the 5-amino-acid membrane-binding domain of SNAP-25 described by Gonzalo et al. [21]. From the immunofluorescence images it is apparent that the plasma membrane localization of wild-type SNAP-25 by itself is directed by palmitoylation, since Cys-negative mutants accumulate in a diffuse cytoplasmic distribution with no evidence of membrane association. However, the distribution of SNAP-25 is changed to a Golgi localization in the presence of syntaxin 1A. Our data show that the trans-membrane domain of syntaxin 1A contains the targeting information necessary for the localization of both syntaxin and SNAP-25.

Syntaxin 1A is a type II membrane protein, and although the mechanism mediating its membrane association has not been specifically resolved, trafficking of syntaxin 1A is probably analogous to the synaptic v-SNARE, VAMP. This synaptic v-SNARE is thought to be first inserted into the endoplasmic reticulum and then translocated via the Golgi before transport to

the presynaptic terminal [13]. The immunofluorescence images suggest that wild-type syntaxin is targeted to the Golgi in non-neuronal cells (results not shown and [28,41]), where it seems to accumulate considerably. This is consistent with the idea that trafficking of t-SNAREs, similarly to the v-SNARE VAMP, requires transit through the Golgi and assembly in vesicles before entering into fast axonal transport [34,42] to localize to axon terminals, a process which is not sustained in non-neuronal cells. In support of this idea, green-fluorescent-protein tagged SNAP-25 was observed to be transported together with *trans*-Golgi-network protein, TGN38, along the axon [12]. In a recent electron microscopy study, SNAP-25 was reported to be localized to the Golgi complex in retinal neurons [43]. Moreover, the palmitoylation of SNAP-25 seems to be sensitive to Brefeldin A, which collapses the Golgi apparatus into tubular structures [20]. This suggests that not only is SNAP-25 translocated in a complex with syntaxin through the Golgi, but that palmitoylation may occur at a later stage after transport to the plasma membrane. Our results, therefore, help to further define the SNARE trafficking pathway by identifying syntaxin 1A as the major element in mediating the localization of SNAP-25 to the Golgi as an initial step towards its final destination at the presynaptic terminal.

Considering coexpression of SNAP-25 with syntaxin is sufficient for the targeting of SNAP-25 to the plasma membrane in neurons, this raises the possibility that palmitoylation could play a role in the function of SNARE complexes in exocytosis. Glycerol density centrifugation of SNARE complexes in the presence of NSF, α -SNAP and non-hydrolysable ATP provides an assay for the formation of the large intermediate 20 S complex of these proteins and also demonstrates the action of NSF in the presence of hydrolysable ATP, which dissociates the 7 S core SNARE complex into its monomeric constituents [26,44]. Our data reveal that when the Cys $\rightarrow$ Ser or Cys $\rightarrow$ Gly mutants of SNAP-25 are included in the SNARE complex this 7 S complex can no longer be disrupted by NSF under *in vitro* conditions, and the complex is unable to participate in neurosecretion *in vivo*.

Further study of the binary and ternary protein interactions between syntaxin 1A, VAMP2 and SNAP-25 demonstrated that cysteine residues or their palmitoylation have no effect on thermostability, thus eliminating the possibility that modification of cysteine residues may reduce the affinities of the SNAREs for each other by destabilizing protein-protein interactions. A possible explanation for the lack of SNARE complex disassembly in the absence of cysteine residues is that NSF and α -SNAP preferentially act on palmitoylated SNARE proteins. Interestingly, Rothman and co-workers have proposed that fatty acylation is, in fact, required at some step of vesicular fusion and trafficking [45]. Palmitoylation of SNAREs, such as VAMP [46], SNAP-25 and synaptotagmin [19], and even α -SNAP itself (P. Washbourne, unpublished work), suggests a global mechanism that may facilitate the transition from the *cis*- to the *trans*-SNARE complex, thus allowing for dissociation of the complex by the action of NSF [47]. In addition, the recent report that VAMP and synaptotagmin are palmitoylated in a developmentally regulated fashion [46], suggests the interesting hypothesis that fatty acylation could serve as a mechanism that has developed to allow the SNAREs to gain their function as mediators of Ca^{2+} -dependent exocytosis once neurons have formed synapses.

Based on these findings it can be further hypothesized that the central cysteine residues of SNAP-25 and potentially their post-translational modification by palmitoyl-CoA play an important role in the SNARE-complex cycle. This is shown to be the case by our assay of exocytosis in PC12 cells and is suggested by a

study in insulin secreting cells [48]. Whereas wild-type SNAP-25b efficiently restores release from PC12 cells, mutants containing the Cys → Ser or Cys → Gly mutations are not able to sustain exocytosis. The reduction in secretion is not due to a reduced presence of Cys-negative SNAP-25 at the plasma membrane, as shown by the immunofluorescence and cell fractionation data, but seems to reside in the fact that these mutants are not able/available to catalyse vesicle fusion. The fact that exocytosis from non-intoxicated cells is not hampered by the presence of the Cys-negative mutants demonstrates that these proteins do not exert dominant-negative effects. This may be surprising considering the effect of the Cys → Ser or Cys → Gly mutations on the ability of NSF to dissociate the 7 S complex. One would expect the Cys-negative mutants to sequester syntaxin and VAMP molecules into an inactive non-recyclable form, thus diminishing the exocytotic output of the cells. A possible explanation for this observation is that the level of the SNARE proteins is not limiting and that several or many independent SNARE complexes (or SNAREpins) probably reside on each vesicle. This would agree with the ring hypothesis for membrane fusion, as was described for influenza virus HA [49], and has been proposed to apply to the analogous SNAREpins in vesicle membrane fusion [7]. Consequently the deficit imposed by the presence of non-functional SNAP-25-containing complexes could be overcome by mass action of the individual functional SNAREpins of the ring structure.

The data presented in the current paper agree with data from the aforementioned study that demonstrates a block in insulin secretion due to the substitution of all four cysteine residues of SNAP-25 for alanines [48]. Gonelle-Gispert et al. [48] conclude that this block is due to a reduced presence of their Cys-negative mutant at the plasma membrane, despite the fact that they were able to drive more of this construct to the membrane by overexpressing syntaxin 1A. We believe, in contrast, that our biochemical data for the NSF-dependent dissociation of SNAP-25 and the Cys-negative mutants provide a novel and more consistent explanation for the block in secretion, thus suggesting a more substantial role for the cysteine residues in SNAP-25 function other than plasma membrane localization.

Our results from the exocytosis assay also complement the recent study by Scales et al. [6], in which they used an acute assay of noradrenaline released from cracked PC12 cells. Whereas they showed that Cys-negative SNAP-25 mutants were effective in participating in an initial round of exocytosis, our data indicate that dissociation and not initial association of Cys-negative SNAP-25 with the core complex is compromised. Thus our results provide further evidence for the necessity of SNARE dissociation by NSF after vesicle fusion and suggest the requirement of palmitoylation in this SNARE component recycling step.

While further investigation will be required to reveal the precise molecular parameters altered by palmitoylation, these studies do suggest how dynamic fatty acylation can affect and perhaps modulate neurotransmission. Moreover, these findings illustrate how the molecular role of palmitoylation within the SNARE complex can serve as a model that may be applied to other important acylated proteins and help reveal a more integral role for this fatty acid modification in the mechanics of protein function other than membrane association.

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