Membrane localization and biological activity of SNAP-25 cysteine mutants in insulin-secreting cells

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SUMMARY

The tSNARE SNAP-25 is expressed in pancreatic β-cells and is involved in the regulated release of insulin. It has been shown previously that SNAP-25 associates with the plasma membrane consequent to palmitoylation of one or more cysteines in the central region of the molecule. The importance of palmitoylation in the biological function of SNAP-25 in exocytosis was not addressed. Furthermore, studies on both SNAP-25 and its non-palmitoylated homologues SNAP-29 and sec9, have suggested an alternative or complementary mechanism for membrane association involving interaction with syntaxin. To address these issues, we have now studied the behavior and biological activity of cysteine mutant SNAP-25 in insulin-secreting (HIT) cells. While 91% of native SNAP-25 was associated with the membrane, this value decreased to 56% for the single cysteine mutant C85/A and to 10% for the double (C85,88/A) and quadruple (C85,88,90,92/A) mutants. The mutant SNAP-25 forms were all found to bind syntaxin 1A with equal efficacy. Over-expression of syntaxin 1A in HIT cells allowed for partial relocalization of both the double and quadruple SNAP-25 cys mutants to the membrane. By introducing a further mutation to the SNAP-25 molecules to render them resistant to botulinum neurotoxin E, it was possible to study their ability to reconstitute regulated insulin secretion in toxin-treated HIT cells. Native SNAP-25 was able to fully reconstitute secretory activity in such cells. Despite the fact that the single cysteine mutant was significantly displaced to the cytosol, it still displayed 82% activity in the secretion reconstitution assay, and a similar discrepancy was seen for the double mutant. Even the quadruple mutant with no remaining cysteines was able to support a minimal level of secretion. It is concluded that both palmitoylation and binding to syntaxin are implicated in membrane association of SNAP-25. This as well as the discrepancy between membrane localization and biological activity of the cysteine mutants, suggests a complex, multi-component process for association of SNAP-25 with the membrane and its recruitment to a biologically productive state.

Key words: SNAP-25, SNARE, Insulin secretion, Membrane localization

INTRODUCTION

SNARE proteins have been shown to play an important role in regulated insulin secretion from pancreatic β-cells (Martin et al., 1995; Regazzi et al., 1996; Sadoul et al., 1995), in much the same way as they are involved in secretion from neurons. Many SNARE proteins, although anchored to a membrane, are not classical trans-membrane proteins synthesized on the RER. The tSNARE syntaxin (plasma membrane) and the vSNARE VAMP (vesicular membrane) both belong to a class of tail-anchored proteins that are post-translationally integrated into the membrane of the endoplasmic reticulum by an as yet unknown mechanism. They are then transported along the secretory pathway to their final destination (Kutay et al., 1995). SNAP-25, which is an intrinsically hydrophilic protein, contains four closely spaced cysteine residues near the center of the molecule (Oyler et al., 1989). Palmitoylation of one or more of these cysteine residues has been shown to be necessary for membrane association of this tSNARE (Lane and Liu, 1997; Veit et al., 1996) and of the closely related, ubiquitously expressed homologue SNAP-23 (Koticha et al., 1999). Membrane-association of SNAP-25 now appears more complex than originally supposed. Other members of the SNAP-25 family of proteins, notably the yeast homologue sec9 and the recently discovered SNAP-29, are thus membrane bound but lack a cysteine domain. They may associate with membranes through interaction with other membrane proteins, like for instance members of the syntaxin family (Brennwald et al., 1994; Steegmaier et al., 1998). Membrane association of SNAP-25 may also be mediated by syntaxin. In fact association of these two molecules appears to occur before anchorage of SNAP-25 to membranes (Vogel et al., 2000). Furthermore, in vitro palmitoylation of SNAP-25 is facilitated by its binding to syntaxin (Veit, 2000).

Based on the above findings, it has been proposed that SNAP-25 becomes localized to the plasma membrane in a multi-step process. The initial events are mediated by binding to syntaxin, but membrane association is consolidated by
palmitoylation of SNAP-25 (Vogel and Roche, 1999). This puts into question earlier studies on the role of palmitoylation of SNAP-25 cysteines and membrane anchorage, to the extent that they were performed in an inappropriate cellular setting (COS cells) devoid of the neuronal syntaxin isoform to which SNAP-25 normally binds or with non-physiological ratios of SNAP-25:syntaxin. There is, furthermore, the need to relate membrane association with the biological function of SNAP-25. These two issues are addressed in the present study in which the localization and biological activity of SNAP-25 cysteine mutants were investigated in insulin-secreting cells.

MATERIALS AND METHODS

Materials

Culture media and serum were from Gibco BRL, Life Technologies AG, Basel, Switzerland and all routine chemicals were from Sigma-Fluka, Buchs, Switzerland unless otherwise stated.

The cDNA coding for human SNAP-25b (Bark and Wilson, 1994) was cloned into the pcDNA3 vector (Invitrogen, Groningen, The Netherlands). For the N-terminal GFP-tagged SNAP-25b construct, EGFP (‘enhanced’ green fluorescent protein from Clontech; Clontech Laboratories GmbH, Heidelberg, Germany) was placed into the pcDNA3 vector containing SNAP-25b using a BamHI site in front of the SNAP-25b cDNA (leading to the insertion of two amino-acids between EGFP and SNAP-25b). The cDNA for human proinsulin was cloned into the pcMV vector and that coding for syntaxin into the pAC vector containing a CMV promoter. In all experiments using botulinum neurotoxin E, only the light chain of the toxin (prepared as recombinant protein; T. Binz, T. Hayashi, S. Yamasaki, and H. Niemann, unpublished work) was used and referred to below as BoNT/E. Recombinant streptolysin-O (SLO; with cys530 mutated to Ala) fused to the N-terminal amino acids; Oyler et al., 1989). The secondary antibody for binding of in vitro translated SNAP-25 mutants to syntaxin was a monoclonal anti-syntaxin (HPC-1, Sigma catalogue number S-0664) and the secondary antibody a goat affinity purified antibody to anti-mouse FITC antibody, diluted 1:1000. Finally cells were stained with 50 μl of SDS-sample buffer containing β-mercaptoethanol. The samples were boiled and subjected to 10% SDS-PAGE and autoradiography. The amount of labeled SNAP-25 bound to the beads had been evaluated by densitometric scanning of the films.

Transfections

For all experiments, cells were used two days after transfection. For the secretion assay, HIT cells were seeded at a density of 4.5×10^6 cells/well in 24-well plates. Transfection was performed using 1 μl of transfectam (Promega) for 5 μg of total DNA/well in a total volume of 0.4 ml of culture medium without FCS for 4-6 hours. The following quantities of DNA were used for the secretion assays: 2.5 μg pCMV human proinsulin plus 0.2 μg pcDNA3-GFP-SNAP-25TR or cysteine mutants plus 2.3 μg pcDNA3 or for control conditions: 2.5 μg pCMV human proinsulin plus 2.5 μg pcDNA3.

For subcellular fractionation, half of a 24-well plate was transfected with 5 μg/well total DNA for each condition: 2.5 μg pcDNA3-GFP-SNAP-25TR or cysteine mutated constructs plus 2.5 μg pcDNA3 or 2.5 μg pAC-syntxin 1A in the case of the co-expression study.

For immunoprecipitation, COS cells were seeded at a density of 10^6 cells per 50 cm^2 Petri dish one day before transfection. Medium was changed 1 hour before transfection and transfection was performed using calcium phosphate with 30 μg of total DNA for each condition: 20 μg of pcDNA3-GFP-SNAP-25TR or cysteine mutants and 10 μg of pcDNA3.

For fluorescence studies, 5×10^6 HIT cells were seeded two days before transfection on glass coverslips coated with poly-L-lysine. For each condition, 2.5 μg of pcDNA GFP-SNAP-25TR or cysteine mutated constructs and 2.5 μg of pcDNA empty vector were used for transfection.

Immunofluorescence

After two days of culture on glass coverslips, HIT cells were fixed for 10 minutes in 4% paraformaldehyde in PBS, washed twice in Dulbecco’s PBS (DBS), permeabilised for 30 seconds in acetone, washed twice with DBS and blocked for 15 minutes in DBS plus 5% BSA before incubation with the monoclonal anti-SNAP-25 antibody (Sternberger Monoclonals Inc., diluted 1:400) for 2 hours at room temperature. Cells were then washed twice in DBS and incubated for 1 hour at room temperature in the dark with the secondary anti-mouse FITC antibody, diluted 1:1000. Finally cells were washed twice in DBS and mounted using Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence images and the fluorescence images of the different GFP-SNAP-25 constructs were taken using a Cool View camera adapted to a fluorescence microscope (Zeiss).

Secretion assay

The secretion assay was performed as described earlier (Gonelle-Gispert et al., 1999) with one change: where indicated, cells to be

primers: 5’CCGGGGTTGCTGGCTCCCGCTAACAAGCTTAAATCC3’ and 5’GGATTTAAGCTTTGAGGCACAGCCACACAGGAGCGCCG3’. All the mutations were confirmed by DNA sequencing.

Binding of in vitro translated SNAP-25 mutants to syntaxin

In vitro translation was performed in the presence of [35S]methionine (NEN Du Pont, Regensburg, Switzerland) according to the protocol supplied by the manufacturer of the translation kit (Promega, Madison, WI, USA). GST-syntxin 1A was immobilized on glutathione-beads as described (Chapman et al., 1994). Seventy μl (dry bed volume) of syntaxin-beads were incubated with 360 μl of buffer B (20 mM Tris, 150 mM NaCl, 2.5 mM CaCl2, 0.5% Triton X-100, 0.1% β-mercaptoethanol) containing 6 μl of the in vitro translated [35S]methionine-labeled SNAP-25 wild-type or mutants. Following overnight incubation at 4°C, the beads were washed four times with 1 ml of buffer B, then resuspended in 50 μl of SDS-sample buffer containing β-mercaptoethanol. The samples were boiled and subjected to 10% SDS-PAGE and autoradiography. The amount of labeled SNAP-25 bound to the beads had been evaluated by densitometric scanning of the films.
treated with BoNT/E were additionally treated with BoNT/E during SLO permeabilization.

The amount of C-peptide released from transfected cells was determined using a human C-peptide ELISA Kit (Dako, Cambridge, UK). For determination of cellular C-peptide content, HIT cells were lysed after the secretion assay in 0.2 ml/well of solubilization buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.5% Triton X-100, pH 7.4) and the C-peptide was determined after centrifugation in the cleared lysate using the same kit. This ELISA recognizes specifically the exogenous human C-peptide and does not cross-react with the endogenous hamster C-peptide of the HIT cells.

Subcellular fractionation
Transfected HIT cells were harvested by trypsinization, and collected by centrifugation. About 1-1.5 \times 10^6 cells were resuspended in 1 ml of hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, pH 7.4). After incubation for 15 minutes on ice, cells were homogenized on ice by 50 strokes in a glass potter. A small fraction of the total homogenate was kept and used as an input. The homogenates were then centrifuged at 100,000 g for 1 hour to obtain the ‘cytosol’ and ‘membrane’ fractions. To exclude contamination of the different fractions, the cytosol was centrifuged a second time for 1 hour and the membrane pellet was rinsed with 0.5 ml of hypotonic buffer followed by a second centrifugation. The membrane pellet was solubilized by suspension and sonication in an equivalent volume of hypotonic buffer containing 0.5% SDS. The total homogenate, as well as the membrane and the cytosolic fractions were mixed with sample buffer, boiled and equal volumes were loaded on SDS-polyacrylamide gels and analyzed by immunoblot.

Immunoprecipitation
Transfected COS cells were scraped from the dish and lysed using 1 ml of solubilization buffer (20 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, 0.5% Triton X-100, pH 7.4). An equivalent number of untransfected HIT cells were lysed in the same buffer. The lysates were then centrifuged at 100,000 g in order to eliminate the Triton insoluble material. Transfected COS cell lysate (150 μl) was mixed with 150 μl of untransfected HIT cell lysate and incubated overnight at 4°C with 3 μl of a monoclonal anti-syntaxin antibody. 75 μl of a 30% slurry of anti-mouse IgG Sepharose beads were added to each condition and incubated for 6 hours at 4°C with agitation. The immunocomplex was pelleted by centrifugation and washed 5 times with 1 ml of solubilization buffer. The beads were boiled for 5 minutes in SDS-Sample buffer, boiled and equal volumes were loaded on SDS-polyacrylamide gels and analyzed by immunoblot.

SDS-PAGE and western blotting
Proteins were separated on 10% polyacrylamide gels under reducing conditions according to Laemmli (1970). After SDS-PAGE, proteins were transferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany) and SNAP-25 was detected using a polyclonal antibody at a dilution of 1:1000.

RESULTS

Cysteine and toxin-resistant mutants of SNAP-25 and GFP-SNAP-25
SNAP-25 contains 4 cysteines (residues 85, 88, 90 and 92) which are thought to be palmitoylated. SNAP-25 with 1 (C85/A), 2 (C85,88/A) or all 4 (C85,88,90,92/A) cysteines mutated to alanine were generated by site directed mutagenesis as depicted in Fig. 1. SNAP-25 is normally cleaved by botulinum neurotoxin E (BoNT/E) between residues 180 and 181 (Fig. 1). The mutation of 3 residues in proximity to this site renders SNAP-25 resistant to BoNT/E (Gonelle-Gispert et al., 1999). We have shown previously, that the toxin-resistant mutant is able to rescue totally Ca²⁺-induced insulin secretion from HIT cells (an insulin secreting cell line) which have been permeabilized and treated with BoNT/E to cleave and inactivate the endogenous SNAP-25. In order to be able to investigate the functional importance of the cysteine residues for regulated insulin secretion, wild-type and the mutant SNAP-25 molecules were further rendered toxin-resistant and are referred to as SNAP-25TR (Fig. 1). For experiments in which it was necessary to distinguish between endogenous and exogenous SNAP-25, or for localization of SNAP-25 and its mutant forms in living cells, fusion proteins were constructed with green fluorescent protein N-terminal to SNAP-25. The toxin-resistant GFP-SNAP-25 fusion protein was fully active in reconstituting insulin secretion from toxin treated cells as described below (Fig. 6A).

Localization of the SNAP-25 cysteine mutants in insulin secreting HIT cells
It has been shown previously that only 14% of SNAP-25

![Fig. 1. Schematic representation of wild-type SNAP-25, and cysteine/toxin-resistant mutants. The 4 cysteine residues and the site of cleavage of SNAP-25 by BoNT/E are as indicated for the wild-type molecule. The toxin-resistant mutants have the suffix 'TR'.](attachment:image.png)
molecules mutated at the first cysteine residue remain associated with the plasma membrane and that a double-cysteine mutant is fully displaced to the cytosol (Lane and Liu, 1997). These studies, however, were performed in COS cells which do not possess a regulated secretory pathway. It has further been shown that syntaxin 1A expressed in cells without a regulated secretory pathway accumulates in the Golgi or the TGN (Rowe et al., 1999). This may indicate that cells without a regulated secretory pathway may not be able to correctly target SNAREs implicated in regulated exocytosis. We therefore wanted to investigate the cellular localization of SNAP-25 cysteine mutants in cells which express both SNAP-25 and its cognate syntaxin endogenously, and which possess a regulated secretory pathway. To this end, GFP-SNAP-25 or cysteine mutants were expressed by transient transfection in insulin-secreting HIT cells. Fluorescence microscopy of fixed cells revealed that GFP-SNAP-25TR (Fig. 2A, c) and endogenous SNAP-25 (Fig. 2A, a) are localized to the same cellular compartments, namely to the plasma membrane as well as to an unidentified perinuclear region. The single mutant GFP-SNAP-25TR Cys 85 (Fig. 2A, d) is localized to some extent at the plasma membrane and the perinuclear region but, as expected, also displays uniform labelling of the cytosol. The GFP-SNAP-25TR double (Fig. 2A, e) and quadruple (Fig. 2A, f) mutants were localized uniformly in the cytosol and almost no fluorescence was distinguishable at the plasma membrane or in the perinuclear region. As a control for the specificity of the localization of the GFP-tagged SNAP-25 molecules we also transfected HIT cells with GFP alone. As shown in Fig. 2A, b, GFP was localized to the cytosol and to the nucleus, to the exclusion of the plasma membrane.

In order to quantify the distribution of SNAP-25 between membrane and soluble cell compartments, transfected HIT cells were fractionated. The total cell homogenate and the crude membrane and cytosol fractions were analyzed by western blot using anti-SNAP-25. Both (endogenous) native SNAP-25 and (transfected) GFP-SNAP-25 were detected by the antibody, and were well resolved according to the difference in molecular size (Fig. 2B). All endogenous SNAP-25 was, as expected, in the membrane fraction (Fig. 2B, lanes 1-3). Although the GFP-SNAP-25TR fusion protein was also largely membrane-associated, approximately 9% was cytosolic (Fig. 2B, lanes 4-6). This was not due to the GFP-tag on SNAP-25 since expression of non-tagged SNAP-25 in HIT cells under similar conditions also gave rise to a minor cytosolic component of SNAP-25 (data not shown). The single cysteine mutant, GFP-SNAP-25TR Cys 85, was significantly displaced from membranes to cytosol, with 44% being cytosolic (Fig. 2B, lanes 7-9) while the double as well as the quadruple cysteine mutants were >90% cytosolic (Fig. 2B, lanes 10-15). The cysteine residues in SNAP-25 are thus important for membrane association in insulin-secreting cells. Even the single cysteine mutant was significantly displaced to the cytosol.
In vitro translated SNAP-25TR and its cysteine mutants bind with the same efficiency to GST-syntaxin beads

It has been hypothesized that SNAP-25 family members, which lack a cysteine domain, like sec9 and SNAP-29, are associated with membranes via binding to syntaxin family members (Brennwald et al., 1994; Steegmaier et al., 1998). Since earlier studies have shown that the syntaxin binding domain of SNAP-25 includes the putative palmitoylation sites (Chapman et al., 1994), the cytosolic localization of SNAP-25 cysteine mutants in HIT cells may have been due to perturbation in its binding to syntaxin. The ability of the SNAP-25 cysteine mutants to bind to syntaxin 1A was therefore tested in vitro. SNAP-25TR and cysteine mutants were in vitro translated in the presence of [35S]methionine and then incubated with GST-syntaxin beads. Beads were washed and boiled in SDS-sample buffer and the amount of bound SNAP-25 was analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3, SNAP-25TR and the different cysteine mutants bound with similar efficiency to GST-syntaxin beads. The observed displacement of the cysteine mutants from cell membranes was thus not due directly to impaired binding to syntaxin.

Co-immunoprecipitation of SNAP-25TR or SNAP-25TR cysteine mutants with syntaxin

In vitro translated proteins are not usually modified post-translationally, and are not thus expected to be palmitoylated. The syntaxin binding capacity of SNAP-25 may, however, be influenced by its degree of palmitoylation. Thus, although no difference in the syntaxin binding capacity of SNAP-25 wild-type and cysteine mutant proteins was observed in vitro, there may be a difference when the proteins are expressed in living cells where palmitoylation takes place. Binding of GFP-SNAP-25TR or cysteine mutants to syntaxin in vivo depends not only on the affinity of the respective molecules for syntaxin but also on their chance of encountering this binding partner and their capacity to compete with endogenous SNAP-25 for syntaxin binding. GFP-SNAP-25TR is localized at the plasma membrane (Fig. 2) and should therefore be able to compete more efficiently with the endogenous SNAP-25 for syntaxin binding than the cysteine mutants which are mainly localised in the cytosol. In order to compare the syntaxin binding capacity of GFP-SNAP-25TR and the cysteine mutants independently of such considerations, co-immunoprecipitation experiments were performed using lysates of COS cells transfected with GFP-SNAP-25TR or the different cysteine mutants mixed with a lysate from untransfected HIT cells. In contrast to COS cells, HIT cells express syntaxin 1A, which should interact not only with SNAP-25 present in the HIT cell lysate but also with the GFP-SNAP-25 fusion proteins from the COS cell lysate. Syntaxin 1A was immuno precipitated with a monoclonal anti-syntaxin antibody and the amount of co-immunoprecipitated SNAP-25 was evaluated on western blots using a polyclonal anti-SNAP-25 antibody. Fig. 4A shows a typical western blot and Fig. 4B the quantification of the amount of co-immunoprecipitated protein from three independent experiments. It was found that GFP-SNAP-25TR single, double and quadruple cys mutants bound as efficiently as the wild-type to syntaxin (Fig. 4B). Thus, even when expressed in cells, no difference between GFP-SNAP-25TR and the three cysteine mutants can be observed in terms of their syntaxin binding capacity.

Overexpression of syntaxin and the soluble SNAP-25 cysteine mutants in HIT cells

It has been shown that chemical deacylation does not release SNAP-25 from the plasma membrane (Gonzalo and Linder, 1998). Thus, unpalmitoylated SNAP-25 is in principle able to stay at the plasma membrane and it has been suggested that this is due to its association with syntaxin. It was in this context intriguing that the double and quadruple cysteine mutants were largely cytosolic. They bind with the same high efficiency to syntaxin as GFP-SNAP-25TR and one would expect that such a strong interaction should be sufficient to result in membrane association of these cysteine mutants. One explanation for this finding could be that there is not enough ‘unengaged’ syntaxin present in HIT cells. If this was the case, overexpression of syntaxin should favor membrane association of the GFP-SNAP-25TR cysteine mutants. This was tested by transfecting HIT cells with GFP-SNAP-25TR or cysteine mutants alone or together with syntaxin (Fig. 5). As already shown in Fig. 2, expression of GFP-SNAP-25TR in HIT cells resulted in partial localization to the cytosol (Fig. 5, lanes 1-3). After co-expression with syntaxin, however, GFP-SNAP-25TR was found exclusively in the membrane fraction (Fig. 5, lanes 4-6). More significantly, co-expression of syntaxin led to partial relocation of both the double (Fig. 5, lanes 7-12) and quadruple (Fig. 5, lanes 13-18) cysteine mutants from the cytosol to the membrane.
membrane fraction. In the case of the quadruple mutant, this indicates that soluble, non-palmitoylated SNAP-25 can associate with membranes if there is a sufficient supply of syntaxin (or more specifically an appropriate ratio of syntaxin:SNAP-25 in the face of SNAP-25 over-expression).

Investigation of the functional activity of the toxin-resistant SNAP-25 cysteine mutants in Ca\(^{2+}\)-stimulated exocytosis from BoNT/E treated HIT cells

Chen et al. (1999) have shown that the carboxy-terminal soluble half of SNAP-25 can rescue exocytosis in toxin treated cells. It may therefore be possible that even soluble SNAP-25 mutants are able to function in the exocytotic process. In order to test this and to assess the importance of cysteine residues in SNAP-25 function, we used the GFP-SNAP-25 TR cysteine mutants in a secretion/reconstitution assay, which we have described previously (Sadoul et al., 1997). GFP-SNAP-25 TR was first tested for its ability to reconstitute insulin secretion in order to exclude artifacts due to the GFP-tag. HIT cells were co-transfected with cDNA for GFP-SNAP-25 TR and human proinsulin. The human proinsulin is used as a marker for the amount of secretion from the subpopulation of transfected cells. The quantity of human C-peptide (a by-product of proinsulin conversion to insulin) secreted from the transfected cells is determined by ELISA using an antibody which recognizes specifically human but not hamster C-peptide (the endogenous product of HIT cells). After permeabilization with streptolysin-O (SLO), HIT cells were treated or not with BoNT/E, and then incubated in a high Ca\(^{2+}\)-buffer to stimulate insulin secretion. Under these conditions, it was possible to test directly whether the GFP-SNAP-25 TR mutant, which is resistant to hydrolysis by the toxin, can replace the cleaved (inactivated) endogenous SNAP-25 in its function. Almost complete rescue of insulin secretion compared to control (SNAP-25 TR without GFP) allowed us to conclude that the GFP-tag did not hinder SNARE-complex formation and the fusion event (Fig. 6A). Intriguingly, the single cysteine mutant, although shown to be 44% cytosolic, was able to reconstitute secretion to 82.3±16.7%. There was similarly a discrepancy between reconstitution activity and membrane localization for the other mutants (Fig. 6B). The double and quadruple mutants being 90% cytosolic reconstitute insulin secretion to 34.5±0.31% and 9.6±0.23%, respectively. Thus, even the quadruple mutant appeared able to support a minimal level of secretion.

DISCUSSION

SNAP-25 is an intrinsically hydrophilic molecule and it has
been suggested that its membrane association depends on palmitoylation of one or more of the 4 cysteine residues situated in the middle of the molecule (Bark, 1993; Hess et al., 1992; Veit et al., 1996). We show here that these cysteines, and by inference their palmitoylation, are not essential for membrane association of SNAP-25. Like other SNAP-25 family members, which lack a palmitoylation domain, SNAP-25 can associate with membranes through interaction with its cognate tSNARE, syntaxin 1A. Our observation that unpalmitoylated SNAP-25 can be associated with membranes through interaction with syntaxin is in agreement with a previous study showing that SNAP-25 binds to syntaxin early after translation and that this initial binding event is necessary to bring SNAP-25 to the plasma membrane where palmitoylation can take place (Vogel et al., 2000). This model is supported by the observation that in vitro palmitoylation of

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**Fig. 5.** Fractionation of HIT cells co-transfected with syntaxin and GFP-SNAP-25TR or GFP-SNAP-25TR cysteine mutants. HIT cells were transfected with GFP-SNAP-25TR or GFP-SNAP-25TR cysteine mutants alone (−syn) or together with syntaxin 1A (+syn). Two days after transfection, cells were collected, homogenized on ice and fractionated by centrifugation into crude membranes and cytosol. Equal aliquots of the total cell homogenate (H), the crude membranes (M), and the cytosol (C) were used for SDS-PAGE and western blot. The immunoblot was revealed with a polyclonal antibody against SNAP-25. White arrow: exogenous GFP-SNAP-25TR; black arrow: endogenous SNAP-25.

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**Fig. 6.** (A) Reconstitution of regulated insulin secretion by SNAP-25TR versus GFP-SNAP-25TR. HIT cells were transiently co-transfected with human proinsulin and the empty vector or SNAP-25TR or GFP-SNAP-25TR. Two days after transfection, cells were permeabilized with SLO and incubated without or with 30 nM BoNT/E (toxin) for 8 minutes. The medium was then replaced by high (stim.) or low (basal) Ca²⁺ containing buffers for a secretion period of 7 minutes. Insulin release from transfected cells was estimated by measuring the amount of human C-peptide released into the medium. (mean ± s.d., n=2). (B) Reconstitution of insulin secretion by the toxin-resistant GFP-SNAP-25 cysteine mutants in SLO-permeabilised HIT cells treated with BoNT/E. HIT cells were transiently co-transfected with human proinsulin and GFP-SNAP-25TR or cysteine mutants. Cells were then treated essentially as described in A except that the cells were treated with BoNT/E also during the permeabilisation period. Data (mean ± s.d., n=3 observations for one experiment) are representative of three independent experiments. For each experiment the value of residual secretion from control cells (empty vector) after toxin treatment (stim/toxin) has been subtracted from all reconstitution values (GFP-SNAP-25TR or cysteine mutants condition stim/toxin). The reconstitution obtained with the different cysteine mutants is expressed as a % of that obtained with GFP-SNAP-25TR.
SNAP-25 is facilitated by its binding to syntaxin (Veit, 2000). Based on the present study, and taking earlier observations into account, it is suggested that SNAP-25 associates with the plasma membrane through interaction with syntaxin 1A and that palmitoylation serves to consolidate its membrane anchorage rather than being an absolute requirement. Under conditions of excessive levels of SNAP-25 in the face of limiting amounts of unengaged syntaxin, there should be displacement of SNAP-25 from the membrane, and this was indeed the case when wild-type GFP-SNAP-25 was overexpressed. It would further be predicted that redressing the imbalance between SNAP-25 and syntaxin would allow all SNAP-25 to be membrane-localized and this was again found to be the case.

One could hypothesize that palmitoylation of SNAP-25 modulates its affinity for syntaxin but it is shown here that SNAP-25/syntaxin interactions do not depend on the palmitoylation state of SNAP-25. This conclusion is based on the observation that SNAP-25 wild-type and cysteine mutants bind with the same efficiency to syntaxin both after in vitro translation (unpalmitoylated state of wild type) as well as after expression in cells (palmitoylated state of wild type). These data are in accord with the earlier observation that a non-palmitoylated SNAP-23 mutant also binds normally to syntaxin (Vogel and Roche, 1999).

The fact that SNAP-25 expressed in COS cells can be co-immunoprecipitated with syntaxin 1A after mixing of the COS cell lysate with a HIT cell lysate indicates that unengaged or ‘SNAP-25-free’ syntaxin 1A must be present in HIT cells. But why is this syntaxin population not able to bind the soluble SNAP-25 cysteine mutants when they are expressed in HIT cells? One possibility is that the syntaxin molecules which are not bound to SNAP-25 are bound to another molecule which hinders SNAP-25/syntaxin interactions. Lysis of cells in a detergent containing buffer could then set free this population of syntaxin which now can bind and co-precipitate the exogenous SNAP-25. Munc-18 has such properties (Fletcher et al., 1999). It has been shown to bind to syntaxin thereby preventing the interaction of syntaxin with SNAP-25. Furthermore, although several studies have shown high affinity binding of Munc-18 and syntaxin 1A, co-immunoprecipitation from cell lysates has proven difficult, indicating that Munc-18 and syntaxin dissociate after cell lysis (Fletcher et al., 1999). This could explain why overexpression of syntaxin 1A is necessary to bring SNAP-25 cysteine mutants to membranes although ‘SNAP-25-free’ syntaxin seems to be present within the cell.

Rothman and colleagues have shown that the cysteine region of SNAP-25 is not essential for the fusion event (Parlati et al., 1999) and Chen et al. (1999) have shown that the soluble carboxy-terminal half of SNAP-25 is able to rescue exocytosis from toxin treated cells. On this basis, it would be predicted that unpalmitoylated SNAP-25 could act in the fusion process after diffusion to the membrane and participation in the SNARE complex. Our results with SNAP-25 mutated at its first cysteine residue show that although this mutation has a strong effect on membrane association of SNAP-25, there is essentially no effect in terms of the functional activity in the secretion event. The double and quadruple cysteine mutants were found to be largely but not exclusively cytosolic, but they nevertheless retained some residual biological activity. As for the single mutant, activity in the reconstitution of the secretion assay was greater than that expected from the percentage of molecules found associated with the membrane fraction.

Taken together, and in conclusion, these data allow for formulation of the following working model. As already proposed by others (Vogel et al., 2000), association of SNAP-25 with the plasma membrane is a multi-step process. Binding to syntaxin is the initial event under physiological circumstances, and is unrelated to palmitoylation. Once the SNAP-25/syntaxin heterodimers reach the cell surface, palmitoylation consolidates membrane anchorage. Of those molecules of SNAP-25 at the cell surface, only a fraction are in a productive biologically active state (presumably participating directly in the SNARE complex or immediately available for complex formation). Recruitment of membrane bound molecules to the active state is a function of the time period during which SNAP-25 is available at the plasma membrane. This hypothesis allows the behavior of the cysteine mutants to be explained. The membrane association of the single cysteine mutant is reduced, presumably due to decreased palmitoylation. This is indicated by the observed displacement of approximately half the molecules to the soluble pool. This mutant is, however, still sufficiently well anchored to the membrane to allow for recruitment of a normal number of molecules to the active state. The single mutant thus displays near full biological activity. The membrane association of the double and quadruple mutants is even lower than that of the single mutant, and the distribution of molecules thus predominantly cytosolic. Recruitment to the active state is severely compromised but not abolished, thus explaining the residual biological activity. Merely determining the percentage of SNAP-25 associated with membranes in the steady-state is thus a poor index of biological activity. It will be interesting in future studies to distinguish at the molecular level between ‘active’ and ‘inactive’ membrane association of SNAP-25.

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