SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) are essential for vesicle docking and fusion. SNAP-25, syntaxin 1A, and synaptobrevin/vesicle-associated membrane protein (VAMP) are SNARE proteins that mediate fusion of synaptic vesicles with the plasma membrane. It has been proposed that interactions of SNAP-25 with syntaxin 1A are required for initial membrane attachment of SNAP-25 (Vogel, K., Cabaniols, J.-P., and Roche, P. (2000) J. Biol. Chem. 275, 2959–2965). However, we have shown previously that residues 85–120 of the SNAP-25 interhelical domain, which do not interact with syntaxin, are necessary and sufficient for palmitoylation and plasma membrane localization of a green fluorescent protein reporter molecule (Gonzalo, S., Greentree, W. K., and Linder, M. E. (1999) J. Biol. Chem. 274, 21313–21318). To clarify the role of syntaxin in membrane targeting of SNAP-25, we studied a SNAP-25 point mutant (G43D) that does not interact with syntaxin. SNAP-25 G43D/green fluorescent protein was palmitoylated and localized at the plasma membrane. Newly synthesized SNAP-25 G43D had the same kinetics of membrane association as the wild-type protein. Furthermore, expression of a cytosolic mutant syntaxin 1A did not interfere with SNAP-25 membrane interactions or palmitoylation in the neuronal cell line NG108-15. Exogenously expressed SNAP-25 targets efficiently to the plasma membrane in cells of neuronal origin but only partially in HeLa cells, a neurosecretion-incompetent line. This phenotype was not rescued when syntaxin 1A was co-expressed with SNAP-25. Our data support a syntaxin-independent mechanism of membrane targeting for SNAP-25.

Membrane trafficking is a fundamental cellular process that must operate with high fidelity to maintain organelle identity, cell function, and viability. Integral membrane proteins referred to as SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors; GFP, green fluorescent protein; MTD, membrane-targeting domain; PHS, phosphate-buffered saline; SNAP-25, synaptosomal protein of 25 kDa; VAMP, vesicle-associated membrane protein; TMD, transmembrane domain) are important elements in this process, participating in the docking and fusion of vesicles with target membranes (1). The hallmark of SNARE proteins is the presence of one or more α-helices in the cytoplasmic domain, which have a propensity to form coiled-coils (reviewed in Refs. 2 and 3). Pairing between cognate SNAREs on opposing membranes results in the formation of a parallel four-helix bundle that brings the membranes together to allow fusion (4, 5). At the neuronal synapse, the SNARE complex is formed between SNAP-25 and syntaxin, localized on the presynaptic membrane, and synaptobrevin/VAMP, localized on the vesicle membrane (1). The central role that SNAP-25 plays in this complex is evident from the impairment of neuronal secretion by botulinum neurotoxin A/E, a toxin that proteolytically inactivates SNAP-25 (6). Moreover, loss of SNAP-25 expression in mice results in embryonic lethality late in gestation with evoked neurotransmitter release absent at the neuromuscular junction and central synapses (7).

Each membrane compartment appears to be associated with a unique set of SNAREs, thereby contributing to the specificity of membrane fusion. The mechanisms by which SNAREs are targeted to their resident membranes are only beginning to be understood. Most syntaxin and synaptobrevin/VAMP family members are classified as tail-anchored proteins based on the presence of a transmembrane segment at the C terminus. Studies of synaptobrevin and other vesicle-associated SNAREs (vSNAREs) demonstrate that these proteins are initially targeted to the endoplasmic reticulum and then sorted to their ultimate destinations (8, 9).

The SNAP-25 family of SNAREs, SNAP-25a and b, SNAP-23, and SNAP-29, is structurally distinct from the tail-anchored SNAREs (3) and thus uses different mechanisms for membrane localization. The tail-anchored SNAREs have a single cytoplasmic coiled-coil domain or SNARE motif, whereas SNAP-25 family members have two SNARE motifs that are connected by an interhelical domain (5). SNAP-25 and SNAP-23 are palmitoylated at a cluster of cysteine residues in the interhelical domain (10–12). Many soluble proteins rely on fatty acylation for membrane association (13, 14), and there is evidence to support a similar functional role for SNAP-25 palmitoylation. Our study of the native protein in PC12 cells revealed that palmitoylation of newly synthesized SNAP-25 coincides temporally with its membrane association (15). Moreover, palmitoylation and membrane association of the newly synthesized protein are sensitive to Brefeldin A, suggesting that these events are coupled and depend on an intact secretory pathway (15). Palmitoylation-defective mutants of SNAP-25 and SNAP-23 are found predominately in the cytoplasm of transfected cells (11, 16, 17). However, palmitoylation-defective forms of SNAP-25 can be targeted to membranes when syntaxin 1A is present (18, 19). SNAP-29 is not a substrate for palmitoylation, yet it behaves as an integral membrane protein.
in transfected cells (20). It has been suggested that SNAP-25 is recruited to membranes through interactions with tail-anchored SNAREs (20).

Proteins such as SNAP-25 that are synthesized on soluble ribosomes and are substrates for palmitoylation must interact with a membrane-bound protein palmitoyltransferase. We proposed that interactions with syntaxin initiate membrane interactions that facilitate palmitoylation (15). However, our subsequent study argued against this hypothesis (17). We found that the first 35 amino acids in the interhelical domain of SNAP-25 are both necessary and sufficient to target a heterologous soluble protein to the plasma membrane in NG108-15 cells (17). The membrane-targeting domain (MTD) begins with the cysteine-rich domain at residue 85 and continues for 35 amino acids to residue 120. Notably, this region is devoid of the o-helical domains of SNAP-25 that interact with syntaxin and synaptobrevin. These and other experiments lead us to propose a model whereby palmitoylation and plasma membrane targeting of SNAP-25 require interaction with an unidentified binding partner that interacts with the interhelical domain.

Two recent reports have argued strongly for a syntaxin-dependent mechanism for membrane targeting of SNAP-25. Vogel et al. (18) demonstrated that newly synthesized SNAP-25 and syntaxin bind to each other while still in the cytoplasm. Initial membrane association of SNAP-25 in the presence of syntaxin was independent of the cysteine residues, suggesting that palmitoylation occurs after syntaxin-mediated membrane association (18). Washbourne et al. (19) reported that the cysteine residues of SNAP-25 are not required for membrane targeting when syntaxin 1A is expressed. Although membrane interactions of the MTD of SNAP-25 are clearly independent of syntaxin interactions, these authors have suggested that the MTD utilizes an alternative trafficking pathway that is not physiologically relevant to intact SNAP-25. In this study, we sought to clarify the role of syntaxin 1A in SNAP-25 localization in neuronal and non-neuronal cell lines. We have also analyzed the localization and trafficking of a point mutant of full-length SNAP-25 that is unable to bind to syntaxin 1A. These studies have allowed us to test our hypothesis that plasma membrane localization of SNAP-25 is independent of syntaxin interactions in the context of the full-length protein.

**EXPERIMENTAL PROCEDURES**

**Materials—**L-[35S]methionine/35S]methionine was purchased from Amersham Biosciences; [3H]palmitate was obtained from PerkinElmer Life Sciences. Cycloheximide was purchased from Sigma and stored at −20 °C as a 2 mg/ml stock in distilled water. Immune complexes were precipitated using Protein G-Sepharose from Amersham Biosciences. HPC-1 monoclonal antibody specific for syntaxin 1A was obtained from mouse ascites as described previously (21, 20). Clonal (Baltimore, MD). HPC-1 monoclonal antibody specific for syntaxin 1A was obtained from mouse ascites as described previously (21, 20). Clonal (Baltimore, MD).

**Construction of SNAP-25 Fusion Plasmids—**HeLa cells—HeLa cells were obtained from Dr. Helen Piwnica-Worms (Washington University, St. Louis, MO) and cultured in high glucose–Dubelcco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5% horse serum, and 2 mM glutamine, 150 units/ml penicillin, 50 units/ml streptomycin, 0.1 mM hypoxanthine, 400 mM aminopterin, and 0.016 mM thymidine. PC12 cells were obtained from Dr. Phyllis Hanson (Washington University, St. Louis, MO) and cultured in high glucose–Dubelcco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, 150 units/ml penicillin, and 50 units/ml streptomycin. HeLa cells were obtained from Dr. Helen Piwnica-Worms (Washington University, St. Louis, MO) and cultured in high glucose–Dubelcco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, 150 units/ml penicillin, and 50 units/ml streptomycin. HeLa cells were analyzed in a 35- or 60-mm tissue culture plates coated with poly-L-lysine until 50–80% confluent. Cells were transfected using LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions and analyzed 24–48 h later.

**Immunoﬂuorescence, Epifluorescence, and Confoal Laser Microscopy—**For epifluorescence, cells were washed in warmed Ringer’s buffer (155 mM sodium chloride, 3 mM potassium chloride, 2 mM calcium chloride, 1 mM magnesium chloride, 3 mM dibasic sodium phosphate, 5 mM Hepes, and 10 mM glucose) post-transfection and mounted onto a microscope slide in a well created with Dow Corning vacuum grease filled with Ringer’s buffer. Cells were immediately visualized using a Zeiss Axioplan microscope coupled to an MRC-1000 laser scanning confocal microscope (Bio-Rad) with a ×63 oil immersion objective. For immunofluorescence, cells were washed with phosphate-buffered saline (PBS) and fixed in a freshly prepared solution of 4% (v/v) paraformaldehyde and 5% (w/v) sucrose for 10 min at room temperature, at 48 h post-transfection. Cells were washed twice with PBS, permeabilized with 1% Triton X-100 in blocking buffer (1% bovine serum albumin w/v, 1% normal goat serum v/v (Invitrogen) in PBS) for 10 min and washed once in blocking buffer. Nonspecific staining was blocked by incubating cells for 45 min at room temperature in blocking buffer. All antibodies were diluted in blocking buffer. Cells were incubated in the appropriate primary antibody for 1 h, washed three times in blocking buffer, and incubated for 1 h with Alexa594-conjugated goat anti-mouse (Molecular Probes, Eugene, OR). After incubation in secondary antibody, cells were washed three times in PBS and mounted onto slides with a drop of Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Cells were examined as above. Confocal images were assembled as montages using Adobe PhotoShop 5.0 and 6.0.

**Radiolabeling and Immunoprecipitation—**Cells were radiolabeled with [35S]methionine and [3H]palmitate as described previously (15). Cells were solubilized in a solution of 1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM sodium chloride, and 2 mM EDTA for 30 min on ice. Nuclear and insoluble material were isolated by centrifugation at 14,000 × g in a Beckman TLA-100.3 rotor (30 min at 4 °C). Lysates were immunoprecipitated with rabbit anti-GFP polyclonal antibody conjugated to Protein G-Sepharose. Immunoprecipitated proteins were analyzed on 13% SDS-polyacrylamide gels. To detect radiolabeled proteins by fluorography, the gels were washed for 30 min in a solution of 1 M sodium salicylate and 15% methanol, dried, and exposed to film.

**Subcellular Fractionation—**Cells grown in 60-mm dishes were transferred with the appropriate constructs. Cells were washed in PBS at 48 h post-transfection and scraped into ice-cold PBS. Cells were collected by centrifugation and suspended in a hypotonic buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2% sucrose) with protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml leupeptin protease inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM N-tosyl-l-phenylalalanine chloromethyl ketone, and 10 μM N-p-tosyl-l-lysine chloromethyl ketone). After a 15-min incubation in ice, cells were homogenized with 19 passes through a ball-bearing homogenizer. Unbroken cells, nuclei, and other debris were pelleted at 800 × g, and the resulting pellet was designated as P1. The post-nuclear supernatant was centrifuged at 100,000 × g for 30 min, yielding a P100 and S100 fraction. The S100 fraction was centrifuged again to remove any residual particulates and mixed with an equal volume of 2× immunoprecipitation buffer. The P100 fraction was washed once in hypotonic buffer and suspended in immunoprecipitation buffer. Equal fractions of the S100 and P100 were either analyzed by immunoprecipitation followed by fluorography or analyzed by immunoblotting.
Non-neuronal Cell Type
Our previous studies of SNAP-25—
A
C
NG108-15
a
b
SNAP-25/GFP
panel b
panel c).
1A and is independent of cysteine residues in SNAP-25.
Unmodified SNAP-25 or syntaxin 1A, initial membrane association of exogenously expressed SNAP-25 depends on the presence of syn-
aptic relationships between SNAP-25 and syntaxin 1A. Two groups have observed that expression of syntaxin 1A with palmitoylation-defective SNAP-25 rescues membrane association (18, 19). We reproduced these findings in NG108-15 cells, observing a redistribution of Cys-SNAP-25 in the amount of SNAP-25 associated with the plasma mem-
brane (Fig. 1, panel e). We conclude from these experiments that expression of heterologous syntaxin 1A in HeLa cells does not significantly impact the localization of wild-type SNAP-25 in HeLa cells.

Heterologous Expression of Syntaxin Results in a Redistribution of Cys-SNAP-25 from the Cytoplasm to Intracellular Membranes—Mutation of the four cysteine residues (Cys−) within the interhelical domain of SNAP-25 results in a protein that cannot be palmitoylated and is cytoplasmic (16) (see Fig. 4, panel b). Two groups have observed that expression of syntaxin 1A with palmitoylation-defective SNAP-25 rescues membrane association (18, 19). We reproduced these findings in NG108-15 cells, observing a redistribution of Cys-SNAP-25/GFP from the soluble to the particulate fraction with exogenously expressed syntaxin 1A (Fig. 2A). The ability of syntaxin 1A to drive membrane interactions of the Cys-SNAP-25 construct was intriguing, and we sought to determine whether this interaction
resulted in plasma membrane localization of the palmitoylation-defective SNAP-25. Syntaxin 1A and Cys–SNAP-25/GFP were cotransfected in NG108-15 cells and localized by indirect immunofluorescence or direct epifluorescence, respectively. Cys-SNAP-25/GFP was found in the cytoplasm and concentrated on intracellular membranes when co-expressed with syntaxin 1A (Fig. 2B, panel d). The plasma membrane distribution of wild-type SNAP-25/GFP was unchanged with heterologous expression of syntaxin 1A (Fig. 2B, panel a). Intriguingly, we observed that in cells in which Cys-SNAP-25/GFP and syntaxin 1A were co-expressed, syntaxin 1A is redistributed from the plasma membrane onto intracellular membranes (Fig. 2B, panel e). We conclude that syntaxin 1A interactions are able to rescue membrane interactions of palmitoylation-defective SNAP-25 but not localization at the plasma membrane.

SNAP-25 G43D Does Not Interact with Syntaxin but Is Palmitoylated and Localizes to the Plasma Membrane—The ability of syntaxin 1A to target palmitoylation-defective SNAP-25 to an intracellular membrane compartment is consistent with the model proposed by Vogel et al. (18), that syntaxin interactions are required for initial membrane targeting of SNAP-25. If this model is correct, then the syntaxin-independent targeting of the SNAP-25 MTD (residues 85–120) to membranes must occur by a distinct mechanism. Because the MTD is a non-native truncated protein, we studied the syntaxin-independent targeting using a model that is more closely related to the native protein. Mutation of glycine 458 to aspartic acid in Sec9p, the SNAP-25 homologue in Saccharomyces cerevisiae, abrogates its interactions with the syntaxin 1 homologue Sso1p in vivo and in vitro (23, 24). The corresponding mutation in SNAP-25 at Gly-43 results in a protein that is unable to bind syntaxin 1A in vitro (25). The glycine at residue 43 accommodates the bulky phenylalanine in syntaxin 1A at residue 216 in the hydrophobic core of the SNARE four-helix bundle. Mutation of Gly-43 to Asp disrupts packing through steric and electrostatic forces (26). To confirm that this mutation also inhibits SNAP-25/syntaxin interactions in cells, we performed co-immunoprecipitation studies (Fig. 3A). Chimeric SNAP-25/GFP constructs were created with the GFP moiety at the C terminus of SNAP-25 and transfected into NG108-15 cells, which express syntaxin 1A. The resulting lysates were immunoprecipitated with an anti-GFP polyclonal antibody and probed for native syntaxin 1A using the HPC-1 monoclonal antibody. Native syntaxin 1A readily co-immunoprecipitated with wild-type and Cys-SNAP-25 but was not detected in immunoprecipitates with SNAP-25 G43D (Fig. 3A, upper panel). The same Western blots probed with anti-GFP antibody showed that all GFP-tagged proteins were present in similar amounts in the immunoprecipitates (Fig. 3A, lower panel).

It has been reported that the interaction of SNAP-25 with syntaxin is important for palmitoylation of SNAP-25 in an in vitro assay (27). To determine whether this is true in vivo, we examined whether palmitate incorporation into SNAP-25 G43D was reduced when compared with the wild-type protein. As shown in Fig. 3B (lower panel), SNAP-25 G43D was palmitoylated in vivo at levels similar to the wild-type protein. Thus, syntaxin interactions are not required for palmitoylation of SNAP-25. We have shown previously that palmitoylation of SNAP-25 is inhibited by Brefeldin A, indicating that modification of the protein is dependent on an intact secretory pathway (15). Palmitoylation of the G43D mutant is inhibited by Brefeldin A (data not shown), verifying that the mutant is palmitoylated similarly to the wild-type protein, thus also requiring an intact secretory pathway.

To study the steady-state localization of SNAP-25 G43D, we examined cells transfected with various SNAP-25/GFP constructs using live cell epifluorescence. NG108-15 cells (Fig. 4, panels a–c) and PC12 cells (Fig. 4, panels d–f) were transfected, mounted in Ringer’s solution, and examined live by confocal microscopy. As expected, wild-type SNAP-25/GFP localized to the plasma membrane (Fig. 4, panels a and d) and Cys–SNAP-25/GFP were predominatey cytosolic (Fig. 4, panels b and e). In both NG108-15 and PC12 cells, the SNAP-25 G43D/GFP chimera localized to the plasma membrane (Fig. 4, panels c and f). These results demonstrate that the steady-state localization of SNAP-25 G43D is at the plasma membrane and that the protein is palmitoylated at levels similar to the wild-type protein. G43D SNAP-25 Has the Same Kinetics of Membrane Attachment as Wild Type—We hypothesized that if SNAP-25 G43D uses the same trafficking pathway as the wild-type protein to achieve plasma membrane localization, then its kinetics of membrane association would be the same as that of the wild-type protein. Previously, we have shown that membrane targeting is coincident with palmitoylation and occurs within 20 min of protein synthesis. Membrane association of the newly synthesized protein coincides with palmitoylation, and both are inhibited by Brefeldin A (15). In this study, we followed the membrane interactions of [35S]methionine pulse-labeled SNAP-25 in NG108-15 cells over a 3-h time course. At the pulse and the three subsequent chase points (15, 60, and 180 min), the cells were collected and fractionated. SNAP-25/GFP was immunoprecipitated from the soluble (S100) and particulate (P100) fractions and analyzed by fluorography. We observed that the G43D mutant associated with membranes with the same kinetics as that of wild-type (Fig. 5, A and B), suggesting that both wild-type and SNAP-25 G43D use the same mechanism to associate with membranes.

Syntaxin 1A-ΔTMD Does Not Inhibit Membrane Association or Palmitoylation of SNAP-25—As an independent test of whether syntaxin 1A influences membrane localization of
SNAP-25, we heterologously expressed a mutant of syntaxin 1A, missing the transmembrane domain (syntaxin 1A ΔTMD), with wild-type SNAP-25/GFP and evaluated SNAP-25 membrane association. Syntaxin 1A ΔTMD is predominately cytosolic (data not shown). If syntaxin 1A is important for SNAP-25 membrane interactions, then the ΔTMD mutant should inhibit SNAP-25 membrane interactions and sequester SNAP-25 in the cytosol. SNAP-25/GFP was expressed with wild-type syntaxin 1A or syntaxin 1A ΔTMD in NG108 cells and radiolabeled with [35S]methionine. As above, cells were collected at the pulse and a 3-h chase point and fractionated. SNAP-25 was immunoprecipitated from the soluble and particulate fractions (S100 and P100) and analyzed by fluorography. The results demonstrate that SNAP-25 co-expressed with wild-type syntaxin 1A trafficked to the membrane with similar kinetics to SNAP-25 expressed with syntaxin 1A ΔTMD (Fig. 6A). Furthermore, palmitate incorporation into SNAP-25 was unaffected by expression of syntaxin 1A ΔTMD (Fig. 6B). These results further support a model in which SNAP-25 binds to membranes shortly after synthesis and is palmitoylated in a syntaxin-independent manner.

**DISCUSSION**

The Role of Syntaxin in Membrane Attachment of SNAP-25—In this study, we examined the role of syntaxin 1A in the membrane interactions of SNAP-25. There are two competing models for this process, and we sought to differentiate between them. We proposed a syntaxin-independent model whereby SNAP-25 is targeted to membranes through interactions that require residues 85–120 within the interhelical domain. This domain of the protein is not involved in SNARE complex formation but contains the palmitoylated cysteine cluster and other sequence motifs that are required for membrane interactions (17). A second model proposes that SNAP-25 binds to syntaxin 1A in the cytoplasm and that the two proteins are initially targeted to membranes through insertion of the C-terminal transmembrane domain of syntaxin (18). Our findings in this study favor the first model.

To determine the role of syntaxin 1A in SNAP-25 membrane attachment, we studied a point mutant in SNAP-25 (G43D) that is unable to bind syntaxin 1A. At the steady state, this protein is localized at the plasma membrane and palmitoylated at levels similar to wild-type SNAP-25. Both the wild-type and mutant proteins display the same kinetics of membrane association as that of wild-type (WT). NG108-15 cells were transfected, pulse-labeled with 100 μCi/ml [35S]methionine for 10 min, and chased for 15, 60, and 180 min in nonradioactive medium containing cycloheximide (10 μg/ml). Cells were collected at 0, 15, 60, and 180 min after the pulse label and fractionated into particulate (P) and soluble (S) fractions. SNAP-25 was immunoprecipitated using the anti-GFP polyclonal antibody. Samples were analyzed by SDS-PAGE and fluorography. The intensity of the bands was measured using a Molecular Dynamics PhosphorImager and ImageQuaNT software. The percent of SNAP-25 in the membrane fraction was calculated by measuring the P100 and S100 fractions and expressing the amount in the P100 as a percentage of the total SNAP-25 in the soluble and membrane fractions. A, graphical representation of three experiments. Each bar represents the mean ± S.E. of three experiments. B, representative fluorograph of the experiment.

**FIG. 4.** SNAP-25 G43D targets to the plasma membrane of PC12 and NG108-15 cells. NG108-15 cells (a–c) and PC12 cells (d–f) were transfected with the appropriate SNAP-25/GFP constructs. After 24 h, cells were washed and mounted live in Ringer’s solution. The cells were visualized using a Zeiss AxioImager confocal microscope. Wild-type SNAP-25/GFP targets to the plasma membrane in both NG108-15 and PC12 cells (a and d). As expected, the Cys-SNAP-25/GFP protein is cytosolic and does not target to the plasma membrane (b and e). SNAP-25 G43D/GFP localized to the plasma membrane in both NG108-15 cells and PC12 cells (c and f).

**FIG. 5.** G43D SNAP-25 has the same kinetics of membrane association as that of wild-type (WT). NG108-15 cells were transfected, pulse-labeled with 100 μCi/ml [35S]methionine for 10 min, and chased for 15, 60, and 180 min in nonradioactive medium containing cycloheximide (10 μg/ml). Cells were collected at 0, 15, 60, and 180 min after the pulse label and fractionated into particulate (P) and soluble (S) fractions. SNAP-25 was immunoprecipitated using the anti-GFP polyclonal antibody. Samples were analyzed by SDS-PAGE and fluorography. The intensity of the bands was measured using a Molecular Dynamics PhosphorImager and ImageQuaNT software. The percent of SNAP-25 in the membrane fraction was calculated by measuring the P100 and S100 fractions and expressing the amount in the P100 as a percentage of the total SNAP-25 in the soluble and membrane fractions. A, graphical representation of three experiments. Each bar represents the mean ± S.E. of three experiments. B, representative fluorograph of the experiment.
SNAP-25 does not target to membranes well in non-neuronal cells and thus may be more susceptible to sequestration in the cytoplasm by the mutant syntaxin. Interestingly, and in agreement with Vogel et al. (18), we observed a recruitment of palmitoylation-defective SNAP-25 from the soluble to the particulate fraction when it was coexpressed with syntaxin 1A (Fig. 2A). However, when visualized by confocal microscopy, Cys-SNAP-25 was associated with intracellular membranes, not the plasma membrane (Fig. 2B, panel d). Thus, the formation of a binary complex between SNAP-25 and syntaxin 1A can rescue membrane association of a palmitoylation-defective mutant but does not allow the protein to be targeted to its resident membrane. Indeed, in the absence of palmitoylation, SNAP-25 was retained on intracellular membranes and sequestered syntaxin 1A (Fig. 2, panels d–f), suggesting that palmitoylation may be required to exit the early exocytic pathway. There is precedent for palmitoylation serving as an export signal from the early exocytic pathway to the plasma membrane. Palmitoylation-defective forms of H- and N-Ras are retained on intracellular membranes rather than being targeted to the plasma membrane (28, 29).

Localization of wild-type SNAP-25 was unaltered by heterologous expression of syntaxin 1A in HeLa cells (Fig. 1B, compare panel c to d), further supporting our model that syntaxin 1A is not the primary factor involved in SNAP-25 membrane association. Native syntaxin 1A is localized on the plasma membrane, however; we observed that in HeLa cells, exogenously expressed syntaxin 1A is concentrated on intracellular membranes as well as at the plasma membrane (Fig. 1B, panel e). Other groups have reported similar results in cells that are neurosecretion-incompetent. In Madin-Darby canine kidney cells, syntaxin 1A was localized almost exclusively on intracellular membranes, whereas SNAP-25 was found at the plasma membrane (30). Consistent with our data, the authors of this study concluded that SNAP-25 and syntaxin 1A have different and separable trafficking pathways (30). Similar results were obtained when syntaxin 1A was expressed in normal rat kidney cells and a PC12 mutant cell line that is missing certain factors required for neurosecretion (31). These authors (31) noted that the Golgi complex was disassembled in these cell types when syntaxin 1A was overexpressed. They concluded that overexpression of syntaxin 1A in neurosecretion-incompetent cells has a profound effect on the secretory pathway. This in turn causes the mislocalization of many proteins that require the secretory pathway for localization, including SNAP-25.

Neuronal Factors for Membrane Targeting of SNAP-25—Transfected SNAP-25 is targeted efficiently to the plasma membrane in cells that express the native protein but not in non-neuronal cells. We found a significant pool of soluble SNAP-25 expressed in HeLa cells when visualized by microscopy with or without co-expressing syntaxin 1A. Our results suggest that when SNAP-25 is expressed in non-neuronal cells, an unidentified factor that facilitates targeting of the protein to the plasma membrane is limiting. We have speculated that such a factor would interact with the membrane-targeting domain of SNAP-25 and facilitate its palmitoylation (17). The interhelical domain of SNAP-23, the ubiquitously expressed SNAP-25 family member, also harbors a plasma membrane-targeting sequence. GFP is efficiently targeted to the plasma membrane when fused to residues 79–123 of SNAP-23, which correspond to the membrane-targeting domain of SNAP-25 (85–120).2 Thus, the function of the interhelical domain is conserved in both proteins, suggesting that SNAP-25 and SNAP-23 share the same mechanism of membrane association. SNAP-23 is ubiquitously expressed but is not as abundant as SNAP-25, one of the major palmitoylated proteins in the nervous system (32). There may not be a need for non-neuronal cells lines to express as much of the hypothetical targeting factor to localize SNAP-23. There are, however, some subtle differences between SNAP-25 and SNAP-23 localization that are cell-type specific (30). Therefore, additional signals may be involved in sorting SNAP-25 and SNAP-23 to discrete locations in specific cell types.

Functional Roles for the Cysteine-rich Cluster in the Interhelical Domain—Two recent studies have shown that the Cys–mutant of SNAP-25 is unable to support regulated exocytosis in PC12 cells (19, 33). Washbourne et al. (19) argued that the failure of the Cys-SNAP-25 mutant to function in this assay was not due to a localization defect but instead was due to a failure of SNARE complex disassembly. However, no evidence was presented to document that the Cys-SNAP-25 protein was localized at the plasma membrane in PC12 cells where it would be required for function. Indeed our study (Fig. 2) of cotransfected syntaxin 1A and Cys-SNAP-25 in NG108-15 cells and that of Washbourne et al. (19) in NIH3T3 cells show that Cys-SNAP-25 and syntaxin 1A are concentrated on intracellular membranes. Therefore, a localization defect cannot be excluded.

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2 S. Gonzalo and M. E. Linder, unpublished observations.
as the underlying explanation for the failure of the Cys-protein to promote exocytosis.

The finding that SNARE complex disassembly is inhibited in cells expressing Cys-SNAP-25 was surprising. The minimal regions of syntaxin, SNAP-25, and syntaptobrevin/VAMP required for SNARE complex assembly and disassembly had been mapped previously using limited proteolysis of ternary SNARE complex and confirmed using complex assembled from recombinant protein fragments (34). The coiled-coil regions of the SNARE complex and confirmed using complex assembled from recombinant protein fragments (34). Washbourne et al. (19) reported that Cys-SNAP-25 protein does not act as a dominant negative in the PC12 exocytosis assay. However, if Cys-SNAP-25 is unable to be disassembled from SNARE complexes, then one would expect the Cys-SNAP-25 protein to act as a dominant negative by forming unusually stable SNARE complexes and preventing the wild-type protein from forming productive SNARE complexes. Therefore, we think it unlikely that defective exocytosis associated with Cys-SNAP-25 is due to a defect in SNARE complex disassembly.

Conclusions—We have reported that SNAP-25 plasma membrane localization and palmitoylation are independent of its interactions with syntaxin 1A. We have also demonstrated that SNARE-25 is not targeted to the plasma membrane as efficiently in neurosecretion-incompetent cells. These results suggest the existence of a neuronal factor that facilitates plasma membrane targeting of SNAP-25 that is limiting in non-neuronal cells. Identification of this factor could have broad implications for trafficking of SNAREs or other palmitoylated proteins.

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