Differential palmitoylation of the endosomal SNAREs syntaxin 7 and syntaxin 8[S]

Yuhong He and Maurine E. Linder[1]

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110

Abstract Palmitoylation is a posttranslational modification that regulates protein trafficking and stability. In this study we investigated whether the endosomal soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins syntaxin 7 and syntaxin 8 are modified with palmitate. Using metabolic labeling and site-directed mutagenesis, we show that human syntaxins 7 and 8 are modified with palmitate through a thioester linkage. Palmitoylation is dependent upon cysteine 239 of human syntaxin 7 and cysteine 214 of syntaxin 8, residues that are located on the cytoplasmic face of the transmembrane domain (TMD). Palmitoylation of syntaxin 8 is minimally affected by the Golgi-disturbing agent brefeldin A (BFA), whereas BFA dramatically inhibits palmitoylation of syntaxin7. The differential effect of BFA suggests that palmitoylation of syntaxins 7 and 8 occurs in distinct subcellular compartments. Palmitoylation does not affect the rate of protein turnover of syntaxins 7 and 8 nor does it influence the steady-state localization of syntaxin 8 in late endosomes. Syntaxin 7 actively cycles between endosomes and the plasma membrane. Palmitoylation-defective syntaxin 7 is selectively retained on the plasma membrane, suggesting that palmitoylation is important for intercompartmental transport of syntaxin 7.—He, Y., and M. E. Linder. **Differential palmitoylation of the endosomal SNAREs syntaxin 7 and syntaxin 8. *J. Lipid Res.* 2009.** 50: **398–404.**

Supplementary key words protein trafficking • fatty acylation • brefeldin A

Palmitoylation refers to the posttranslational addition of palmitate to cysteine residues in proteins (1–3). Two features of palmitoylation distinguish it from other covalent lipid modifications that occur in the cytoplasm or on the cytoplasmic face of membranes. First, palmitoylation is reversible. Palmitate is added to proteins through a reversible thioester linkage, whereas myristate and prenyl groups are attached through stable amide and thioether linkages, respectively. Second, palmitoylation is a modification of both integral and peripheral membrane proteins, whereas N-myristoylation and prenylation almost exclusively are modifications of proteins peripherally associated with membranes.

A key function for lipid attachment to otherwise soluble proteins is to promote their interactions with membranes. Integral membrane proteins are permanently inserted into membranes by virtue of their transmembrane domains (TMDs). For many receptors and ion channels, palmitoylation of cytoplasmic tails creates additional sites of protein-membrane attachment (3). In other integral membrane proteins, however, the site of palmitoylation is often at one or more cysteine residues near the interface of the cytoplasm and the membrane. The functional consequences of palmitoylating integral membrane proteins are unclear; although evidence is emerging that palmitoylation may impact protein stability (4, 5).

Several members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family of proteins are known to be substrates for palmitoylation (6, 7). SNARE proteins are mediators of membrane-fusion events in the secretory pathway and endosomal network. The hallmark of SNARE proteins is the SNARE motif, a conserved stretch of 60–70 amino acids, which has a propensity to form coiled-coils. Pairing between cognate SNAREs on opposing membranes results in the formation of a parallel four-helix bundle, which brings the membranes together to allow fusion. Most SNAREs are anchored to membranes by a C-terminal TMD. However, several SNAREs lack TMDs. The neuronal protein synaptic-associated protein of 25 kDa (SNAP-25) and its ubiquitously expressed homolog SNAP-23 bind to membranes through a stretch of palmitoylated cysteine residues present in the interhelical domain that connects two SNARE motifs (8–10). Ykt6 associates with membranes through a palmitoylated and farnesylated motif located at the C terminus (11).

Yuhong He was supported by a postdoctoral fellowship from the American Heart Association (Heartland Affiliate). Work in the authors' laboratory was supported by NIH (GM51466).

Manuscript received 10 July 2008 and in revised form 14 October 2008.

Published, JLR Papers in Press, October 31, 2008.
DOI 10.1194/jlr.M800360-JLR200

Abbreviations: BFA, brefeldin A; GFP, green fluorescent protein; SNAP-25, synaptic-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors; TMD, transmembrane domain.

[1] To whom correspondence should be addressed.
e-mail: mlinder@wustl.edu

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure.

Palmitoylation of SNAREs with TMDs has been reported for multiple yeast SNAREs (4, 12). First described for Snc1 and Snc2, SNAREs required for fusion of Golgi-derived vesicles with the plasma membrane, a recent survey of the yeast palmitoylproteome revealed that 8 of the 23 *S. cerevisiae* SNAREs were palmitoylated (13). These 8 SNAREs have one or more juxtamembranous Cys residues, whereas the remaining 15 SNARE proteins lack them. The eight palmitoylated *S. cerevisiae* SNAREs reside in the trans-Golgi network, endosomes and the plasma membrane, which are membranes with a rich sterol content. The functional consequences of palmitoylation of the TMD SNAREs are not clear except for Tlg1, which regulates membrane trafficking between endosomes and the Golgi. Palmitoylation of Tlg1 protects it from ubiquitination and subsequent degradation in the yeast vacuole (4).

It has not been established whether palmitoylation of TMD SNAREs is conserved in mammals. In this study we examined whether syntaxins 7 and 8 are modified with palmitate. Syntaxins 7 and 8 are localized in early and late endosomes and can traffic through the plasma membrane (14). A SNARE complex of syntaxin 7 and syntaxin 8, vti1b and VAMP8 mediates homotypic fusion of late endosomes (15–17). Evidence suggests that syntaxins 7 and 8 also mediate heterotypic fusion of late endosomes with lysosomes in conjunction with vti1b and VAMP7 (18, 19). We found that syntaxins 7 and 8 are palmitoylated and investigated the functional consequences of this modification.

EXPERIMENTAL PROCEDURES

Materials

[9,10-^{3}H] Palmitate (31.0 Ci/mM) was purchased from Perkin-Elmer Life Sciences and [^{35}S] methionione (>1000 Ci/mM) was from GE Healthcare. Hydroxylamine, Brefeldin A (BFA) and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). BFA was stored at −20°C as 2 mg/ml stock solutions in DMSO. Cycloheximide was stored at −20°C as a 2 mg/ml stock in distilled water. Sources for antibodies are as follows: CD63 (H5C6) mouse monoclonal antibody and LAMP-1 (H4A3) mouse monoclonal antibody (Developmental Studies Hybridoma Bank, The University of Iowa); CD46 mouse monoclonal antibody (gift from Dr. Douglas M. Lublin, Washington University School of Medicine); EEA1 mouse monoclonal antibody (BD Biosciences); goat anti-mouse Alexa Fluo 546-conjugated secondary antibody (Invitrogen). Green fluorescent protein (GFP) antibodies were generated and affinity purified as described (20) and coupled to protein G-conjugated beads (GE Healthcare) (21).

Site-directed mutagenesis and vector construction

Plasmids for the expression of the human EGFP-syntaxin 7 and myc-syntaxin 8/pcDNA3 were the generous gifts of Dr. Jerry Kaplan (University of Utah Health Sciences Center) and Dr. Richard Scheller (Genentech). The EGFP-syntaxin 8 expression plasmids used for the studies described herein were generated by subcloning syntaxin 8 from pcDNA3 into the *Eco*RI and BamHI sites of the pEGFP-C2 vector (Invitrogen). All of the point mutations were created using a QuikChange mutagenesis kit (Stratagene) and verified by nucleotide sequence analysis. Site-directed mutagenesis was performed using the polymerase chain reaction and the oligonucleotide ML1445 5′ CAAATCCAGAAAAACCCTGCTCATCATCATTCTTATC 3′, and ML1446 5′ GATAAGAATGATGATGAGCAGGGTTTTTCTGGATTTG 3′ to change the syntaxin 7 cDNA to code for a leucine instead of cysteine at residue 239 (C239L). Oligonucleotides ML1447 5′ CACCATGATCATCCCAGCAGAGGCTGACTTTCTG 3′, ML1448 5′ CACCATGATCATCCCAGC AGAGGCTGACTTTCTG 3′ changed the syntaxin 8 cDNA to code for an alanine instead of a cysteine at residue 214 (C214A).

Cell culture and transfection protocols

HeLa cells were cultured in high glucose DMEM supplemented with 10% bovine growth serum, 2 mM glutamine, 150 units/ml penicillin, and 50 units/ml streptomycin. The cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Typically, 3×10^5 cells were transfected with 1 μg of DNA and 3 μl of lipofectamine 2000.

Metabolic labeling with [^{3}H]palmitate

For labeling with [^{3}H]palmitate, Hela cells (in a 35 mm dish) were transiently transfected with wild-type or mutant syntaxin 7 and 8 overnight. The cells were washed twice with serum free DMEM containing Na pyruvate and nonessential amino acids, then incubated for 4 h in 0.6 ml [^{3}H]palmitate labeling media (DMEM containing 0.5 mCi/ml [^{3}H]palmitate, 10% dialyzed fetal calf serum, 1% DMSO, Na pyruvate, and nonessential amino acids).

Immunoprecipitation

Transfected Hela cells were washed with 1 ml of ice-cold PBS and lysed in 0.5 ml buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.5% SDS and protease inhibitors 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml lima bean protease inhibitor, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride). The cell pellets were homogenized using a 25-gauge needle and syringe and incubated at 4°C using end-over-end rotation for 2 h. Insoluble material was removed by centrifugation at 100,000 *g* in a Beckman TLA-100.3 rotor (20 min at 4°C). The supernatants were immunoprecipitated with GFP antibody covalently coupled to protein G-Sepharose (15 μl packed beads). Immunoprecipitated proteins were subjected to SDS-PAGE and analyzed by Coomassie Blue staining, immunoblotting and fluorography (22).

Hydroxylamine treatment

Hydroxylamine treatment was performed according to Bizzozero (23). The immunoprecipitates were divided in half and resolved on separate SDS gels. Following staining with Coomassie Blue, the gels were soaked for 5–10 h in 20–40 vols of fresh hydroxylamine (1 M hydroxylamine, 50% isopropanol, pH7.0) or as a control, 1 M Tris-HCl, pH7.0 containing 50% isopropanol. Both gels were washed four times in 50% isopropanol for a total of 2 days and prepared for fluorography.

Half-life determination of syntaxins 7 and 8

Hela cells were seeded in 35 mm dishes in DMEM media with 10% bovine growth serum and 2 mM glutamine, transfected with syntaxin 7 or 8, wild-type or C239L or C214A mutant plasmids, and incubated overnight. Prior to labeling, the cells were incubated for 1 h in methionine-free medium. Cells expressing syntaxin 7 or 8 were metabolically labeled with 50 μCi/ml of [^{35}S] methionine for 2 h. The medium was replaced with complete DMEM. Cells were harvested at 0, 4, 8, 20, 28, and 36 h and processed for immunoprecipitation and fluorography as previously

described. [^{35}S]methionine labeled proteins were quantified with a Phosphorimager (Molecular Dynamics Storm 860).

BFA treatment

BFA (final concentration of 10 μg/ml) suspended in DMSO was applied to the transfected Hela cells in the [^{3}H]palmitate labeling medium. Control cells were incubated with DMSO.

Immunofluorescence and confocal microscopy

Transfected cells on coverslips were fixed for 15 min with 4% paraformaldehyde and permeabilized using 1% Triton X-100. For CD46 immunofluroescence, the cells were fixed but not permeabilized. Nonspecific binding was blocked using 1% (w/v) BSA for 1 h at room temperature. Cells were then incubated for 1 h with the primary antiserum (anti-EEA1, -CD63, -LAMP1, -CD46, or -myc). Alexa Fluo 546-conjugated secondary antibodies were subsequently added and incubated for 1 h at room temperature. Slides were mounted in Vectorshield and confocal immunofluorescence microscopy was performed using a Zeiss LSM-10 laser-scanning microscope. Images were collected at room temperature and represent single equatorial planes obtained with a × 63 objective. The only image processing was adjustment of brightness and/or contrast in Adobe Photoshop.

RESULTS

Syntaxin 7 and 8 are palmitoylated

To determine whether syntaxin 7 and 8 are palmitoylated, Hela cells expressing GFP-tagged forms of the protein were radiolabeled with [^{3}H]palmitate. Radioactivity was detected in immunoprecipitates of GFP-syntaxin 7 and GFP-syntaxin 8 (**Fig. 1B**). Covalent attachment of palmitate into proteins occurs primarily through a thioester linkage to cysteine residues and is susceptible to hydrolysis by neutral hydroxylamine (24). Treatment of radiolabeled GFP-syntaxin 7 and GFP-syntaxin 8 with hydroxylamine led to a loss of the tritium signal on the fluorograms, whereas the protein levels remained unchanged (Fig. 1C), establishing that the acyl moiety on the syntaxins 7 and 8 was attached via a thioester bond.

Cys239 of syntaxin 7 and Cys214 of syntaxin 8 are at the boundary of the cytoplasmic domain and the C-terminal TMD (Fig. 1A). To test whether these residues are required for palmitoylation, we replaced C239 of syntaxin 7 with leucine and C214 of syntaxin 8 with alanine. Serine is typically substituted for cysteine when making palmitoylation mutants. However, we chose to avoid introducing a polar residue juxtaposed to the TMD (4) and selected the hydrophobic residues based on nearby sequences (Fig. 1A). Neither GFP-syntaxin 7(C239L) nor GFP-syntaxin 8(C214A) incorporated radioactive palmitate, demonstrating that palmitoylation is dependent on a cysteine residue adjacent to the TMD (Fig. 1C).

To determine whether palmitoylation of syntaxins 7 and 8 occurs only on nascent or on mature protein, we performed radiolabeling of cells with [^{3}H]palmitate in the presence of a protein synthesis inhibitor. There was a substantial reduction, but not complete inhibition of palmitate incorporation into GFP-syntaxin 7 and GFP-syntaxin 8 (Fig. 1D). By contrast, N-Ras palmitoylation was unaffected by cycloheximide treatment (data not shown), consistent with the rapid and stoichiometric turnover of palmitate that has been characterized previously (25, 26). The data suggest that during a 4-hour labeling period, most of the palmitate is incorporated into nascent protein. However, the residual palmitate incorporation in the absence of new protein synthesis suggests that there may be slow turnover of palmitate on mature syntaxins. This point will be addressed further in the Discussion.

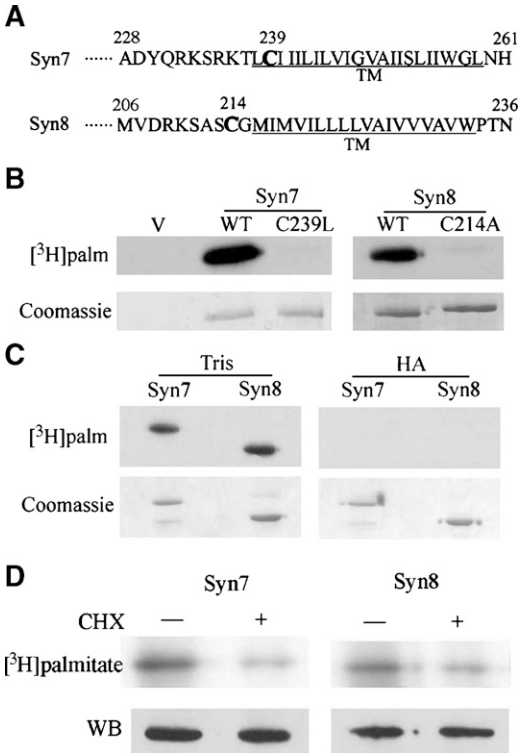

Fig. 1. Palmitoylation of syntaxin 7 and syntaxin 8 at juxtamembranous cysteine residues. A: The C-terminal sequence of human syntaxins 7 and 8. Numbers refer to amino acid sequence position. The palmitoylated cysteines of syntaxin 7 (Cys239) and syntaxin 8 (Cys214) are in boldface type. B: Green fluorescent protein (GFP)-tagged syntaxin 7 and 8 and the corresponding mutants were ectopically expressed in Hela cells and incubated with [^{3}H]palmitate. The proteins were immunoprecipated with anti-GFP antibody covalently coupled to Protein G Sepharose, resolved by SDS-PAGE and then visualized by fluorography and staining with Coomassie Blue. C: Radiolabeled syntaxins 7 and 8 were separated by SDS-PAGE. The gels were incubated either in 1M hydroxylamine (pH7.0) or 1M Tris-HCl (pH7.0) as a control and visualized by fluorography and staining with Coomassie Blue. The data shown in B and C are the representatives of at least three independent experiments. D: GFP-syntaxin 7 or GFP-syntaxin 8 was transfected into Hela cells. One day later, the cells were pretreated with vehicle or cycloheximide (CHX) (20 μg/ml) for 1 h, followed by radiolabeling with [^{3}H]palmitate (0.5 mCi/ml) for 4 h with or without cycloheximide. Cell lysates were processed for immunoprecipitation and visualized by fluorography and Western blot (WB). The data shown are representative of two independent experiments.

Syntaxin 7 and syntaxin 8 palmitoylation displays differential sensitivity to BFA

We next sought to determine where syntaxin 7 and syntaxin 8 are palmitoylated in cells. By analogy to synapto-

brevin (27), syntaxin 7 and syntaxin 8 are presumed to be tail-anchored proteins that are posttranslationally inserted into the ER membrane prior to their trafficking through the Golgi and sorting into the endosomal compartment. The fungal metabolite BFA blocks transport through the secretory pathway. In BFA-treated cells, there is a collapse of Golgi cisternae into a mixed ER-Golgi compartment and tubulation of the trans-Golgi network and endosomal membranes (28, 29). Treatment of cells with BFA results in the retention of newly synthesized H-ras (30), the delta opioid receptor (31) and the viral hemagglutin proteins on intracellular membranes (32), but does not affect their palmitoylation. This suggests that the palmitoyltransferases that modify these proteins are present in the mixed ER/Golgi compartment. By contrast, SNAP-25 palmitoylation is inhibited by BFA and other agents that block movement through the secretory pathway (33), suggesting that its palmitoyltransferase may be localized distal to the early secretory pathway.

As shown in **Fig. 2**, palmitoylation of GFP-syntaxin 7 was substantially inhibited by BFA, whereas GFP-syntaxin 8 palmitoylation was only slightly decreased. The inhibitory effect of BFA on syntaxin 7 palmitoylation was not due to decreased protein expression, as equal amounts of GFP-tagged protein were present in the immunoprecipitates in the presence or absence of BFA (Fig. 2). The differential sensitivity to BFA suggests that palmitoylation of syntaxin 7 and syntaxin 8 occurs in different subcellular compartments.

Palmitoylation does not affect protein turnover of syntaxin 7 and syntaxin 8

Palmitoylation protects the yeast SNARE protein Tlg1 from ubiquitination and degradation (4). In the absence of palmitoylation, the protein half-life of Tlg1 is reduced substantially. To investigate whether the stability of the syntaxin 7 and 8 are affected by palmitoylation, we measured the protein turnover rates of wild-type and palmitoylation-defective mutants of syntaxin 7 and 8 in pulse-chase experiments. As shown in **Fig. 3**, GFP-syntaxin 7(C239L) and GFP-syntaxin 8(C214A) had similar turnover rates to those of the wild-type proteins. These results suggested that palmitoylation does not influence the stability or degradation of the syntaxin 7 and syntaxin 8.

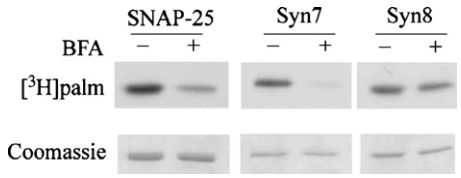

Fig. 2. Brefeldin A (BFA) differentially inhibits palmitoylation of syntaxin 7 and syntaxin 8. Synaptic-associated protein of 25 kDa (SNAP-25), syntaxin 7, or syntaxin 8 were ectopically expressed in Hela cells and radiolabeled with [^{3}H] palmitate in the presence or absence of 10 μg/ml BFA. Following immunoprecipitation, the proteins were analyzed by SDS-PAGE and visualized by fluorography or staining with Coomassie Blue. The data shown are the representatives of three independent experiments.

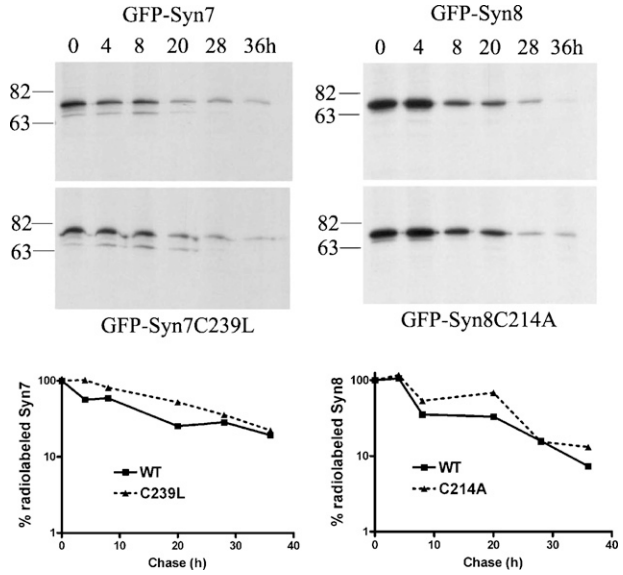

Fig. 3. The protein half-life of wild-type syntaxins 7 and 8 and their corresponding palmitoylation-defective mutants are similar. Hela cells were transfected with wild-type or mutant syntaxins 7 and 8. After an overnight incubation, the cells were labeled with [^{35}S]methionine and chased with complete DMEM for indicated times. Syntaxins were immunprecipitated and processed for fluorography (upper panels) or quantified by phosphor scanning (graphs). Values were normalized to the intensity at 0 h of chase. The data shown are from a single experiment and are representative of three experiments with GFP-tagged syntaxins and one experiment with myc-tagged syntaxins.

Palmitoylation affects the steady state localization of syntaxin 7 but not syntaxin 8

To study if palmitoylation plays a role in the steady-state localization of syntaxin 7 and syntaxin 8, GFP-tagged forms of wild-type and palmitoylation-deficient syntaxins were expressed in HeLa cells and examined by confocal microscopy. GFP-syntaxin 7 overlapped most prominently with EEA1, an early endosome marker and to some extent with CD63, a late endosomal marker, and the lysosomal marker LAMP1 (**Fig. 4A**). The diffuse reticular fluorescence extending throughout the cell likely represents nascent protein in the ER (Fig. 4A). There was a striking shift in the localization of palmitoylation-defective syntaxin 7 to the plasma membrane (Fig. 4A). GFP-syntaxin 8 displayed prominent colocalization with CD63 and LAMP1 and less overlap with EEA1 (Fig. 4B). There was no apparent difference in the localization of wild-type and the palmitoylation-defective mutant of GFP-syntaxin 8 (Fig. 4B). To further investigate whether mutation of the palmitoylation site alters the subcellular localization of syntaxin 7 and syntaxin 8, we coexpressed myc-tagged wild-type protein with the GFP-tagged palmitoylation mutant (see supplementary Fig. I). Again, we observed that palmitoylation-defective syntaxin 7 was more abundant in the plasma membrane, whereas GFP-syntaxin 8 localization was unaffected by the loss of palmitoylation.

Syntaxin 7 is known to traffic through the plasma membrane (14). To confirm quantitatively that there was an accumulation of palmitoylation-defective syntaxin 7 at the

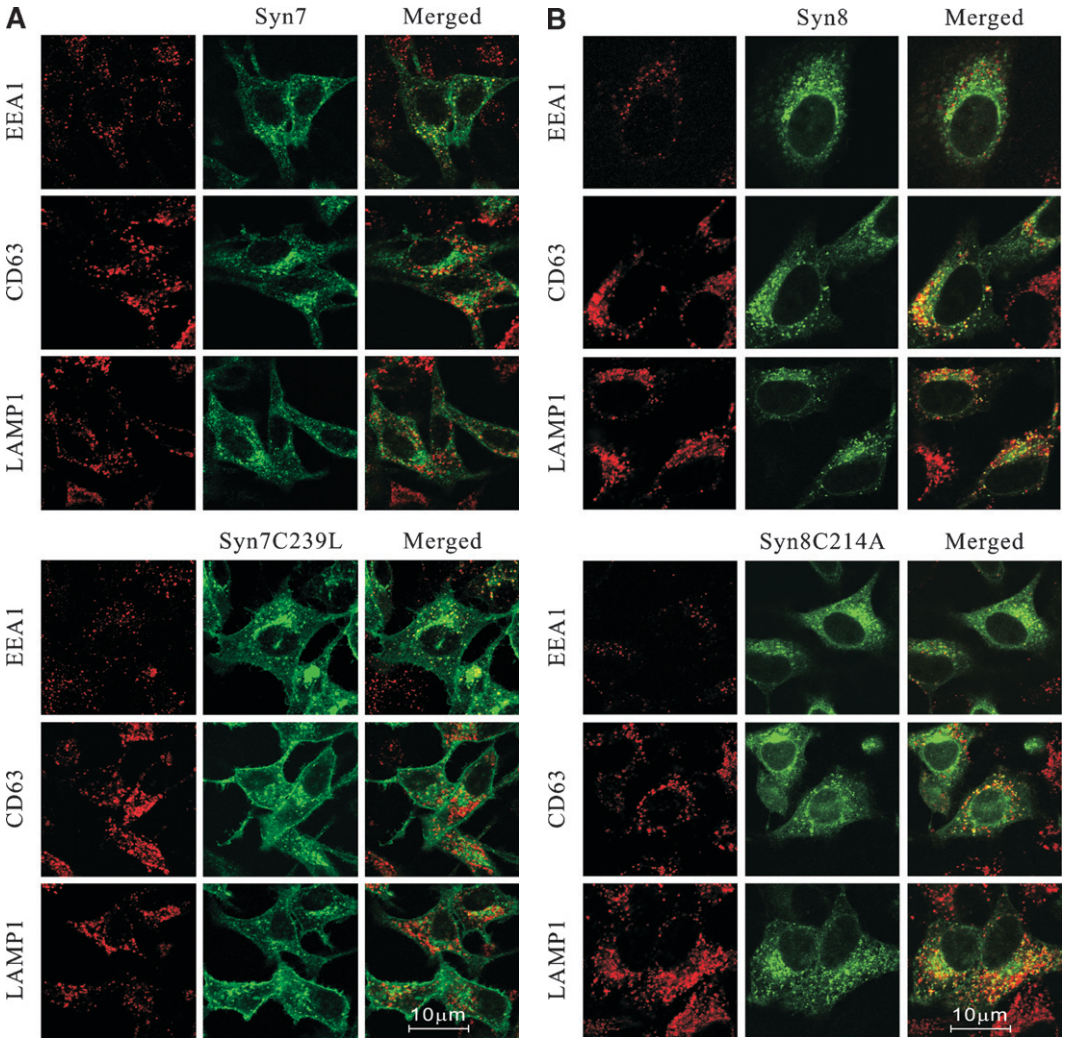

Fig. 4. Localization of wild-type and palmitoylation-defective syntaxins 7 and 8. Hela cells were transfected with GFP-tagged wild-type or mutant syntaxin 7 (A) or syntaxin 8 (B). One day later, cells were processed for GFP epifluorescence and immunofluorescence with anti-EEA1, anti-CD63, or anti-LAMP1 (red). The cells were examined by confocal microscopy. Yellow represents the overlapping areas in merged images.

plasma membrane, we scored cells transfected with GFP-syntaxin 7 for costaining with CD46, a plasma membrane marker (**Fig. 5**). Wild-type syntaxin 7 was localized at the plasma membrane in 34 ± 6% of the cells, whereas syntaxin 7(C239L) was present on the plasma membrane in 58 ± 7% of the cells (>100 cells counted in three independent experiments). These results suggest that palmitoylation is required for normal trafficking of syntaxin 7 through the plasma membrane.

DISCUSSION

The results presented herein demonstrate that the endosomal SNARE proteins syntaxin 7 and syntaxin 8 are palmitoylated at a cysteine residue near the junction of the cytoplasmic and TMD. It is anticipated that this modification will be conserved in many other mammalian SNARE proteins based on the presence of cysteine residues near or in the TMD. In yeast, SNAREs associated with the early secretory pathway are not palmitoylated, whereas SNAREs present in the plasma membrane and the endosomal network are modified (4, 13). This dichotomy suggests that palmitoylation may facilitate sorting of TMD SNAREs into thicker, sterol-rich membranes. Based on sequence, the mammalian SNAREs do not fit this model. Syntaxin 3 and Syntaxin 2 (isoform 2) are localized in the plasma membrane and do not have cysteines within the last 50 amino acids of the proteins, whereas Bet1 and GS15, SNAREs associated with the intermediate and Golgi compartments, have cysteine residues near the cytoplasmic end of the TMD (7). Further documentation of the mammalian palmitoylproteome will be required to identify the full complement of palmitoylated SNAREs.

Palmitoylation of proteins can be stable or dynamic. Viral glycoproteins and caveolin are examples of proteins that are stably palmitoylated, and no palmitate incorporation is detected in the absence of new protein synthesis

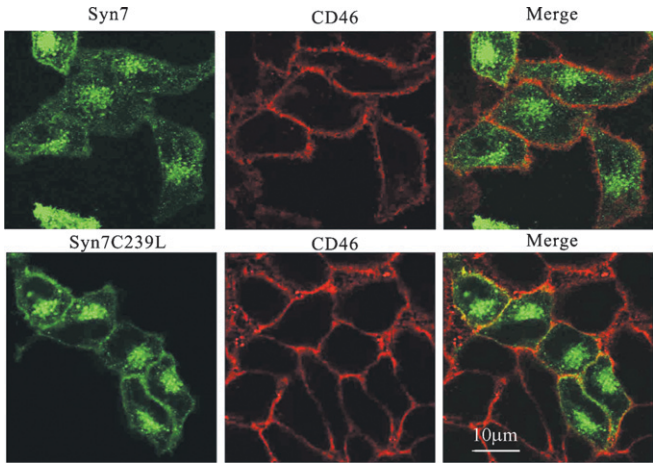

Fig. 5. Plasma membrane localization of wild-type and palmitoylation-defective syntaxin 7. Hela cells were transfected with wild-type or mutant GFP-tagged syntaxin 7. One day later, cells were processed for GFP epifluorescence and immunofluorescence with the antibodies to the cell surface protein CD46 (red). The cells were examined by confocal microscopy. Yellow represents the overlapping areas in merged images.

(32, 34). In contrast, many signaling proteins cycle between membrane compartments using an acylation/deacylation cycle that occurs on the time course of minutes (26). Accordingly, palmitate incorporation is unaffected by the presence of protein synthesis inhibitors. With syntaxin 7 and syntaxin 8, we found that there was a substantial reduction of palmitate incorporation in the presence of cycloheximide, but it was not complete. We suggest three possible explanations for palmitate incorporation into syntaxin 7 or syntaxin 8 in the absence of new protein synthesis. First, palmitoylation could be turning over at a slow rate. Second, there could be two pools of syntaxin: one that is stably palmitoylated and another that undergoes palmitate turnover. Third, syntaxin 7 or syntaxin 8 might be modified more than 1 h after synthesis.

Palmitoylation is emerging as a mechanism to regulate protein stability. The yeast SNARE Tlg1 is protected from ubiquitination and degradation when palmitoylated (4). Exit of the integral membrane proteins CHS3 (35) and LRP6 (36) from the ER is also dependent upon modification with palmitate. We tested whether palmitoylation had an impact on the protein half-life of syntaxin 7 or syntaxin 8. Blocking palmitoylation by mutation of the modified cysteine did not shorten the protein half-life of syntaxin 7 or syntaxin 8, nor did it result in accumulation of the protein in the endoplasmic reticulum or Golgi apparatus. Thus, palmitoylation does not appear to be required for syntaxins 7 and 8 to bypass the quality control machinery of the cell, as is the case for the yeast SNARE Tlg1.

The differential sensitivity of syntaxin 7 and syntaxin 8 palmitoylation to BFA was surprising. We anticipated that syntaxin 7 and syntaxin 8 would undergo similar posttranslational processing with palmitate. The presumptive biosynthetic pathway for both proteins is posttranslational insertion into the ER and transport to the endosomal network via the secretory pathway. However, there must be some divergence in the trafficking of syntaxin 7 and syntaxin 8 because we observed that syntaxin 7 was more prevalent in early endosomes, whereas syntaxin 8 was associated with late endosomes at steady state. We also observed that when palmitoylation was prevented by mutation, syntaxin 7 and syntaxin 8 displayed different phenotypic consequences. Syntaxin 7 accumulated in the plasma membrane, whereas the steady-state localization of syntaxin 8 was unperturbed. Hence, syntaxin 7 and syntaxin 8 sample different membrane compartments, each potentially with its own complement of palmitoyltransferases.

The palmitoylation of proteins known to be modified early in the secretory pathway is unaffected by BFA treatment (30–32). Similarly, syntaxin 8 might be modified in the ER or Golgi. The BFA sensitivity of syntaxin 7 palmitoylation is similar to that of SNAP-25 (33). Recent studies in *Drosophila* point to HIP14 (DHHC17) as the palmitoyltransferase that modifies SNAP-25 (37, 38). The localization of HIP14 in vesicular structures distant from the cell body may explain the requirement for an intact secretory pathway for SNAP-25 palmitoylation. We anticipate that syntaxin 7 is palmitoylated by palmitoyltransferase localized in a post-Golgi compartment.

An interesting phenotype was observed for the syntaxin 7 palmitoylation mutant. In the absence of palmitoylation, syntaxin 7 accumulated on the plasma membrane. Although localized primarily in early and late endosomes, syntaxin 7 is known to traffic through the plasma membrane (14). It is unknown whether this represents nascent syntaxin 7 that is delivered to the plasma membrane and then sorted to endosomes or if syntaxin 7 recycles from the plasma membrane to endosomes. In either case, the absence of palmitoylation results in the accumulation of syntaxin 7 at the plasma membrane, suggesting that it is important for efficient clearing from the plasma membrane and delivery to endosomes. It is notable that Swf1, the palmitoyltransferase responsible for yeast SNARE palmitoylation, has recently been shown to localize in cortical puncta and colocalize with actin patches (39). Actin patches form on the plasma membrane at sites of endocytosis and bud off with endocytic vesicles (40). It is interesting to speculate that syntaxin 7 encountering its cognate palmitoyltransferase at sites of endocytosis might underlie its recruitment to an endocytic vesicle for transit to early endosomes. Future studies will address which of the 23 mammalian palmitoyltransferases are responsible for syntaxin 7 and syntaxin 8 and help resolve the question of where syntaxins 7 and 8 are palmitoylated in the cell.

Note Added in Proof

Evidence that endogenous syntaxin 7 and syntaxin 8 are palmitoylated was recently published in a global analysis of the neuronal palmitoyl-proteome (Kang R., J. Wan, P. Arstikaitis, H. Takahashi, K. Huang, A. O. Bailey, J. X. Thompson, A. F. Roth, R. C. Drisdel, R. Mastro, W. N. Green, J. R. Yates 3rd, N. G. Davis, and A. El-Husseini. 2008. Neural palmitoyl-proteomics reveals dynamic synaptic palmitoylation. *Nature.* 456: 904–909).

We thank Drs. Richard Scheller, Rytis Prekeris, Rob Piper, and Jerry Kaplan for providing plasmids, and Dr. Douglas Lublin for providing CD46 antibodies.

REFERENCES

1. Linder, M. E., and R. J. Deschenes. 2007. Palmitoylation: policing protein stability and traffic. *Nat. Rev. Mol. Cell Biol.* **8:** 74–84.
2. Resh, M. D. 2006. Palmitoylation of ligands, receptors, and intracellular signaling molecules. *Sci. STKE.* **2006:** re14.
3. Greaves, J., and L. H. Chamberlain. 2007. Palmitoylation-dependent protein sorting. *J. Cell Biol.* **176:** 249–254.
4. Valdez-Taubas, J., and H. Pelham. 2005. Swf1-dependent palmitoylation of the SNARE Tlg1 prevents its ubiquitination and degradation. *EMBO J.* **24:** 2524–2532.
5. Abrami, L., S. H. Leppla, and F. G. van der Goot. 2006. Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. *J. Cell Biol.* **172:** 309–320.
6. Jahn, R., and R. H. Scheller. 2006. SNAREs–engines for membrane fusion. *Nat. Rev. Mol. Cell Biol.* **7:** 631–643.
7. Hong, W. 2005. SNAREs and traffic. *Biochim. Biophys. Acta.* **1744:** 120–144.
8. Veit, M., T. H. Sollner, and J. E. Rothman. 1996. Multiple palmitoylation of synaptotagmin and the t-SNARE SNAP-25. *FEBS Lett.* **385:** 119–123.
9. Lane, S. R., and Y. Liu. 1997. Characterization of the palmitoylation domain of SNAP-25. *J. Neurochem.* **69:** 1864–1869.
10. Vogel, K., and P. A. Roche. 1999. SNAP-23 and SNAP-25 are palmitoylated in vivo. *Biochem. Biophys. Res. Commun.* **258:** 407–410.
11. Fukasawa, M., O. Varlamov, W. S. Eng, T. H. Sollner, and J. E. Rothman. 2004. Localization and activity of the SNARE Ykt6 determined by its regulatory domain and palmitoylation. *Proc. Natl. Acad. Sci. USA.* **101:** 4815–4820.
12. Couve, A., V. Protopopov, and J. E. Gerst. 1995. Yeast synaptobrevin homologs are modified posttranslationally by the addition of palmitate. *Proc. Natl. Acad. Sci. USA.* **92:** 5987–5991.
13. Roth, A. F., J. Wan, A. O. Bailey, B. Sun, J. A. Kuchar, W. N. Green, B. S. Phinney, J. R. Yates 3rd, and N. G. Davis. 2006. Global analysis of protein palmitoylation in yeast. *Cell.* **125:** 1003–1013.
14. Prekeris, R., B. Yang, V. Oorschot, J. Klumperman, and R. H. Scheller. 1999. Differential roles of syntaxin 7 and syntaxin 8 in endosomal trafficking. *Mol. Biol. Cell.* **10:** 3891–3908.
15. Antonin, W., D. Fasshauer, S. Becker, R. Jahn, and T. R. Schneider. 2002. Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. *Nat. Struct. Biol.* **9:** 107–111.
16. Antonin, W., C. Holroyd, D. Fasshauer, S. Pabst, G. F. Von Mollard, and R. Jahn. 2000. A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. *EMBO J.* **19:** 6453–6464.
17. Ward, D. M., J. Pevsner, M. A. Scullion, M. Vaughn, and J. Kaplan. 2000. Syntaxin 7 and VAMP-7 are soluble N-ethylmaleimide-sensitive factor attachment protein receptors required for late endosome-lysosome and homotypic lysosome fusion in alveolar macrophages. *Mol. Biol. Cell.* **11:** 2327–2333.
18. Mullock, B. M., C. W. Smith, G. Ihrke, N. A. Bright, M. Lindsay, E. J. Parkinson, D. A. Brooks, R. G. Parton, D. E. James, J. P. Luzio, et al. 2000. Syntaxin 7 is localized to late endosome compartments, associates with Vamp 8, and Is required for late endosome-lysosome fusion. *Mol. Biol. Cell.* **11:** 3137–3153.
19. Pryor, P. R., B. M. Mullock, N. A. Bright, M. R. Lindsay, S. R. Gray, S. C. Richardson, A. Stewart, D. E. James, R. C. Piper, and J. P. Luzio. 2004. Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Rep.* **5:** 590–595.
20. Seedorf, M., M. Damelin, J. Kahana, T. Taura, and P. A. Silver. 1999. Interactions between a nuclear transporter and a subset of nuclear pore complex proteins depend on Ran GTPase. *Mol. Cell. Biol.* **19:** 1547–1557.
21. Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory.
22. Loranger, S. S., and M. E. Linder. 2002. SNAP-25 traffics to the plasma membrane by a syntaxin-independent mechanism. *J. Biol. Chem.* **277:** 34303–34309.
23. Bizzozero, O. A. 1995. Chemical analysis of acylation sites and species. *Methods Enzymol.* **250:** 361–379.
24. Magee, A. I., A. H. Koyama, C. Malfer, D. Wen, and M. J. Schlesinger. 1984. Release of fatty acids from virus glycoproteins by hydroxylamine. *Biochim. Biophys. Acta.* **798:** 156–166.
25. Magee, A. I., L. Gutierrez, I. A. McKay, C. J. Marshall, and A. Hall. 1987. Dynamic fatty acylation of p21N-ras. *EMBO J.* **6:** 3353–3357.
26. Rocks, O., A. Peyker, M. Kahms, P. J. Verveer, C. Koerner, M. Lumbierres, J. Kuhlmann, H. Waldmann, A. Wittinghofer, and P. I. Bastiaens. 2005. An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science.* **307:** 1746–1752.
27. Kutay, U., G. Ahnert-Hilger, E. Hartmann, B. Wiedenmann, and T. Rapoport. 1995. Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *EMBO J.* **14:** 217–223.
28. Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz. 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. *J. Cell Biol.* **116:** 1071–1080.
29. Robinson, M. S., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* **69:** 129–138.
30. Apolloni, A., I. Prior, M. Lindsay, R. Parton, and J. Hancock. 2000. H-ras but Not K-ras Traffics to the Plasma Membrane through the Exocytic Pathway. *Mol. Cell. Biol.* **20:** 2475–2487.
31. Petaja-Repo, U. E., M. Hogue, T. T. Leskela, P. M. Markkanen, J. T. Tuusa, and M. Bouvier. 2006. Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *J. Biol. Chem.* **281:** 15780–15789.
32. Veit, M., and M. F. Schmidt. 1993. Timing of palmitoylation of influenza virus hemagglutinin. *FEBS Lett.* **336:** 243–247.
33. Gonzalo, S., and M. E. Linder. 1998. SNAP-25 palmitoylation and plasma membrane targeting require a functional secretory pathway. *Mol. Biol. Cell.* **9:** 585–597.
34. Parat, M. O., and P. L. Fox. 2001. Palmitoylation of caveolin-1 in endothelial cells is post-translational but irreversible. *J. Biol. Chem.* **276:** 15776–15782.
35. Lam, K. K., M. Davey, B. Sun, A. F. Roth, N. G. Davis, and E. Conibear. 2006. Palmitoylation by the DHHC protein Pfa4 regulates the ER exit of Chs3. *J. Cell Biol.* **174:** 19–25.
36. Abrami, L., B. Kunz, I. Iacovache, and F. G. van der Goot. 2008. Palmitoylation and ubiquitination regulate exit of the Wnt signaling protein LRP6 from the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* **105:** 5384–5389.
37. Huang, K., A. Yanai, R. Kang, P. Arstikaitis, R. R. Singaraja, M. Metzler, A. Mullard, B. Haigh, C. Gauthier-Campbell, C. A. Gutekunst, et al. 2004. Huntingtin-interacting protein HIP14 is a palmitoyl transferase involved in palmitoylation and trafficking of multiple neuronal proteins. *Neuron.* **44:** 977–986.
38. Stowers, R. S., and E. Y. Isacoff. 2007. Drosophila huntingtin-interacting protein 14 is a presynaptic protein required for photoreceptor synaptic transmission and expression of the palmitoylated proteins synaptosome-associated protein 25 and cysteine string protein. *J. Neurosci.* **27:** 12874–12883.
39. Dighe, S., and K. Kozminski. 2008. Swf1p, a member of the DHHC-CRD family of palmitoyltransferases, regulates the actin cytoskeleton and polarized secretion independently of its DHHC motif. *Mol Biol Cell.* **19:** 4454–4468.
40. Moseley, J. B., and B. L. Goode. 2006. The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol. Mol. Biol. Rev.* **70:** 605–645.