Roles of the cytoplasmic and transmembrane domains of syntaxins in intracellular localization and trafficking

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SUMMARY

Syntaxins are target-soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors (t-SNAREs) involved in docking and fusion of vesicles in exocytosis and endocytosis. Many syntaxin isoforms have been isolated, and each one displays a distinct intracellular localization pattern. However, the signals that drive the specific intracellular localization of syntaxins are poorly understood. In this study, we used indirect immunofluorescence analysis to examine the localization of syntaxin chimeras, each containing a syntaxin transmembrane domain fused to a cytoplasmic domain derived from a different syntaxin. We show that the cytoplasmic domains of syntaxins 5, 6, 7 and 8 have important effects on intracellular localization. We also demonstrate that the transmembrane domain of syntaxin 5 is sufficient to localize the chimera to the compartment expected for wild-type syntaxin 5. Additionally, we find that syntaxins 6, 7 and 8, but not syntaxin 5, are present at the plasma membrane, and that these syntaxins cycle through the plasma membrane by virtue of their cytoplasmic domains. Finally, we find that di-leucine-based motifs in the cytoplasmic domains of syntaxins 7 and 8 are necessary for their intracellular localization and trafficking via distinct transport pathways. Combined, these results suggest that both the cytoplasmic and the transmembrane domains play important roles in intracellular localization and trafficking of syntaxins.

Key words: Syntaxin, Cytoplasmic domain, Transmembrane domain, Di-leucine-based motif, Intracellular localization, Antibody uptake experiment

INTRODUCTION

In eukaryotic cells, protein transport along the exocytotic and endocytotic pathways is mediated via various types of transport vesicles that transit between intracellular compartments. These transport vesicles bud from a donor membrane compartment and fuse with a target membrane compartment (Palade, 1975; Pryer et al., 1992; Rothman and Warren, 1994; Rothman and Wieland, 1996; Schekman and Orci, 1996). *N*-ethylmaleimide-sensitive factor (NSF; yeast homologue Sec18p) and soluble NSF attachment proteins (SNAPs; yeast homologue Sec17p) have been shown to participate in many different transport events (Clary et al., 1990; Graham and Emr, 1991; Griff et al., 1992). SNAP receptors (SNAREs) are thought to coordinate the actions of NSFs and SNAPs, thus directing the process of intracellular vesicle fusion (Rothman and Warren, 1994). This hypothesis predicts that the docking of transport vesicles to target membrane compartments is mediated by the specific pairing of vesicle-SNAREs (v-SNAREs; members of the vesicle-associated membrane protein (VAMP) or synaptobrevin family present on transport vesicles) with target-SNAREs (t-SNAREs; members of the syntaxin and SNAP-25 families present on target membrane compartments) (Bennett and Scheller, 1993; Söllner et al., 1993a; Söllner et al., 1993b; McNew et al., 2000; Parlati et al., 2000; Fukuda et al., 2000). The presence of many isoforms of t- and v-SNAREs that uniquely localize to distinct membrane compartments may provide a basis for the specificity of the docking step in different transport pathways (Bennett and Scheller, 1993).

Many members of the mammalian syntaxin family have been identified; each localizes to specific membrane compartments along the exocytotic and endocytotic pathways. The first group of syntaxins identified (syntaxins 1A (HPC-1), 2, 3 and 4) is predominantly restricted to the plasma membrane; these syntaxins mediate constitutive and regulated vesicle transport events at the cell surface (Inoue et al., 1992; Bennett et al., 1993; Gaisano et al., 1996; Low et al., 1996). By contrast, syntaxin 18 localizes to the endoplasmic reticulum (ER) (Hatsuzawa et al., 2000), syntaxins 5, 6, 10, 11 and 16 localize to different subcompartments within the Golgi apparatus (Dascher et al., 1994; Bock et al., 1997; Watson and Pessin, 2000; Tang et al., 1998a; Valdez et al., 1999; Tang et al., 1998c) and syntaxins 7, 8, 12 and 13 are found in the post-Golgi endosomal population (Wong et al., 1998; Nakamura et al., 2000; Prekeris et al., 1999; Subramaniam et al., 2000; Tang et al., 1998b; Prekeris et al., 1998). Because of the importance of t-SNAREs in docking and fusion of transport vesicles, the roles of syntaxins in intracellular localization must be established in order to understand the mechanism of transport specificity.

The functional domains of several syntaxins have been characterized with regard to their roles in intracellular localization. First, both the transmembrane and cytoplasmic

domains of Sed5p, a yeast homologue of syntaxin 5, which is required for transport of vesicles between the ER and the Golgi apparatus, have been shown to be important for localization to the cis-Golgi network (CGN) (Hardwick and Pelham, 1992; Banfield et al., 1994). Second, in mammalian cells, two isoforms of syntaxin 5 (42 kDa and 35 kDa) are believed to arise from the same gene through alternative translation initiation sites. The 42 kDa form has an N-terminal cytoplasmic extension containing a type II ER retrieval motif and localizes to the ER, whereas the 35 kDa form localizes to the CGN, as does Sed5p (Hui et al., 1997). Third, it has recently been shown that syntaxin 6 has two independent cytoplasmic regions that are responsible for its localization to the trans-Golgi network (TGN) (Watson and Pessin, 2000). One of the domains has a tyrosine-based motif that can function as a plasma membrane internalization signal (Bos et al., 1993; Humphrey et al., 1993; Banting and Ponnambalam, 1997; Bonifacino and Dell'Angelica, 1999). Finally, the yeast syntaxin Vam3p, which localizes to vacuoles, has a di-leucine-based motif in its cytoplasmic domain (Darsow et al., 1998). This motif is a sorting signal present in endosomal/lysosomal-targeting proteins (Darsow et al., 1998; Pond et al., 1995; Tang and Hong, 1999). However, the details of the mechanisms by which syntaxins localize to specific intracellular venues are poorly understood.

In this study, we used chimeric syntaxins to identify the syntaxin polypeptide domains that direct intracellular localization. We constructed chimeras combining the cytoplasmic and transmembrane domains of different syntaxins, and we compared their sites of intracellular localization to those of wild-type syntaxins.

MATERIALS AND METHODS

Materials and antibodies

Brefeldin A (BFA) (Sigma) was prepared as a 1 mg/ml stock solution in methanol, and used at a final concentration of 5 μg/ml. Monoclonal rat anti-hemagglutinin (HA) antibody (3F10) and monoclonal mouse anti-c-myc antibody (9E10) were purchased from Roche Diagnostics Corp. Polyclonal rabbit anti-β-COP antibody and monoclonal mouse anti-TGN38 antibody were purchased from Affinity Bioreagents, Inc. Monoclonal mouse anti-EEA1 antibody was purchased from Transduction Laboratories. Polyclonal rabbit anti-lamp-1 antibody was a kind gift from Kenji Akasaki (Fukuyama University, Hiroshima, Japan). All fluorescent secondary antibodies were obtained from Jackson Immunoresearch Laboratories.

cDNA cloning and plasmid construction

A cDNA encoding the full length of human syntaxin 1A was obtained by using the polymerase chain reaction (PCR) on total RNA isolated from the human neuroblastoma cell line NB-1. In addition, cDNAs encoding the full-length human syntaxins 5, 6, 7 and 8 were obtained by PCR of human liver and kidney cDNA libraries (Life Technologies Inc.). Although two splicing isoforms of syntaxin 5 exist, we obtained only the short (35 kDa) form, which localizes to the CGN. Primer sets were designed from sequences found in GenBank.

Chimeras were generated as follows. The cytoplasmic domain of syntaxin 1A was connected to the presumed transmembrane domain of syntaxin 5 (amino acids 280-301), 6 (amino acids 235-255), 7 (amino acids 237-261) or 8 (amino acids 215-236), resulting in syn1-5, syn1-6, syn1-7, and syn1-8, respectively. The transmembrane domain of syntaxin 1A (amino acids 266-288) was connected to the cytoplasmic domain of syntaxin 5, 6, 7 or 8, resulting in syn5-1, syn6-1, syn7-1 and syn8-1, respectively. Similarly, the transmembrane domain of syntaxin 5 was connected to the cytoplasmic domain of syntaxin 6, resulting in syn6-5.

QuickChange™ Site-Directed Mutagenesis (Stratagene) was used to generate mutations in the putative di-leucine-based motifs found at amino acids 162-168 of syntaxin 7 and at amino acids 77-83 of syntaxin 8. The syn7-mut mutation of syntaxin 7 contained Leu167-Ile168→Ala-Ala substitutions. The syn8-mut mutation of syntaxin 8 contained Leu82-Leu83→Ala-Ala substitutions.

For intracellular localization analysis, syntaxin cDNAs were subcloned into the pcDNA3-HAN vector (Shin et al., 1997) for expression in mammalian cells as fusion proteins in which the N-termini were fused to an HA tag. For antibody uptake experiments, syntaxin cDNAs were subcloned into the pcDNA3-myc3C vector for expression as fusion proteins in which the C-termini were fused to three c-myc tags.

Cell culture

PC12h cells (Watanabe et al., 1999) were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium containing 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% fetal calf serum and 5% horse serum. Rat Clone 9 hepatocytes were cultured at 37°C in 5% CO_2 in nutrient mixture F12 medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin, supplemented with 10% fetal calf serum.

Indirect immunofluorescence analysis

For electroporation, a 60-80% confluent culture of PC12h cells was trypsinized and 10^6 cells were resuspended with 10 μg DNA in 400 μl Dulbecco's modified Eagle's medium containing 4 mM L-glutamine. After incubation on ice for 10 minutes, the cells were transferred to a Bio-Rad Gene Pulser cuvette (0.4 cm electrode gap) and electroporated with one shock at 250 mV, 960 μF, using a Bio-Rad Pulse Controller (Bio-Rad Laboratories). The cells were then incubated on ice for 10 minutes, resuspended in serum-containing medium and plated on polyethylenimine-coated Lab-Tek II chamber slides (Nunc). After incubation for 24 hours, the cells were treated with 20 mM sodium butyrate for 24 hours (for intracellular localization analysis) or for 48 hours (for antibody uptake experiments). Rat Clone 9 cells grown on eight-well Lab-Tek II chamber slides were transfected using FuGene6™ transfection reagent (Roche Diagnostics Corp.) and incubated for 12 hours (for intracellular localization analysis) or 24 hours (for intracellular localization analysis and antibody uptake experiments).

The above cells were processed for indirect immunofluorescence analysis as described previously (Torii et al., 1995). Briefly, cells were fixed and permeabilized with methanol at –20°C for 5 minutes. In some experiments, cells were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. Where indicated, the cells were treated with 5 μg/ml BFA for 30 minutes or incubated with 8 μg/ml monoclonal mouse anti-c-myc antibody 9E10 for 3 hours before fixing. The fixed and permeabilized cells were incubated with monoclonal rat anti-HA antibody 3F10 and the indicated antibodies against organelle marker proteins, and were sequentially incubated with secondary antibodies for the intracellular localization analysis. For detection of c-myc-tagged syntaxins in antibody uptake experiments, fixed and permeabilized cells were incubated with FITC-conjugated anti-mouse IgG. The stained cells were observed with a confocal laser-scanning microscope (LSM 410 invert; Carl Zeiss).

RESULTS

Syntaxin cytoplasmic domains are important for intracellular localization

We first analyzed the intracellular localization of HA-tagged

wild-type syntaxins 5, 6, 7 and 8, each of which was transiently transfected into Clone 9 cells (Fig. 1). In agreement with previous studies, HA-tagged syntaxins 5 and 6 localized to the perinuclear Golgi regions, overlapping with CGN marker β-COP and TGN marker TGN38, respectively (Bock et al., 1997; Banfield et al., 1994; Hui et al., 1997). In studies of syntaxins 7 and 8, which have been previously shown to localize to endosomes (Wong et al., 1998; Nakamura et al., 2000; Prekeris et al., 1999; Subramaniam et al., 2000), we found that HA-tagged syntaxin 7 colocalized with EEA1 (a marker for early endosomes), but not with lamp-1 (a marker for late endosomes and lysosomes), whereas HA-tagged syntaxin 8 colocalized with both EEA1 and lamp-1. Similar localization patterns were also obtained in PC12h cells (Fig. 2).

Next, in order to identify the syntaxin domains responsible for specific intracellular localization, we generated chimeras that combined the cytoplasmic domain of syntaxin 1A with the transmembrane domain of syntaxin 5, 6, 7 or 8 (syn1-5, syn1-6, syn1-7 and syn1-8, respectively), and compared the intracellular localization of these chimeras with that of wild-type syntaxins. At first, we transfected syntaxin 1A into Clone 9 cells as a control for these chimeras. When syntaxin 1A was expressed in Clone 9 cells, it could not be localized at the plasma membrane (data not shown). Similarly, when syn1-6, syn1-7 and syn1-8 were expressed in Clone 9 cells, they had a distribution like that of wild-type syntaxin 1A (data not shown). However, in PC12h cells, syntaxin 1A could be localized at the plasma membrane (Fig. 2i). According to these findings, we used PC12h cells to analyze the intracellular localization of syntaxin 1A and the cytoplasmic domain of syntaxin-1A-containing chimeras. We consider that PC12h cells are not suitable for immunofluorescence analysis because their soma is too small to study with microscopy. Hence we mainly used Clone 9 cells except for intracellular localization analysis of wild-type syntaxin 1A and the cytoplasmic domain of syntaxin-1A-containing chimeras. For all the constructs of the syntaxins, we compared the intracellular localizations in PC12h cells and in Clone 9 cells (data not shown). When wild-type syntaxin 5 was expressed in PC12h cells, it was localized to the CGN (Fig. 2a). Similarly, when syn1-5 was expressed, it was also localized to the CGN (Fig. 2b). This result suggests that the transmembrane domain of human syntaxin 5 is sufficient for specific localization to the CGN, as previously shown for the yeast homologue Sed5p (Banfield et al., 1994).

In contrast to our result for syn1-5, syn1-6 localized to the plasma membrane when expressed in PC12h cells, like wild-type syntaxin 1A, although wild-type syntaxin 6 was localized to the TGN (Fig. 2c,d). Similar results were also obtained for cells expressing syn1-7 or syn1-8; in both cases, the chimeras were localized at the plasma membrane, whereas the wild-type syntaxins 7 and 8 were localized to endosomes (Fig. 2e-h). These results suggest that the cytoplasmic domains of syntaxins 6, 7 and 8 are important for their intracellular localization. Furthermore, these results suggest that the transmembrane domains of syntaxins 6, 7 and 8 are necessary for their transport to the plasma membrane but not for specific intracellular localization.

To examine further the role of the cytoplasmic domain, we generated chimeras in which the transmembrane domain of syntaxin 1A replaced the syntaxins' normal transmembrane domains, resulting in syn5-1, syn6-1, syn7-1 and syn8-1. We then determined the intracellular localization of these chimeras when expressed in Clone 9 cells (Fig. 3). When syn5-1 was expressed, it colocalized with β-COP as well as wild-type syntaxin 5. When syn6-1 was expressed, it colocalized with TGN38 as did wild-type syntaxin 6. Likewise, when syn7-1 and syn8-1 were expressed, they were localized to endosomes similar to wild-type syntaxins 7 and 8, respectively (compare Fig. 3 with Fig. 1). Similar results were also obtained in PC12h cells (data not shown). Therefore, these results indicate that both the transmembrane and cytoplasmic domains of syntaxin 5 are important for its intracellular localization, and that the cytoplasmic domains of syntaxins 6, 7 and 8 are important for their specific intracellular localization.

Immunofluorescence analysis of chimeras in BFA-treated cells

To confirm the role of the syntaxin 5 transmembrane domain, we generated chimeras containing the transmembrane domain of syntaxin 5, transfected these chimeras into Clone 9 cells and analyzed their intracellular localization. As shown in Fig. 4d, syn6-5 was found in the perinuclear Golgi region, as are syntaxin 5, syn5-1 and syn1-5 (PC12h cells) (Fig. 4a-c). Similar results were obtained in PC12h cells and for syn7-5 and syn8-5 (data not shown). Next, to examine whether both the cytoplasmic and transmembrane domains of syntaxin 5 contribute to its retention in the CGN, we analyzed the intracellular localization of syntaxin 5 chimeras in BFA-treated cells. Treatment with BFA causes rapid disassembly of proteins trafficking between the ER and the Golgi apparatus and redistributes these proteins into the ER (Lippincott-Schwartz et al., 1989). By contrast, BFA treatment causes membrane proteins resident in the TGN to disassemble and redistribute to the microtubule organizing center (MTOC) (Reaves and Banting, 1992). The effect of BFA on membrane flow can therefore be used to determine whether a protein of interest is associated with the Golgi complex or with the TGN. In Fig. 4, the intracellular localization of wild-type and chimeric syntaxins is shown in the absence (Fig. 4a-f) or presence (Fig. 4a′-f′) of BFA. Within 30 minutes of BFA treatment, syn5-1, syn1-5 and syn6-5 were redistributed into the ER, like wild-type syntaxin 5 (Fig. 4a′-d′), whereas syntaxin 6 and syn6-1 were redistributed to the MTOC (Fig. 4e′,f′). These results indicate that the cytoplasmic and transmembrane domains of syntaxin 5 are independently able to restrict exocytotic trafficking so that wild-type and chimeric syntaxin 5 proteins stay in the CGN and are not transported to the TGN.

Syntaxin 8 cycles through the plasma membrane

Syntaxin 7 is present not only in endosomes, but also at the plasma membrane, where it cycles through the plasma membrane (Prekeris et al., 1999). The cytoplasmic domain of syntaxin 6 has recently been shown to possess a tyrosine-based motif that can function as a plasma membrane internalization signal. Mutations or deletions in this motif in syntaxin 6 caused an increase in the amount of the syntaxin 6 found at the plasma membrane, indicating that syntaxin 6 also cycles through the plasma membrane (Watson and Pessin, 2000). Hence, we examined whether syntaxin 8 is also present at the plasma membrane, and whether it also cycles through the plasma membrane. Clone 9 cells were transfected with

syntaxin 5, 6, 7 or 8 and incubated for 12 hours for low-level expression (Fig. 5a-d) or 24 hours for overexpression (Fig. 5a′-d′). When syntaxin 5 was overexpressed, it localized only to the CGN (Fig. 5a′), confirming that the cytoplasmic and transmembrane domains of syntaxin 5 restrict its exocytotic trafficking to the CGN. By contrast, when syntaxins 6, 7 and 8 were overexpressed, they were found on the plasma membrane (Fig. 5b′-d′). This staining, although faint, was

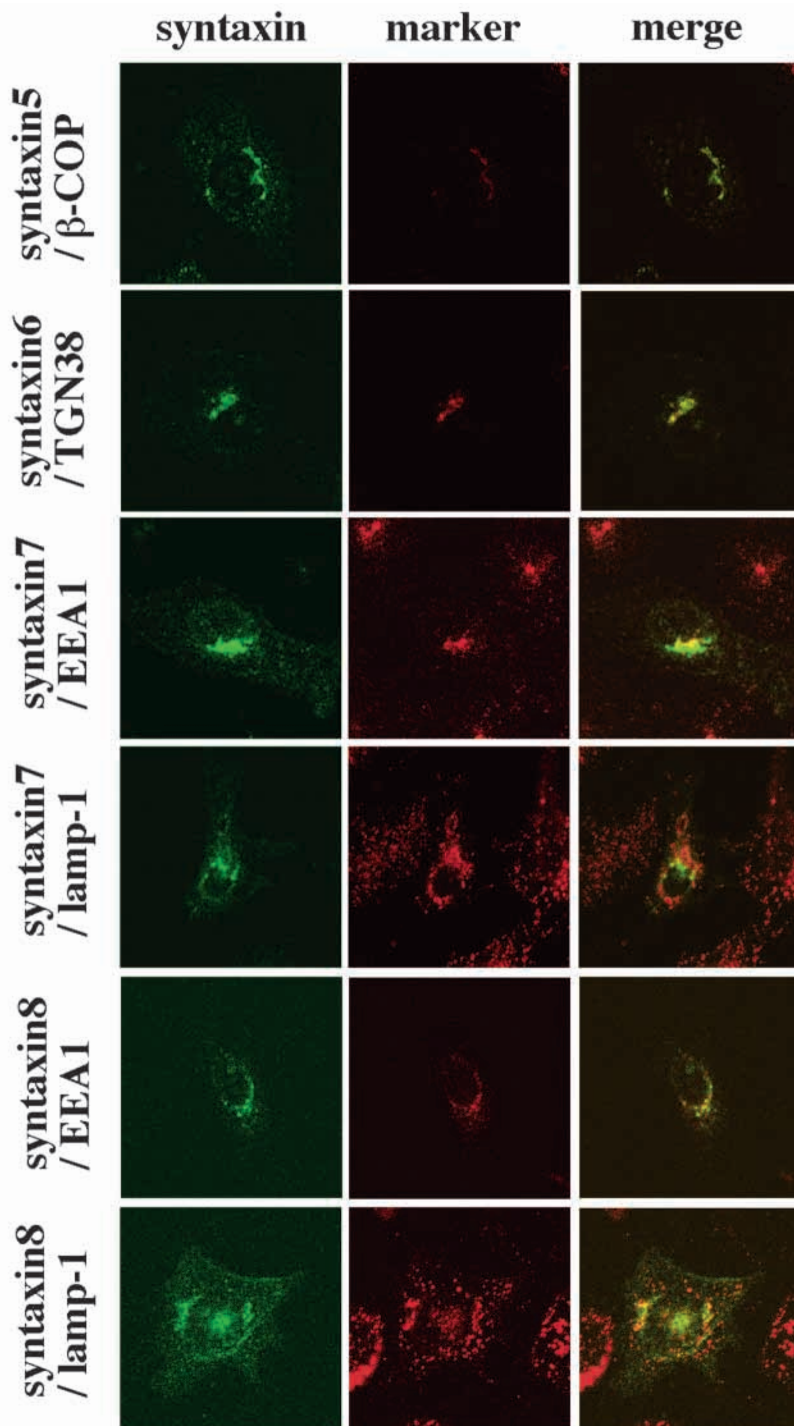


Fig. 1. Intracellular localization of wild-type syntaxins. Clone 9 cells were transfected with HA-tagged wild-type syntaxins 5, 6, 7 and 8 as described in Materials and Methods. After incubation for 12 hours, the cells were fixed and stained with anti-HA antibody and antibodies against indicated marker proteins. Syntaxins 5 and 6 overlapped with β-COP and TGN38, respectively. Syntaxin 7 was colocalized with EEA1 but not with lamp-1, and syntaxin 8 was partially colocalized with both EEA1 and lamp-1.

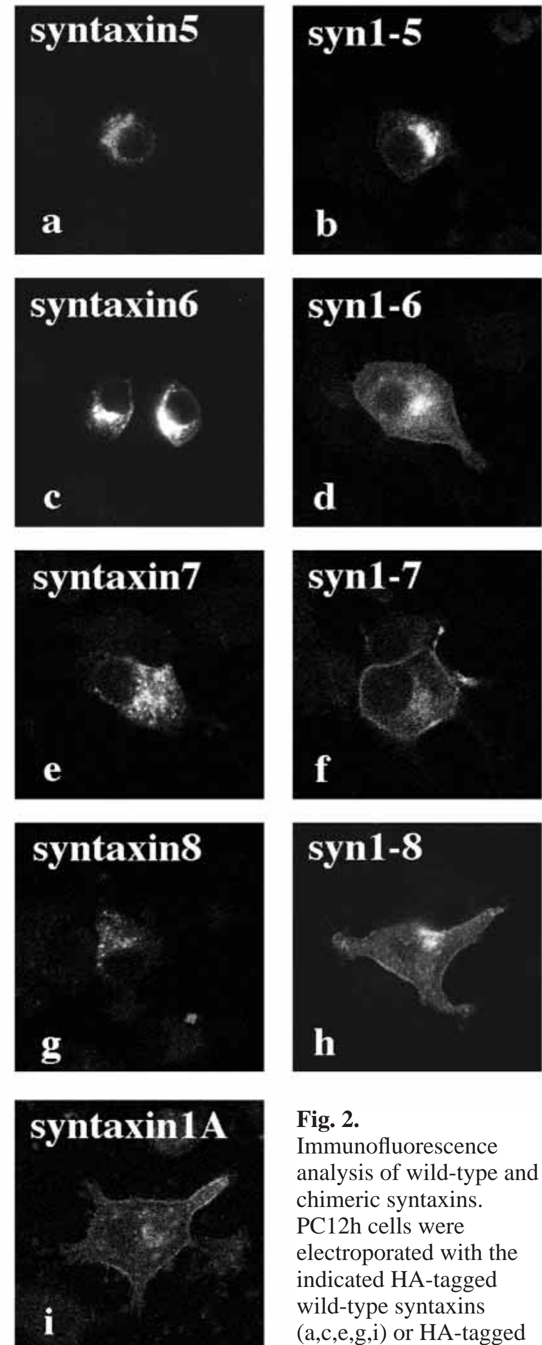


Fig. 2. Immunofluorescence analysis of wild-type and chimeric syntaxins. PC12h cells were electroporated with the indicated HA-tagged wild-type syntaxins (a,c,e,g,i) or HA-tagged syntaxin 1A chimeras containing the transmembrane domain of syntaxin 5, 6, 7 or 8 (b,d,f,h, respectively), as described Materials and Methods. After incubation for 24 hours, the cells were treated for an additional 24 hours with 20 mM sodium butyrate. The cells were fixed and stained with anti-HA antibody. Syn1-5 localized to the CGN, as did wild-type syntaxin 5. Other chimeras were localized at the plasma membrane, but did not localize to the same compartments as did their wild-type counterparts containing the same transmembrane domains.

found in low-expressing cells (Fig. 5b-d; arrows), suggesting that syntaxin 8 may cycle through the plasma membrane, as do syntaxins 6 and 7.

To assess this possibility, we carried out antibody uptake experiments to analyze the internalization of syntaxin 8. Because most syntaxins do not contain a large enough luminal domain for antibody binding, we transiently expressed wild-type and chimeric syntaxins in which the C-termini were fused to three c-myc tags. When these tagged proteins are inserted into a membrane, the c-myc tags situate within the lumen of the appropriate compartment (Chao et al., 1999). Thus, after exocytosis, the c-myc tags are exposed on the extracellular surface of cells so that they may interact with anti-c-myc antibody in the medium. Incubation of syntaxin-8-expressing cells, as well as syntaxin-6- or syntaxin-7-expressing cells, with anti-c-myc antibody resulted in efficient labeling of intracellular compartments (Fig. 6a,d,g). Antibody uptake was dependent on protein cycling through the plasma membrane, as cells expressing syntaxin 5 did not uptake the anti-c-myc antibody (data not shown). These results indicate that syntaxin 8 does cycle through the plasma membrane.

We also investigated whether both the cytoplasmic and transmembrane domains must be present in order for syntaxins 6, 7 and 8 to cycle through the plasma membrane. Incubation of anti-c-myc antibody with cells expressing syn6-1, syn7-1 or syn8-1 also resulted in efficient labeling of intracellular compartments (Fig. 6b,e,h, respectively). However, incubation of anti-c-myc antibody with cells expressing syn1-6, syn1-7 or syn1-8 resulted in labeling at the cell surface only (Fig. 6c,f,i, respectively), indicating that the cytoplasmic domains of syntaxins 7 and 8 have an important role in internalization from the plasma membrane, as well as syntaxin 6.

Di-leucine-based motifs have distinct roles in intracellular localization and trafficking of syntaxins 7 and 8

The above results suggest that the cytoplasmic domains of syntaxins 7 and 8 contain internalization signals. Di-leucine-based motifs are important sorting signals present in endosomal/lysosomal-targeting proteins and are typically composed of an acidic residue (aspartic acid or glutamic acid) followed by a pair of leucine residues (Darsow et al., 1998; Pond et al., 1995; Tang and Hong, 1999). Recently, it was shown that many syntaxins that localize to the TGN or to endosomes have putative di-leucine-based motif(s) in their cytoplasmic domains. Like these syntaxins, both syntaxins 7 and 8 contain a putative di-leucine-based motif in their cytoplasmic domains (Tang and Hong, 1999). Therefore, we examined the roles of these putative di-leucine-based motifs in syntaxins 7 and 8 by ascertaining the effects of mutations within these motifs. These wild-type and mutated motifs are shown in Fig. 7.

Clone 9 cells were transfected with the indicated mutant syntaxins and incubated for 12 hours (for low-level expression) or 24 hours (for overexpression and antibody uptake experiments). Cells expressing syn7-mut displayed intracellular localization similar to that of wild-type syntaxin 7, although syn7-mut was localized at the plasma membrane

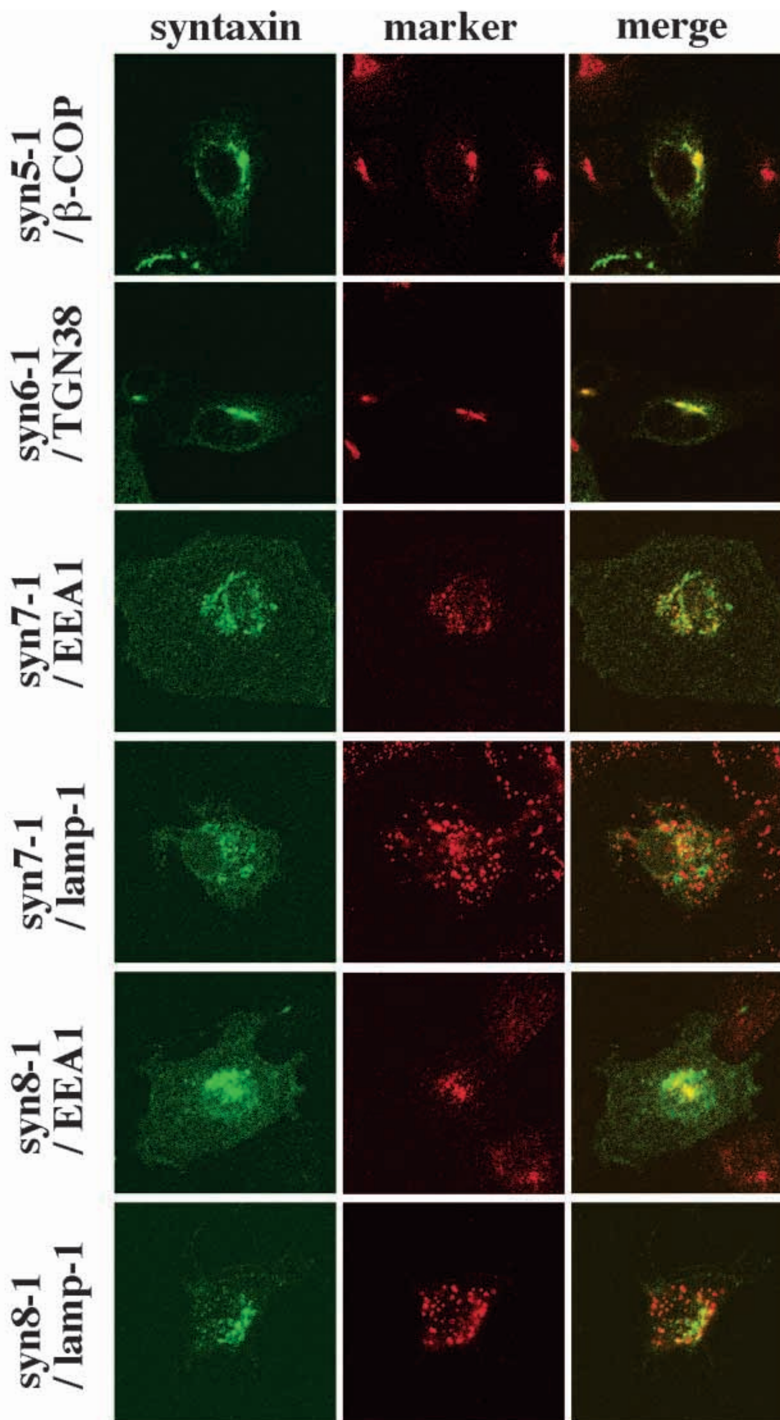
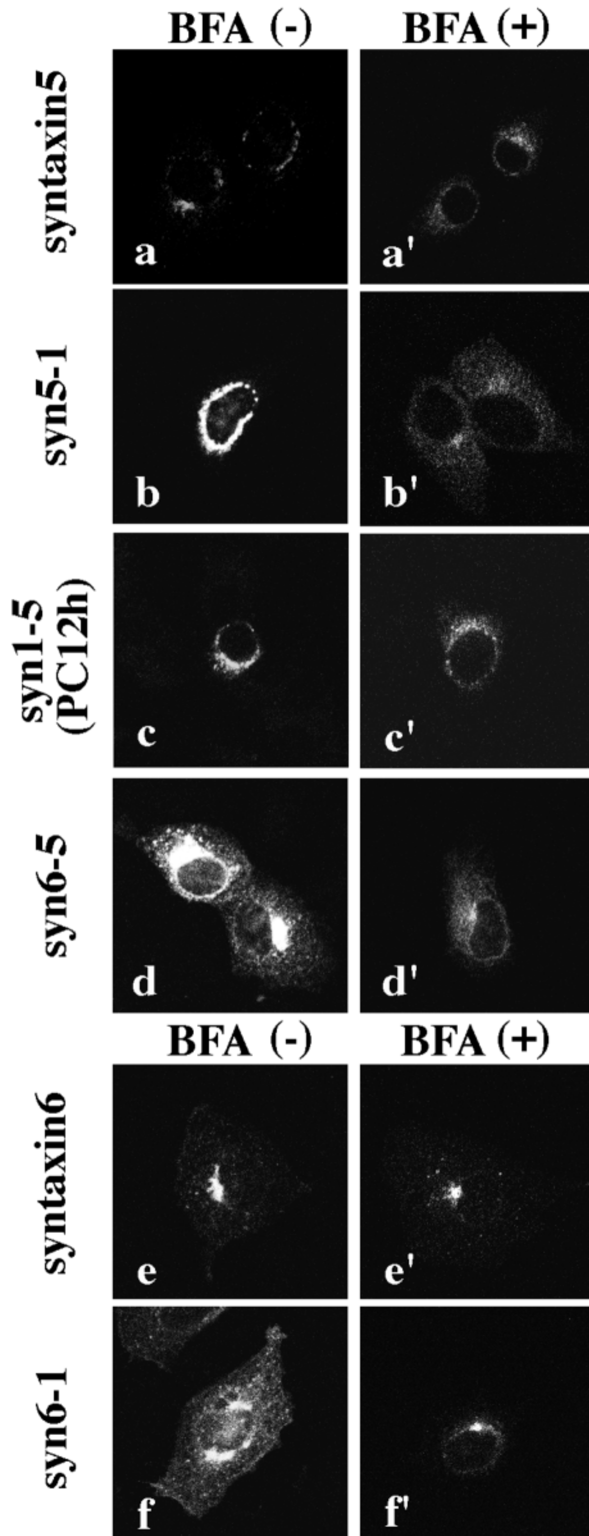


Fig. 3. The cytoplasmic domains are important for the intracellular localization of syntaxins. Clone 9 cells were transfected with HA-tagged chimeras composed of the transmembrane domain of syntaxin 1A and the cytoplasmic domain of either syntaxin 5, 6, 7 or 8. After incubation for 12 hours, the cells were fixed and stained with anti-HA antibody and antibodies against indicated marker proteins. Each chimera localized to the same compartment(s) as did its wild-type counterpart with the same cytoplasmic domain.



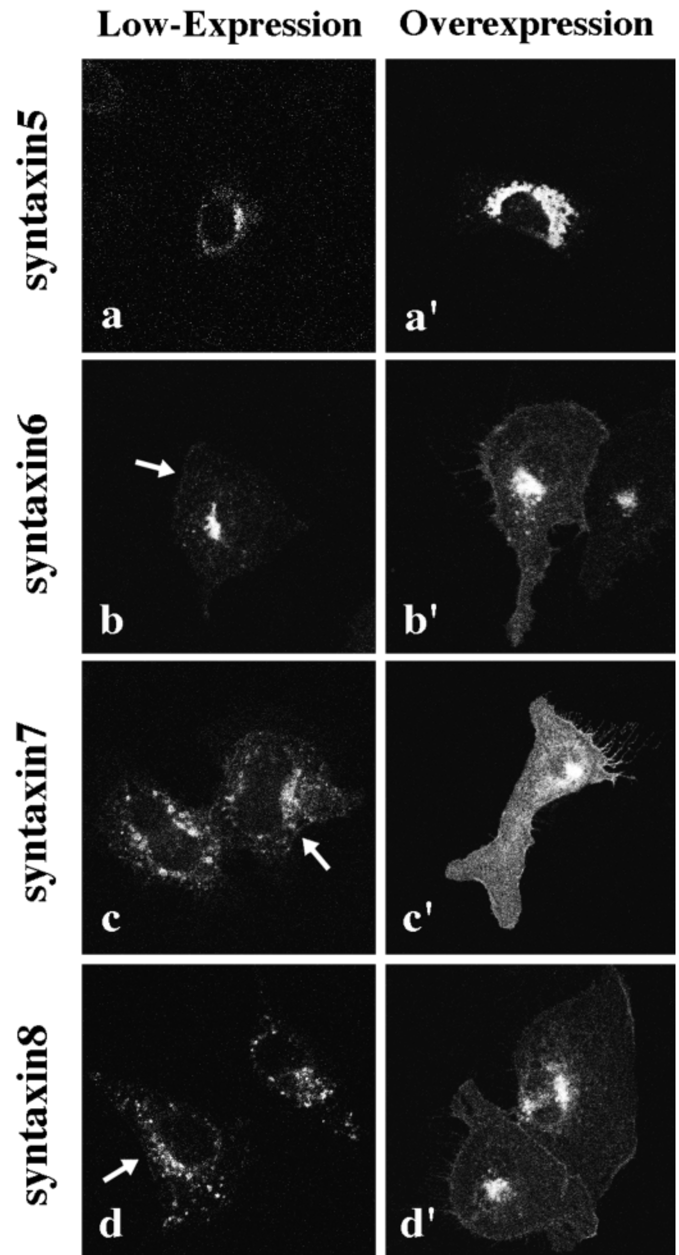


Fig. 5. Syntaxin 8 is present at the plasma membrane. Clone 9 cells were transfected with HA-tagged wild-type syntaxins and incubated for 12 hours for low-level expression (a-d) or 24 hours for overexpression (a′-d′) before fixing. The cells were stained with anti-HA antibody. In low-expression cells, syntaxins 6, 7 and 8 were faintly labeled at the plasma membrane (b-d; arrows) besides their specific intracellular localizations. In overexpressing cells, syntaxins 6, 7 and 8, but not syntaxin 5, were present at the plasma membrane.

Fig. 4. Immunofluorescence analysis of chimeric syntaxins in BFA-treated cells. Clone 9 cells were transfected with HA-tagged syntaxin 5 (a,a′), syn5-1 (b,b′), syn6-5 (d,d′), syntaxin 6 (e,e′), and syn6-1 (f,f′) and PC12h cells were transfected with HA-tagged syn1-5 (c,c′) in the absence (a-f) or presence (a′-f′) of BFA. After incubation for expression, the cells were treated with 5 μg/ml BFA for 30 minutes before fixing. The cells were stained with anti-HA antibody. In BFA-treated cells, chimeras containing either the cytoplasmic domain or the transmembrane domain of syntaxin 5 were redistributed from the CGN into the ER, as was syntaxin 5 (a′-d′). By contrast, in BFA-treated cells, syntaxin 6 and syn6-1 were redistributed from the TGN to the MTOC (e′,f′).

more effectively than wild-type syntaxin 7 in low-level expression (Fig. 7a,b). In antibody uptake experiments, however, cells expressing syn7-mut were labeled at the cell surface and were not labeled in any intracellular compartments (Fig. 7c). These results indicate that the putative di-leucine-based motif in the cytoplasmic domain of syntaxin 7 is important for its internalization.

When syn8-mut was expressed at low levels in Clone 9 cells, it was localized to the perinuclear Golgi region (Fig. 7d). Moreover, in overexpressing cells, this mutant was localized only to the perinuclear Golgi region and little was found at any intracellular compartments and the plasma membrane (Fig. 7e). Antibody uptake experiments for syn8-mut-expressing cells showed labeling neither at the cell surface nor at any intracellular compartments (Fig. 7f). These results were distinctly different from those observed for wild-type syntaxin 8, suggesting that this mutated motif is normally involved in the exocytotic trafficking of syntaxin 8 from the TGN. Furthermore, these results indicate that the di-leucine-based motifs in the cytoplasmic domains of syntaxins 7 and 8 have distinct roles in intracellular localization and trafficking.

DISCUSSION

In this study, we examined whether the cytoplasmic domain or the transmembrane domain of various syntaxins directs their intracellular localization. By using indirect immunofluorescence analysis of wild-type and chimeric syntaxins in Clone 9 cells and PC12h cells, we found that syntaxins 5, 6, 7 and 8, and syn5-1, syn6-1, syn7-1 and syn8-1 were localized to the CGN, TGN and endosomes in both Clone 9 cells and PC12h cells. However, syntaxin 1A was not localized to the plasma membrane when expressed in Clone 9 cells, but only when it was expressed in PC12h cells. Moreover, we found that the cytoplasmic domain of syntaxin 1A-containing chimeras (syn1-6, syn1-7 and syn1-8) were not localized at the plasma membrane and were distributed in the same way as wild-type syntaxin 1A in Clone 9 cells. We further surveyed their intracellular localization in MDCK and HeLa cells and found that there is no plasma membrane staining in these cell lines (data not shown). Although we could not conclude where syntaxin 1A and the cytoplasmic domain of syntaxin 1A-containing chimeras exist in Clone 9 cells, it is likely that the cytoplasmic domain of syntaxin 1A contains a signal, with which any factor(s) in PC12h cells interacts to localize syntaxin 1A to the plasma membrane.

We determined that both the cytoplasmic and transmembrane domains of syntaxin 5 are involved in its specific intracellular localization to the CGN. These results do not conflict with results shown for the yeast homologue Sed5p (Banfield et al., 1994). By contrast, we did not find any evidence that the transmembrane domain of syntaxin 6, 7 or 8 directs specific intracellular localization of these molecules. In Fig. 2d and Fig. 5b′, syn1-6 and wild-type syntaxin 6 seemed to be localized to the TGN as well as to the plasma membrane, so that the transmembrane domain of syntaxin 6 might contain a TGN-localization signal. However, if the transmembrane domain of syntaxin 6 is involved in its specific intracellular localization to the TGN, syntaxin 6 should be found only at the TGN but not at the plasma membrane even in overexpressing cells. We obtained apparent plasma membrane staining in syn1-6-expressing and wild-type syntaxin-6-expressing cells. Therefore, we considered that the transmembrane domain of syntaxin 6 has no or, if any, a weak role for its specific intracellular localization. We further demonstrated that the transmembrane domains of syntaxins 6, 7 and 8, but not of syntaxin 5, as such allow trafficking to the plasma membrane. These findings indicate that the transmembrane domain of syntaxin 5 does not permit escape

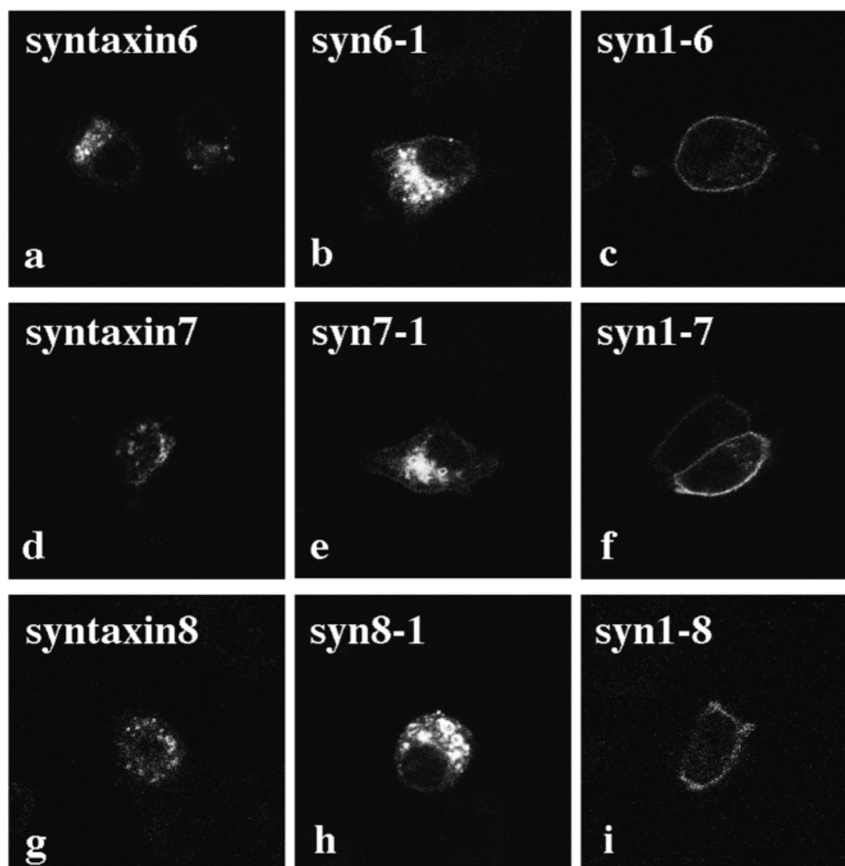


Fig. 6. The cytoplasmic domains of syntaxins 7 and 8 direct their internalization from the plasma membrane. PC12h cells were electroporated with c-myc-tagged wild-type syntaxins (a,d,g), transmembrane domain chimeras (b,e,h), or cytoplasmic domain chimeras (c,f,i), and were processed for antibody uptake experiments. After incubation for 24 hours, the cells were treated for an additional 48 hours with 20 mM sodium butyrate. The cells were incubated for 3 hours in the presence of mouse anti-c-myc antibody before fixing. The cells were fixed and stained with FITC-conjugated anti-mouse IgG. Incubation of wild-type syntaxin-expressing cells with anti-c-myc antibody resulted in efficient labeling of intracellular compartments. Similar results were obtained in cells expressing syn6-1, syn7-1 or syn8-1. However, incubation of cells expressing syn1-6, syn1-7 or syn1-8 with anti-c-myc antibody resulted in labeling only at the plasma membrane.

from the early transport pathway. One possibility for the retention of the transmembrane domain of syntaxin 5 would be the formation of oligomeric structures with itself and/or other resident proteins of the compartment. Alternatively, it has been proposed, although not yet proven, that the length of the transmembrane domain in membrane proteins determines their fate. In support of this proposal, proteins that localize to the ER and Golgi apparatus generally possess shorter transmembrane domains than do plasma membrane proteins (Letourneur and Klausner, 1992; Rayner and Pelham, 1997). Nonetheless, we were unable to find a correlation between intracellular localization of syntaxins and the length of their transmembrane domains. Therefore, a more precise studies are necessary to estimate the role of the transmembrane domains of syntaxins.

Here, we showed that the cytoplasmic domains of syntaxins 6, 7 and 8 can direct intracellular localization and internalization from the plasma membrane. Like wild-type syntaxins 6, 7 and 8, the chimeras syn6-1, syn7-1 and syn8-1 were shown to be localized either to the TGN or to endosomes and to cycle through the plasma membrane. However, when these cytoplasmic domains were combined with the syntaxin 5 transmembrane domain, producing syn6-5, syn7-5 and syn8-5, these chimeras were retained at the CGN rather than being transported to either the TGN or the endosomes. These results again indicate that the transmembrane domain of syntaxin 5 suppresses transport at the CGN, whereas the transmembrane domains of syntaxins 6, 7 and 8 allow transport to the plasma membrane. These results further suggest that the cytoplasmic domains of syntaxins 6, 7 and 8 can function as localization signals only when these proteins are delivered, at the least, to a post-CGN compartment. It seems likely that the role of syntaxin transmembrane domains is to constrain trafficking within the transport pathway, and the role of syntaxin cytoplasmic domains is to then determine the final localization fate of syntaxins.

To identify the localization and internalization signals in the cytoplasmic domains of syntaxins 7 and 8, we examined putative di-leucine-based motifs. Di-leucine-based motifs are thought to play a role in endocytosis (Pond et al., 1995; Letourneur and Klausner, 1992) and have been found to bind to the clathrin adapter complexes AP-1, AP-2 (Heilker et al., 1996) and AP-3 (Darsow et al., 1998; Höning et al., 1998). The AP-1 adapter complex mediates the formation of clathrin-coated vesicles at the TGN and is involved in protein transport from the TGN. By contrast, the AP-2 clathrin adapter complex mediates the formation of clathrin-coated vesicles at the plasma membrane and is involved in endocytosis (Pearse and Robinson, 1990). In these experiments, we showed that the putative di-leucine-based motif of syntaxin 7 (amino acids 162-168) is involved in its internalization (Fig. 7; Fig. 8). Therefore, it seems likely that this di-leucine-based motif binds to the AP-2 clathrin adapter complex and that syntaxin 7 internalizes from the plasma membrane via a clathrin-mediated pathway.

To examine the role of the putative di-leucine-based motif (amino acids 77-83) in the cytoplasmic domain of syntaxin 8, we determined the effect of mutating this motif on intracellular localization and internalization. In these experiments, we showed that syn8-mut was localized predominantly to the perinuclear Golgi region in overexpressing cells, but not to endosomes or to the plasma membrane (Fig. 7; Fig. 8). This result indicates that the di-leucine-based motif present at amino acid residues 77-83 is involved in trafficking syntaxin 8 from the TGN to the plasma membrane or endosomes, suggesting that this motif may bind to the AP-1 and AP-3 adapter complexes, respectively. The AP-1 clathrin adapter complex is known to distribute in the Golgi region, and it is involved in protein transport from the TGN via a clathrin-mediated pathway. The AP-3 adapter complex has also been shown to distribute in the Golgi region, and it is involved in protein

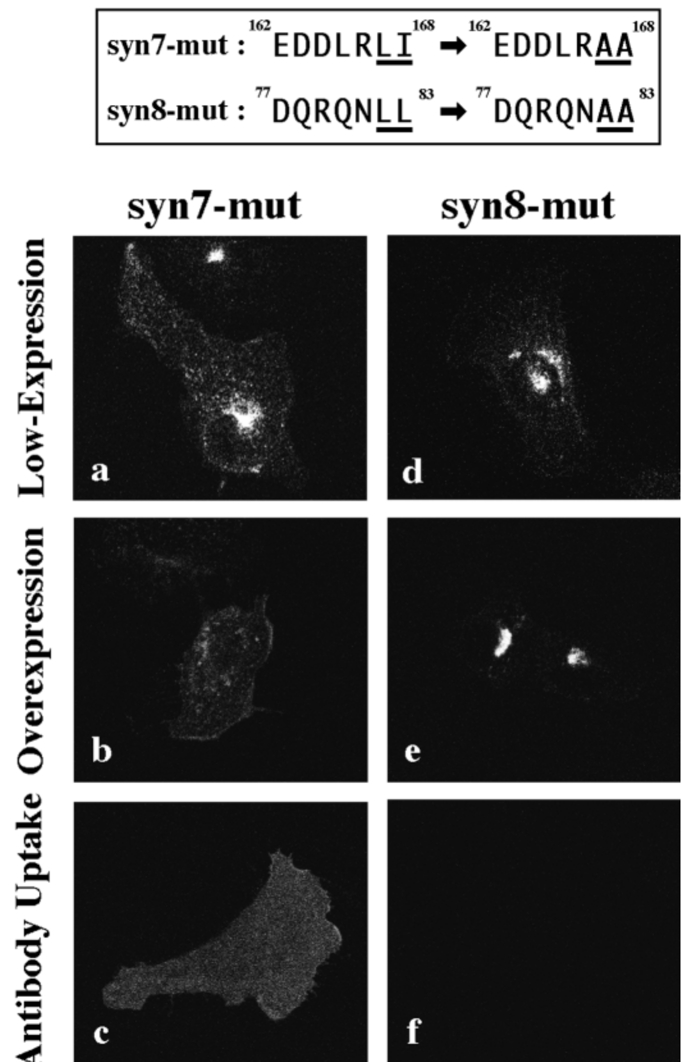


Fig. 7. Di-leucine-based motifs are important for the intracellular localization and trafficking of syntaxins 7 and 8. A putative di-leucine-based motif is present in the cytoplasmic domains of syntaxin 7 (amino acids 162-168) and syntaxin 8 (amino acids 77-83). Mutations in these motifs were made as indicated by underlining. Clone 9 cells were transfected with syn7-mut (a,b) or syn8-mut (d,e), each of which was fused to an N-terminal HA tag. For intracellular localization analysis, the cells were incubated for 12 hours (a,d) or 24 hours (b,e) and stained with anti-HA antibody. For antibody uptake experiments, Clone 9 cells were transfected with mutants in which the C-termini were fused to three c-myc tags (c,f). After 24 hours incubation, the cells were incubated for 3 hours in the presence of mouse anti-c-myc antibody before fixing. The cells were fixed and stained with FITC-conjugated anti-mouse IgG. (a-c) Syn7-mut was localized to endosomes and at the plasma membrane. In antibody uptake experiments, labeling was found only at the plasma membrane. (d-f) Syn8-mut was localized to the perinuclear Golgi region. No staining was observed in antibody uptake experiments.

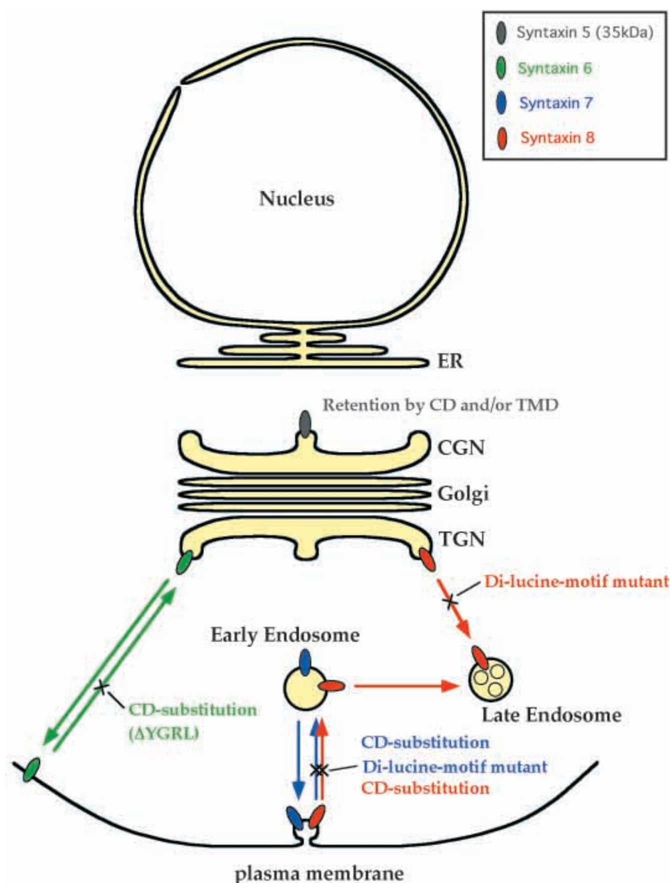


Fig. 8. Schematic representation of vesicular transport of syntaxins between intracellular compartments. The 35 kDa isoform of syntaxin 5 used in this study is retained at the CGN by virtue of either the cytoplasmic domain (CD) or the transmembrane domain (TMD). Substitution of the cytoplasmic domain (CD-substitution), but not the transmembrane domain, of syntaxins 6, 7 and 8 with that of syntaxin 1A prevents their internalization. The di-leucine-based motifs in the cytoplasmic domains of syntaxins 7 and 8 have important, but distinct, roles in intracellular localization. Mutation of the di-leucine-based motif of syntaxin 7 (amino acids 162-168) inhibited internalization of syntaxin 7. By contrast, mutation of the di-leucine-based motif of syntaxin 8 (amino acids 77-83) resulted in accumulation of syntaxin 8 at the perinuclear Golgi region. The transport pathways of syntaxins 5, 6, 7 and 8 are denoted by gray, green, blue and red, respectively.

transport from the TGN to endosomal/lysosomal compartments. It has also been reported that most syntaxin-8-positive membranes lack a clathrin coat, suggesting that syntaxin 8 travels to endosomes via a non-clathrin-mediated pathway (Prekeris et al., 1999). Whether the AP-3 complex associates with clathrin, as do the AP-1 and AP-2 complexes, has been a matter of much debate (Newman et al., 1995; Simpson et al., 1997; Dell'Angelica et al., 1997a; Dell'Angelica et al., 1997b; Dell'Angelica et al., 1998). It seems likely that the di-leucine-based motif at amino acid residues 77-83 of syntaxin 8 may bind to the AP-3 adapter complex for trafficking from the TGN, rather than to the AP-1 clathrin adapter complex.

As illustrated in Fig. 8, we have shown that the cytoplasmic domains of syntaxins 5, 6, 7 and 8 are important for intracellular localization. The 42 kDa syntaxin 5 isoform, which localizes to the ER, has an N-terminal cytoplasmic extension containing a type II ER retrieval motif (Hui et al., 1997). However, although the 35 kDa syntaxin 5 isoform used in this study has no identified signal motifs in its cytoplasmic domain, both the wild-type 35 kDa isoform and the syn5-1 chimera localized to the CGN. This discrepancy raises the possibility that the cytoplasmic domain of syntaxin 5 contains an unidentified intracellular localization signal for retention in the CGN. By contrast, we showed that syntaxin 8 cycles through the plasma membrane by virtue of its cytoplasmic domain. However, we could not find any internalization signal motifs in the cytoplasmic domain of syntaxin 8. A candidate for the plasma membrane internalization signal was the di-leucine-based motif at amino acids 77-83 of syntaxin 8. We showed instead that this motif is involved in trafficking syntaxin 8 from the TGN, whereas that of syntaxin 7 (amino acids 162-168) is involved in internalization. Therefore, there may be an unidentified internalization signal in the cytoplasmic domain of syntaxin 8. Alternatively, the di-leucine-based motif may function in both exocytotic and endocytotic pathways. Additional strategies for identifying signal motif(s) may be required to address these issues.

In conclusion, we have shown here that both the cytoplasmic and the transmembrane domains of syntaxins contribute to their specific localization. We suggest that TGN/endosomal syntaxins cycle through the plasma membrane. Moreover, our results indicate that the cytoplasmic domains of these syntaxins are important for their specific intracellular localization and internalization. In particular, di-leucine-based motifs appear to play distinct roles in intracellular localization and trafficking of syntaxins 7 and 8. Because many syntaxins may contain di-leucine-based motif(s) or other intracellular localization signal motif(s), further studies will enable us to understand better the mechanism of specific intracellular localization of syntaxins, as well as the specific pairing of SNARE proteins.

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