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Identification of the Carboxyl-Terminal Membrane-Anchoring Region of HPC-1/Syntaxin 1A with the Substituted-Cysteine-Accessibility Method and Monoclonal Antibodies

Kei Suga*[,1], Tetsuo Yamamori[2] and Kimio Akagawa[1]

[1]Department of Physiology, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611; and [2]Division of Speciation Mechanisms, National Institute for Basic Biology, Okazaki, Aichi 444-8585

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HPC-1/syntaxin 1A is a member of the syntaxin family, and functions at the plasma membrane during membrane fusion as the target-soluble *N*-ethylmaleimide–sensitive factor–attachment protein receptor (t-SNARE). We identified the membrane-anchoring region of HPC-1/syntaxin 1A, and examined its role in anchoring of a protein to the plasma membrane. A series of mutants was created from a cysteine-less mutant of HPC-1/syntaxin 1A by substitution of each residue at the C-terminus with cysteine. The accessibility of the thiol-groups in each mutant was analyzed *in vivo*. The cysteine (C145) within the N-terminal cytosolic segment was labeled, but not that at C271 or C272, or any of those introduced at the C-terminus. The addition of additional residues to the C-terminal tail of HPC-1/syntaxin 1A allowed labeling by thiol-specific reagents. A monoclonal antibody directed against the C-terminal tail peptide did not react with the protein located at the plasma membrane. In addition, subcellular fractionation and immunocytochemical analyses with various transmembrane mutants showed that the C-terminal tail comprising eight amino acids is essential for anchoring of HPC-1/syntaxin 1A to the plasma membrane. These results indicate that the C-terminal membrane-anchoring region, which comprises 23 amino acids, does not traverse the lipid-bilayer and that the C-terminal tail is essential for anchoring of HPC-1/syntaxin 1A to the plasma membrane.

Key words: HPC-1/syntaxin 1A, monoclonal antibody, plasma membrane, substituted-cysteine-accessibility method, tail-anchored protein.

Abbreviations: BM, 3-(*N*-maleimidylpropionyl)biocytin; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactoside; SCAM, substituted-cysteine-accessibility method; SM, 4-acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid; SNAP-25, 25-kDa synaptosomal–associated protein; SNARE, soluble *N*-ethylmaleimide–sensitive factor–attachment protein receptor; VAMP2, vesicle-associated membrane protein/synaptobrevin 2.

Syntaxins are target-soluble *N*-ethylmaleimide–sensitive factor–attachment protein receptors (t-SNAREs) that are involved in the intracellular trafficking of vesicles. Initially, HPC-1/syntaxin 1A was identified as a 35-kDa, neuronal-specific membrane protein (*1–3*). HPC-1/syntaxin 1A functions as a central component in neurotransmitter release at the presynaptic plasma membrane, where it forms complexes with other proteins, such as the 25-kDa synaptosomal-associated protein (SNAP-25) and vesicle-associated membrane protein 2 (VAMP/synaptobrevin 2). The structure and formation of these complexes have been studied extensively (reviewed in Ref. *4*). These proteins assemble into a four-stranded helical bundle (*5–8*) and form the synaptic vesicle membrane-fusion machinery, which is called the "SNARE complex" (*9*, *10*). It has been suggested that the assembly of this complex drives membrane fusion by drawing the vesicle and target membranes together (*11*, *12*), and that the transmembrane domains of VAMP2 and HPC-1/syntaxin 1A form part of a fusion pore to overcome the energetic barrier to lipid bilayer fusion (*13*, *14*). However, the mechanisms underlying fusion pore formation are not yet fully understood.

HPC-1/syntaxin 1A is classified as a tail-anchored protein with four α-helices (the Ha, Hb, Hc, and H3 domains) and a C-terminal hydrophobic region, which is assumed to be a single transmembrane region (Fig. 1A). Insertion of the C-terminal hydrophobic region of HPC-1/syntaxin 1A into membranes is thought to be required for regulating function by stabilizing SNARE complex formation and for membrane fusion. Several studies, concerning the predicted transmembrane domain, have shown that the C-terminal hydrophobic region plays important roles in protein-protein interactions and membrane fusion (*14–19*). However, the C-terminal membrane-anchoring region of HPC-1/syntaxin 1A has not been experimentally identified or precisely characterized. In order to better understand the process of membrane fusion during exocytosis, it is necessary to characterize the membrane-anchoring region of HPC-1/syntaxin 1A. Previously, we reported significant roles for the cytoplasmic and transmembrane

*To whom correspondence should be addressed. Tel: +81-422-47-5511, Fax: +81-422-47-4801, E-mail: ksuga@kyorin-u.ac.jp

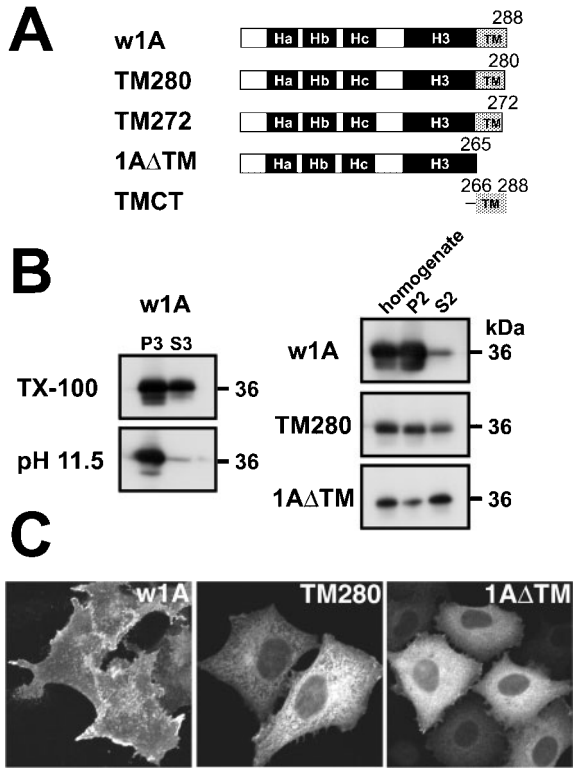

Fig. 1. **Subcellular fractionation and immunocytochemical analyses of the membrane-anchoring region of HPC-1/syntaxin 1A.** (A) Schematic representation of HPC-1/syntaxin 1A and its transmembrane (TM)-mutants. The Ha, Hb, Hc, and H3 domains of syntaxin are indicated in black. The putative transmembrane (TM) domain is shown in gray. (B) Homogenates, as well as membrane (P2) and cytosolic (S2) fractions were obtained from cells that expressed w1A (w1A-pcDNA3.1) and from the TM-truncation mutants. P2 fractions were suspended in PBS containing 1% Triton X-100 or 100 mM bicarbonate buffer (pH 11.5). Samples were extracted for 30 min, and then centrifuged at 100,000 ×*g* for 2 h to obtain supernatant (S3) and pellet (P3) fractions. Aliquots of 4 μg of each fraction were subjected to SDS–PAGE and Western blotting with the anti–HPC-1/syntaxin 1A polyclonal antibody. The w1A protein was integrated into membranes in an alkali-resistant manner, but was extracted with 1% Triton X-100 (B, left). The w1A protein was associated exclusively with the membrane fraction (P2 fraction). However, the amount of the TM280 mutant protein in the cytosolic fraction (S2 fraction) increased. Complete deletion of the membrane-anchoring region (1AΔTM) decreased the amount of protein in the membrane fraction, and increased the amount of protein in the cytosolic fraction. (C) HeLa cells were transfected with w1A or a TM-truncation mutant plasmid and then fixed after 24 h. The cells were then stained with a mAb (14D8) that recognizes the N-terminal region of HPC-1/syntaxin 1A. The w1A protein was localized to the plasma membrane. In contrast, TM280 was found predominantly in the cytosol and partially in the ER. The 1AΔTM mutant protein was only located in the cytosol.

domains of syntaxin family members in specific intracellular localization (*20*, *21*). HPC-1/syntaxin 1A is initially inserted into the endoplasmic reticulum (ER), and then targeted to the plasma membrane (*20*). We sought to clarify the role of the C-terminal hydrophobic region in anchoring of HPC-1/syntaxin 1A to the plasma membrane. Thus, we investigated the structural features of the membrane-anchoring region using the substituted-cysteine-accessibility method (SCAM). In addition, we produced monoclonal antibodies directed against the C-terminal tail peptide and used them to identify this region immunocytochemically. We also performed subcellular fractionation and imunocytochemical analyses with various truncation mutants to examine the role of this region in anchoring to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—4-Acetamido-4′-maleimidylstilbene-2, 2′-disulfonic acid (stilbenedisulfonate maleimide), and 3-(*N*-maleimidylpropionyl) biocytin (biotin maleimide) were purchased from Molecular Probes (Eugene, OR). The mouse monoclonal anti-myc antibody (9E10), rat monoclonal anti-HA antibody (3F10), and polyethylene glycol were purchased from Roche Diagnostics Corporation (Mannheim, Germany). Hemocyanin was from Sigma Chemical Company (St. Louis, MO). *M*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was from Pierce (Rockford, IL). TiterMax Gold was from CytRx Corporation (Norcross, GA). The complete and incomplete Freund's adjuvants were from Difco (Detroit, MI). All other reagents were of the highest grade available, unless otherwise noted. The rabbit polyclonal anti–HPC-1/syntaxin 1A and mouse monoclonal anti–HPC-1/syntaxin 1A (14D8) antibodies were prepared as described previously (*22*, *23*).

Plasmid Construction and Site-Directed Mutagenesis—A full-length cDNA fragment that encodes the gene for rat HPC-1/syntaxin 1A (*3*) was inserted into the pcDNA3.1 mammalian expression vector (Invitrogen, Groningen, Netherlands) to create w1A-pcDNA3.1 (w1A). For the construction of a cysteine-less mutant of HPC-1/syntaxin 1A (Cys-less), the cysteine residue at amino acid (aa) position 145 of w1A-pcDNA3.1 was substituted with an alanine by site-directed mutagenesis. A series of nine mutants (Fig. 2A) was created from Cys-less by substitution of the C-terminal residues with cysteine (1Acys-mutants; A281C-G288C) (Fig. 1A). A myc tag (SGREQKLISEEDLNGAA) was attached to the C terminus of HPC-1/syntaxin 1A, just upstream of the stop codon (1Amyc-pcDNA3.1). The cysteine residue at aa 145 of 1Amyc-pcDNA3.1 (1Amyc) was substituted with alanine to create a cysteine-less mutant of 1Amyc (1AmycCys-less) (Fig. 3A). Substitutions in 1AmycCys-less of the arginine residue at aa 291, and the alanine at aa 304 with cysteine led to the creation of R291C and A304C, respectively (1AmycCys-mutants; R291C and A304C). All of the mutations were generated with QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA), using appropriate synthetic oligonucleotides. In order to tag HPC-1/syntaxin 1A at the N terminus with HA tag (HA-1A), full-length HPC-1/syntaxin 1A cDNA was subcloned into the pcDNA3-HAN vector (*21*). Oligonucleotides 5′-GATCCACCATCGGGGGCATCTTTGGATAG-3′ and 5′-AATTCTATCCAAAGATGCCCCCGATGGTG-3′ were annealed into duplexes that were flanked by *Bam*HI- and *Eco*RI-compatible ends, and the resulting fragment was ligated into the *Bam*HI and *Eco*RI sites of the pGEX2TK vector to generate LCT-pGEX2TK. The HPC-1/syntaxin 1A cDNA fragment for amino acids 1–280 was produced by PCR using 5′-primers that con-

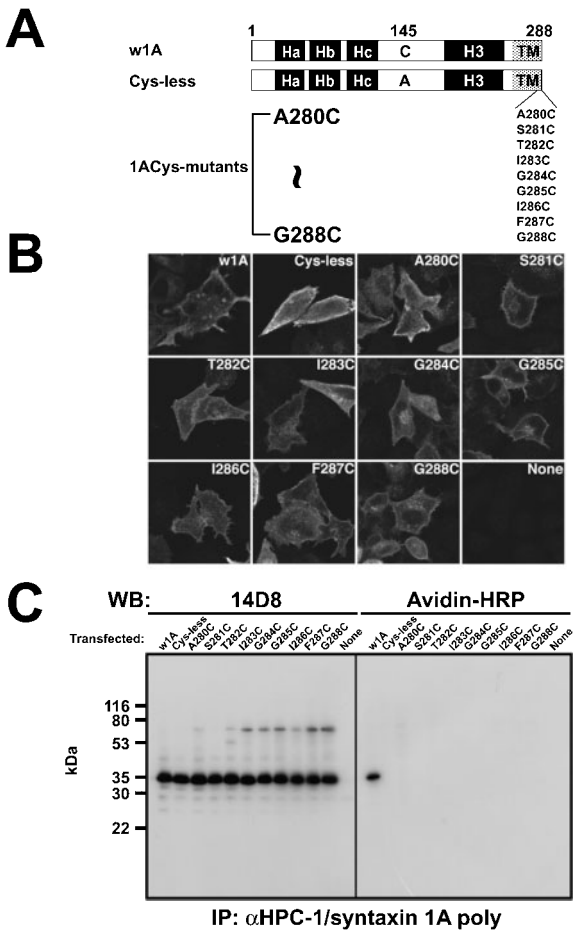

Fig. 2. **Labeling with biotin-maleimide of the 1ACys-less mutant, which contains an introduced Cys at the C-terminus of HPC-1/syntaxin 1A.** (A) Schematic representation of the wild-type HPC-1/syntaxin 1A (w1A) and cysteine-mutants. A series of nine mutants was created from a cysteine-less mutant (C145A) of HPC-1/syntaxin1A (Cys-less) by substitution of each residue within the C-terminus with cysteine (1ACys-mutants). (B) HeLa cells were transfected with the indicated mutated plasmids, fixed after 44 h, and then stained with the anti–HPC-1/syntaxin 1A polyclonal antibody. The presence of mutant proteins on the plasma membrane was confirmed by indirect immunofluorescence, as described in "EXPERIMENTAL PROCEDURES." (C) HeLa cells expressing mutant proteins were incubated with 100 μM BM. The cells were lysed, immunoprecipitated, and then processed for SDS–PAGE and Western blotting, as described in "EXPERIMENTAL PROCEDURES." A representative of four independent experiments is shown. The w1A protein was labeled with BM, but the 1ACys-mutant proteins (A280C-G288C) were not labeled (right panel), despite the fact that a considerable amount of each mutant protein was immunoprecipitated (left panel).

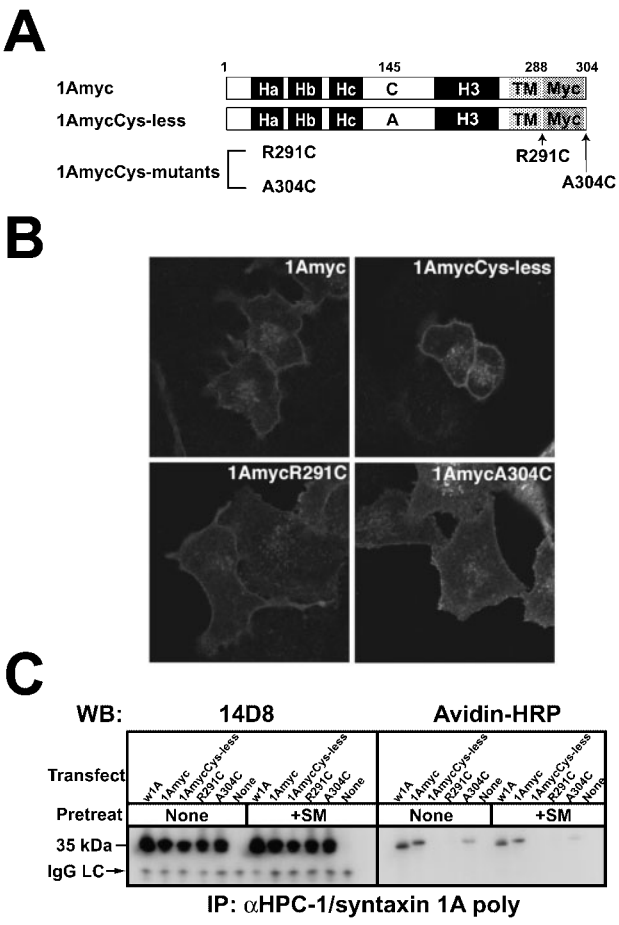

Fig. 3. **Biotin-maleimide labeling of the 1AmycCys-less mutant, which contained a substituted Cys residue at either the N- or C-terminus of the myc tag.** (A) Schematic representation of myc-tagged HPC-1/syntaxin 1A (1Amyc) and mutants thereof. The myc tag was introduced at the C-terminus of w1A as described in "EXPERIMENTAL PROCEDURES." Two mutants were created from the cysteine-less mutant of 1Amyc (1AmycCys-less) by substitution of residues at the junction and at the C-terminus of the myc tag (1AmycCys-mutants). (B) HeLa cells were transfected with the indicated mutant plasmids, fixed after 44 h, and then stained with the anti-HPC-1/syntaxin 1A polyclonal antibody. The presence of mutant proteins on the plasma membrane was confirmed by the indirect immunofluorescence method. (C) HeLa cells expressing mutant proteins were incubated in the presence or absence of 100 μM SM, washed, and subsequently labeled with 100 μM BM. The cells were lysed, immunoprecipitated, and then processed for SDS–PAGE and Western blotting. A representative of three independent experiments is shown. 1Amyc and A304C were labeled with BM, but the 1AmycCys-less and R291C mutant proteins were not labeled (right panel), despite the fact that a considerable amount of each mutant was immunoprecipitated (left panel). In addition, the labeling of A304C by BM was inhibited by pretreatment of the cells with SM. The addition of the C-terminal tag to HPC-1/syntaxin 1A allows labeling with BM, and inhibition of this labeling by SM.

tained an additional *Bgl*II site, and 3′-primers that contained an additional stop codon sequence and an *Eco*RI site just downstream of the codon for aa 280. The PCR product was digested with *Bgl*HI and *Eco*RI, and then cloned into the *Bam*HI and *Eco*RI sites of the pGEX2TK and pcDNA3.1 vectors to produce TM280-pGEX2TK and TM280-pcDNA3.1, respectively. Transmembrane-truncation mutants (TM272-pGEX2TK, TM272-pcDNA3.1, 1AΔTM-pGEX2TK, and 1AΔTM-pcDNA3.1) were constructed in a similar manner. The full-length cDNA of HPC-1/syntaxin 1A, and the fragment that encodes aa 266–288 were produced by PCR using 5′-primers that contained an additional *Bgl*II site, and 3′-primers that contained an additional *Eco*RI site just downstream of the stop codon. The PCR product was digested and inserted into the *Bam*HI and *Eco*RI sites of the pGEX2TK and pcDNA3.1 vectors to generate 1A-

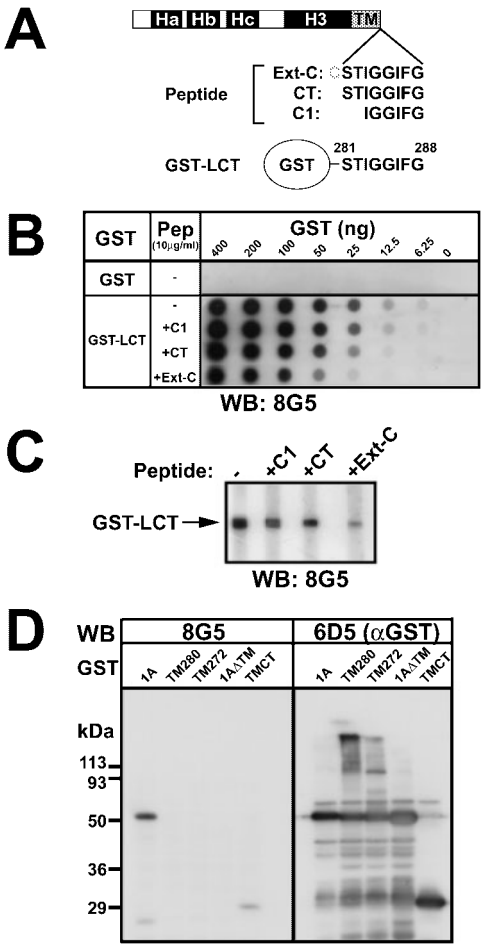

Fig. 4. **Production of a monoclonal antibody against the C-terminal tail of HPC-1/syntaxin 1A.** (A) Schematic representation of synthetic peptides and GST-fusion proteins of HPC-1/syntaxin 1A. The Ext-C peptide, which was conjugated to hemocyanin (KLH-Ext-C), and GST-LCT were used for immunization and the screening of hybridomas for appropriate antibodies. (B) Competition analysis of epitopes recognized by the antibody on dot-blot analysis under non-denaturing conditions. The indicated amounts of purified GST and GST-LCT were blotted onto Immobilon P membranes and then probed with mAb 8G5 IgG, following pre-incubation with the indicated peptides at a final concentration of 10 μg/ml. (C) Competition analysis of epitopes recognized by the antibody on Western blot analysis under SDS-denaturing conditions. Blotted membranes were probed with mAb 8G5 IgG, following pre-incubation with each peptide. The antibody recognized the N-terminal portion of the CT-peptide under both denaturing and non-denaturing conditions. (D) *E. coli* was transformed with each mutant and then induced with IPTG. The cells were sonicated, and lysates were prepared by sonication in cracking buffer [10 mM Na-phosphate (pH 7.0), 6 M urea, 1% SDS, 1% 2-mercaptoethanol]. The lysates were diluted and subjected to SDS–PAGE, followed by Western blotting with the indicated antibodies. Anti–C-terminal mAb 8G5 recognized the full-length HPC-1/syntaxin 1A fused to GST (1A) and the TM domain fused to GST (TMCT) expressed in *E. coli* (left panel). Mutants that lacked the C-terminal amino acids (TM280 and TM272) were not recognized by mAb 8G5 (left panel). The presence of each mutant protein on the blotted membrane was confirmed by the anti-GST mAb (6D5, right panel).

pGEX2TK and 1A-pcDNA3.1, and TMCT-pGEX2TK and TMCT-pcDNA3.1, respectively. The presence of the desired mutation and the identities of the inserted sequences were verified for all of the constructs, both by direct sequencing using ABI373A Sequencer with a BigDye filter (Applied Biosystems) and by digestion with appropriate restriction enzymes.

Expression and Purification of GST-Fusion Proteins—The HPC-1/syntaxin 1A cDNA fragments were cloned into the pGEX2TK vector (Amersham Biosciences, Uppsala, Sweden), and then transformed into *Escherichia coli* strain BL21 (Stratagene) to express the recombinant glutathione-*S*-transferase (GST) fusion proteins. The bacteria were grown in LB medium at 37°C in the presence of 100 mg/liter ampicillin, induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG), and then cultured for four hours at 37°C for GST-LCT, or overnight at 27°C for GST-1A, GST-TMCT, and the other transmembrane (TM)-truncation mutants (GST-TM280, -TM272, and –1AβTM). The bacteria were harvested by centrifugation, resuspended in cracking buffer [10 mM Na-phosphate, (pH 7.0), 6 M urea, 1% SDS, 1% 2-mercaptoethanol], and then sonicated to obtain the lysates that were used in the experiment shown in Fig. 4D. Lysates were prepared from the *E. coli* strains that were transformed with GST-LCT by sonicating the cells in lysis buffer (50 mM EDTA in PBS plus protease inhibitor cocktail). The lysates were extracted with 1% Triton X-100 and the cell debris was removed by centrifugation. GST-LCT was purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Biosciences), which was suspended in lysis buffer.

Production of Monoclonal Antibodies—An oligopeptide that corresponded to aa 281 to 288 of HPC-1/syntaxin 1A (Ext-C) was coupled *via* an additional N-terminal cysteine to hemocyanin (KLH-Ext-C) using cross-linking reagent MBS. KLH-Ext-C and GST-LCT were used for immunization. Six- to 8-week-old BALB/c mice were immunized with KLH-Ext-C by i.p. injection, initially in combination with complete Freund's adjuvant (50 μg protein/mouse), and 2 weeks later in combination with incomplete Freund's adjuvant (50 μg protein/mouse). GST-LCT emulsified with an equal volume of TiterMax Gold was also used for immunization. Spleen cells were fused with PAI mouse myeloma cells using polyethylene glycol 3 days after the final booster. The hybridomas were selected in HAT medium, and cultured in RPMI-1640 medium containing 20% fetal calf serum (FCS). Culture supernatants derived from the hybridoma cultures were screened by either ELISA, using the synthetic peptides or the recombinant proteins (Fig. 4A) as an immobilized ligand, or Western blotting. Positive hybridoma cells were cloned from a single colony that was derived by the limiting dilution method. Antibody classes were determined with Mouse Monoclonal Antibody Isotyping Kit RPN29 (Amersham Biosciences). Ascites fluid was purified from BALB/c mice that had been implanted intraperitoneally with the hybridoma. The IgG fraction was obtained by affinity chromatography on protein G-Sepharose 4FF (Amersham Biosciences). Anti-GST monoclonal antibody 6D5 was also generated during the production of the anti-GST-LCT antibodies.

Cell culture and Transfection—COS7 cells and PC12h cells were cultured at 37°C under 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM), which comprised 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml

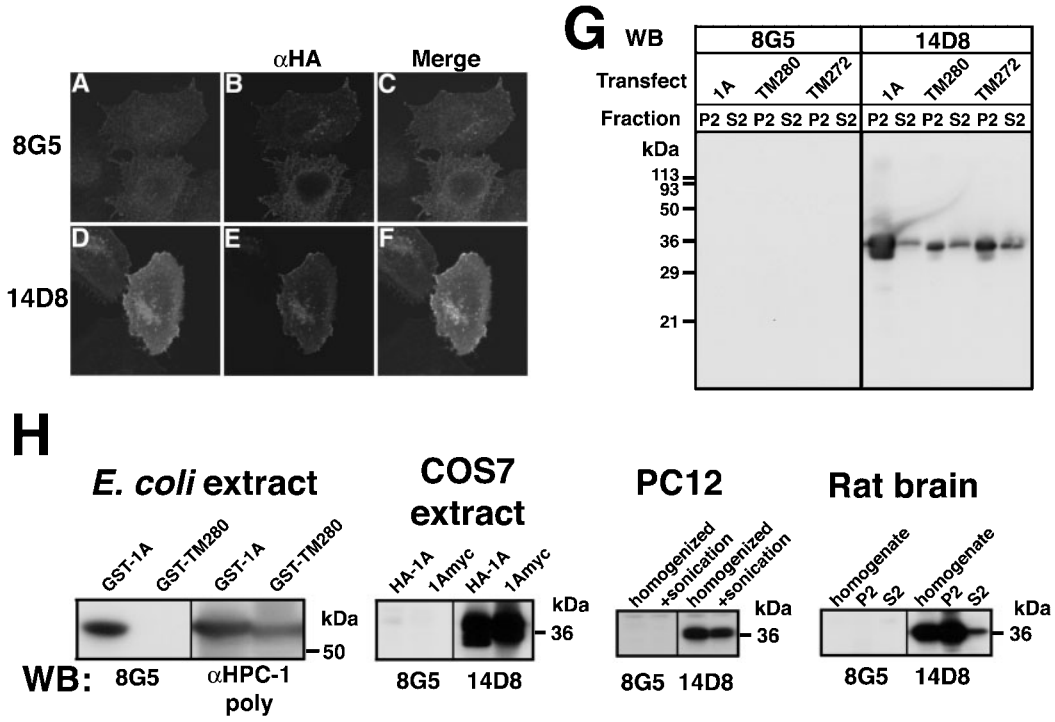

Fig. 5. **Immunocytochemical and Western blot analyses of the C-terminal tail of HPC-1/syntaxin 1A using monoclonal antibodies.** (A–F) Immunocytochemical analysis of the C-terminal tail of HPC-1/syntaxin 1A. HeLa cells were transfected with N-terminally HA-tagged HPC-1/syntaxin 1A (HA-1A), fixed after 24 h, and then stained with the indicated antibodies (A and D) together with the anti-HA antibody (B and E). The proteins showed identical localization with the anti-HA antibody and mAb 14D8, which recognizes the N-terminal portion of HPC-1/syntaxin 1A (D–F). However, mAb 8G5 did not react with HA-1A, which was located at the plasma membrane, unlike in the case with the anti-HA antibody (A–C). (G) HeLa cells were transfected with the indicated plasmids. The cells were lysed, fractionated, and then subjected to SDS–PAGE, followed by Western blotting using the indicated antibodies. Unexpectedly, mAb 8G5 did not react with the C-terminal tail of HPC-1/syntaxin 1A expressed in HeLa cells. (H) Western blot analyses were performed with native HPC-1/syntaxin 1A from PC12 cells, rat brain, and COS7 cells transfected with the indicated plasmids. Subcellular fractionation and extraction were carried out as described in "EXPERIMENTAL PROCEDURES." In contrast to mAb 14D8, mAb 8G5 did not react with the C-terminal tail of HPC-1/syntaxin 1A expressed in either PC12 cells, rat brain, or transfected COS7 cells, despite its reactivity with HPC-1/syntaxin 1A expressed in *E. coli*.

streptomycin, and 10% FCS. HeLa cells were cultured in DMEM, which comprised 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, non-essential amino acids, and 10% FCS. On the day before transfection, the HeLa cells were inoculated into a 6-well plate for the labeling experiment and into a 35-mm dish with an attached coverslip for immunocytochemical analysis. HeLa and COS7 cells were transfected using FuGene6 Transfection reagent (Roche Diagnostics) with 2 μg of plasmid DNA encoding either the wild-type or mutant HPC-1/syntaxin 1A.

Labeling with Biotin Maleimide—Labeling assays were performed as described by Loo and Clarke (*24*) with minor modifications. The cells were cultured for 40–44 h after transfection, and then washed three times with PBSCM (PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$). The cells were pre-incubated with stilbenedisulfonate maleimide (SM) in PBSCM at 37°C for 30 min for blocking, washed three times with PBSCM, and then biotinylated by incubation with 100 μM biotin maleimide (BM) in PBSCM. BM was stored in dimethylsulfoxide (DMSO). The concentration of DMSO in the labeling medium was less than 1% (v/v). After incubation for 30 min at 37°C, the cells were washed three times with PBSCM and once with PBS.

Extract Preparation—Cells that had been transfected with the TM-truncation mutant plasmids were harvested with a cell scraper, recovered by centrifugation, lysed in hypotonic buffer [10 mM Tris-HCl, 0.5 mM EDTA, (pH 7.4)] containing a protease inhibitor cocktail (Wako Chemicals, Osaka), and then sonicated for 30 s. An equal volume of 2× PBS-NPTX (2× PBS containing 0.4% Nonidet P-40 and 0.4% Triton X-100) was added, followed by shaking at room temperature for 2.5 h. Insoluble material was removed by centrifugation at 15,000 ×*g* for 10 min, and the supernatant was used as the extract sample.

Subcellular Fractionation—HeLa cells, PC12h cells, and rat brain were homogenized in a buffer comprising 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and a protease inhibitor cocktail (Wako Chemicals) using a Teflon homogenizer. Each homogenate was centrifuged at 800 ×*g* for 10 min, and the resulting supernatant (S1) was centrifuged for a further 2 h at 100,000 ×*g* in a Beckman TLA45 rotor (S2). The membrane fraction was obtained by suspending the pellet in homogenization buffer (P2). The protein concentration was determined by

the Bradford method, using bovine serum albumin (BSA) as the standard.

Immunoprecipitation, SDS–PAGE, and Western Blotting—Extracts of wild-type and mutant cells expressing derivatives of HPC-1/syntaxin 1A were immunoprecipitated with the rabbit polyclonal anti-HPC-1/syntaxin 1A antibody. The immunocomplexes were precipitated with protein G-Sepharose (Amersham Biosciences), and then solubilized in SDS sample buffer at 95°C for 5 min. Samples were subjected to SDS–PAGE according to the method of Laemmli (*25*), and then transferred onto Immobilon P membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skimmed milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature, and subsequently labeled with the monoclonal mouse anti-HPC-1/syntaxin 1A (14D8) antibody for 1.5 h. After washing in PBS-T, the blots were labeled with horseradish peroxidase–conjugated goat anti–mouse IgG (Vector Laboratories, Burlingame, CA) for 30 min. Biotinylated proteins were detected using avidin-conjugated horseradish peroxidase (Vector). Immunoreactive bands were visualized on X-ray film using enhanced chemiluminescence reagents (ECL; Amersham Biosciences). Each assay was carried out for at least three times.

Immunocytochemistry—HeLa cells were transfected with pcDNA3.1 plasmids containing either the wild-type or a mutant HPC-1/syntaxin 1A, and then cultured for 24 h. The cells were washed with PBS and then fixed with 3.7% paraformaldehyde in PBS for 15 min. After fixation, the cells were permeabilized with 0.1% Triton X-100 in PBS containing 5% normal goat serum (NGS-PBSTX). The cells were then incubated with 5 μg/ml of the anti-HPC-1/syntaxin 1A antibody in NGS-PBSTX for 1.5 h at room temperature. After this incubation, the cells were washed and incubated for 1 h with either the rhodamine-labeled anti–rabbit IgG antibody (Cappel, Aurora, OH) or the Alexa Flour 488–conjugated anti–mouse IgG antibody (Molecular Probes). The mouse monoclonal anti–c-myc antibody (9E10) and the rat monoclonal anti-HA antibody (3F10) were used for the detection of myc-tagged and HA-tagged HPC-1/syntaxin 1A constructs, respectively. Cy3-labeled anti–rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) was used as the secondary antibody for the anti-HA antibody. The stained cells were observed with a confocal laser-scanning microscope (LSM 410, Carl Zeiss). Images were collected as TIFF files and processed using Adobe Photoshop software (Mountain View, CA).

RESULTS

The C-Terminal Tail of HPC-1/Syntaxin 1A is Important for Integration into the ER and for Localization to the Plasma Membrane—Previously, we reported that HPC-1/syntaxin 1A was initially inserted into the endoplasmic reticulum (ER), and was targeted subsequently to the plasma membrane *via* a secretory pathway (*20*). If the C-terminal tail is important for anchoring to membranes, truncation of the C-terminus of HPC-1/syntaxin 1A should lead to failure of integration into the ER and accumulation in the cytosol. Therefore, we examined the role of the C-terminal tail in the anchoring to membranes.

The wild-type HPC-1/syntaxin 1A (w1A) of 35 kDa was present in the homogenate of transfected HeLa cells. On the other hand, no 35-kDa cross-reactive protein was detected in the homogenate of untransfected HeLa cells (data not shown), which indicates that HeLa cells lack endogenous HPC-1/syntaxin 1A. TM-truncation mutants of HPC-1/syntaxin 1A (Fig. 1A) were expressed in mammalian cells, and then insertion of these mutant proteins into membranes was examined by subcellular fractionation and immunocytochemical studies. The w1A protein was localized to the plasma membrane (Fig. 1C, left panel), and associated exclusively with the membrane fraction (P2 fraction; Fig. 1B, upper right panel). As shown in the left panel of Fig. 1B, there was a tight association between w1A and the membrane, since w1A could be extracted from the P2 fraction with Triton X-100, but not with bicarbonate buffer (pH 11.5). In contrast, the proportions of the TM280 and 1AΔTM mutant proteins in the cytosol fraction (S2 fraction) increased (Fig. 1B, right panel). However, the TM280 mutant protein was absent from the plasma membrane, but was present mainly in the cytosol, and partially in the ER (Fig. 1C, middle panel). Co-localization of TM280 with an ER-marker (SERCA2; sarco/endoplasmic reticulum Ca^{2+}-ATPase 2) was observed when the double-immunofluorescence method was used (data not shown). Complete removal of the membrane-anchoring region (1AΔTM mutant) resulted in localization of the protein to the cytosol as reported (Fig. 1C, right panel) (*20*). These results suggest that the C-terminal tail is important for the initial integration into the ER membrane and the subsequent targeting of HPC-1/syntaxin 1A to the plasma membrane. Thus, in order to clarify the role of the C-terminal tail in anchoring HPC-1/syntaxin 1A to the plasma membrane, we investigated the structural features of the region using the substituted-cysteine-accessibility method (SCAM).

The C-Terminal Tail of HPC-1/Syntaxin 1A Does Not Traverse the Lipid-Bilayer—Wild-type HPC-1/syntaxin 1A contains three intrinsic cysteine residues at aa 145, 271, and 272. The cysteine at aa 145 is assumed to be cytoplasmic, and the other two cysteine residues are predicted to be located in the lipid-bilayer. It has been suggested that the cysteines at aa 271 and aa 272 might affect cytoplasmic protein-protein interactions and the modulation of membrane channels (*17*, *26*). Accordingly, the naturally occurring cysteine residue at aa 145 of w1A was substituted with an alanine residue to produce a cysteine-less mutant of HPC-1/syntaxin 1A (Cys-less). We used Cys-less as the prototype to create cysteine mutants of the C-terminal tail of w1A. As illustrated in Fig. 2A, each of the nine residues within the C-terminus was changed to a cysteine residue by oligonucleotide-mediated site-directed mutagenesis. Thus, we produced a series of nine HPC-1/syntaxin 1A mutants (1ACys-mutants; A281C-G288C). Expression of the Cys-less protein on the plasma membranes of HeLa cells was assessed by indirect immunofluorescence using the anti-HPC-1/syntaxin 1A polyclonal antibody (Fig. 2B). As was seen in the case of w1A (Figs. 1C and 2B), the nine mutated proteins were located in the plasma membrane. Single amino acid substitutions at the C-terminus of HPC-1/syntaxin 1A did not affect the intracellular locali-

zation, which indicates that these mutants can be used for *in vivo* labeling experiments.

In vivo labeling experiments were performed to identify the putative transmembrane residues that were exposed to the exoplasmic space. The w1A, Cys-less, and nine Cys-mutants were cultured for 40–44 h after transfection. The cells were then incubated with membrane-permeable sulfhydryl-specific reagent biotin maleimide (BM). The cells were lysed and immunoprecipitated, and then the efficacy of labeling was assessed by Western blotting. BM (100 μM) was used to label the cysteine residues (C145) in the cytosol (*24*). The w1A strain, but not the Cys-less mutant, was labeled with BM, which indicated a specific modification of the cysteine residues by BM (Fig. 2C, right panel). In addition, the absence of biotinylation of intrinsic cysteine residues (C271 and C272) in the Cys-less mutant revealed that this region was hidden in the lipid bilayer. Although significant amounts of the 35-kDa mutant protein were immunoprecipitated by the anti-HPC-1/syntaxin 1A polyclonal antibody (Fig. 2C, left panel), the 1ACys-mutants (A280C-G288C) were not labeled by BM. Thus, the C-terminus of HPC-1/syntaxin 1A is probably embedded in the lipid-bilayer, in the same way as the region that contains C271 and C272. The cysteine (C145) in the putative cytoplasmic domain of w1A was not labeled when pretreated with membrane-impermeable sulfhydryl-specific reagent stilbene maleimide (SM) (data not shown). Next, we constructed several mutants tagged with the myc epitope at the C-terminus of HPC-1/syntaxin 1A (1Amyc; Fig. 3A) to see if the added amino acids would: (a) make HPC-1/syntaxin 1A accessible to the external aqueous milieu; (b) allow labeling with BM; and (c) lead to inhibition of labeling by membrane-impermeable sulfhydryl-specific reagent, SM. The myc tag was introduced at the C-terminus of w1A as described in "EXPERIMENTAL PROCEDURES." As shown in Fig. 3A, two mutants were created from the Cys-less mutant of 1Amyc (1AmycCys-less) by substitution of individual residues at the junction of the C-terminus of HPC-1/syntaxin 1A and the myc tag, or at the C-terminus of the myc tag (1ACys mutants R291C and A304C, respectively). The presence of these mutant proteins on the plasma membranes of HeLa cells was confirmed by indirect immunofluorescence (Fig. 3B). Cells that expressed the mutant proteins were incubated in the presence or absence of 100 μM SM, washed, and subsequently labeled with 100 μM BM. As shown in Fig. 3C (right panel), 1Amyc and A304C were labeled with BM, but the 1AmycCys-less and R291C mutants were not, despite the fact that considerable amounts of protein from these mutants were immunoprecipitated by the polyclonal anti-HPC-1/syntaxin 1A antibody (Fig. 3C, left panel). No labeling was observed with the 1AmycCys-less mutant and, more surprisingly, the R291C mutant was not labeled. This indicates that when at least three additional amino acids are ligated to the C-terminus of HPC-1/syntaxin 1A they are likely to be sequestered in the membrane lipids. In addition, the labeling of A304C by BM was inhibited by pretreatment with 100 μM SM, which indicates that the C-terminus end of myc-tagged HPC-1/syntaxin 1A was exposed to the extracellular space. Taken together with the results shown in Fig. 2, these results suggest that the C-terminal membrane-anchoring region of HPC-1/syntaxin 1A does not traverse the lipid-bilayer, but is embedded in the plasma membrane.

Analysis of the C-Terminal Tail of HPC-1/Syntaxin 1A with Monoclonal Antibodies—We adopted an alternative approach to further clarify the results of the *in vivo* labeling experiments. We generated monoclonal antibodies (mAbs) against a synthetic peptide that comprised the 8 C-terminal amino acids of HPC-1/syntaxin 1A. Fusions of this peptide with carrier proteins KLH and GST (Fig. 4A) were used for immunization. We obtained four independent hybridoma clones that produced antibodies against the C-terminus of the protein (data not shown). Of these, mAb 8G5 recognized the N-terminal portion of the CT peptide (Fig. 4A) under both denaturing and non-denaturing conditions (Fig. 4, B and C, respectively), as revealed by competitive analysis with a series of synthetic peptides.

To further establish the specificity of mAb 8G5, various transmembrane (TM)-mutants of HPC-1/syntaxin 1A (Fig. 1A) were generated as GST-fusion proteins in *E. coli*. GST-1A, GST-TM280, GST-TM272, GST-1AΔTM, and GST-TMCT migrated at the expected molecular weights of 60, 59.3, 58.5, 57.8, and 29.3 kDa, respectively, when probed with the anti-GST mAb 6D5 (Fig 4D, right panel). On the other hand, mAb 8G5 recognized GST-1A and GST-TMCT migrated at the expected molecular weights of 60 and 29.3 kDa, respectively (Fig 4D, left panel). TM-truncation mutants that lacked the C-terminal amino acids, *i.e.*, GST-TM280, -TM272, and –1AΔTM, were not recognized by mAb 8G5. These results demonstrate the specificity of mAb 8G5 for the C-terminus of HPC-1/syntaxin 1A. In addition, this mAb 8G5 exhibits its reactivity under both denaturing and non-denaturing conditions (Figs. 4, B–D), as revealed by dot-blot, Western blot and ELISA analyses with series of synthetic peptides and fusion proteins.

Immunocytochemical analyses were performed with mAb 8G5 to further confirm that the C-terminal tail of HPC-1/syntaxin 1A was embedded in the plasma membrane. HeLa cells were transfected with the N-terminally HA-tagged HPC-1/syntaxin 1A (HA-1A), fixed, permeabilized, and then double-labeled with the indicated antibodies (Fig. 5, A and D) in combination with the anti-HA antibody 3F10 (Fig. 5, B and E). Co-localization was observed on staining with the anti-HA antibody and mAb 14D8, which recognizes the N-terminal portion of HPC-1/syntaxin 1A (Fig. 5, D–F). In agreement with previous studies, mAb 8G5 did not react with HA-tagged HPC-1/syntaxin 1A in the plasma membranes of HeLa cells (Fig. 5, A–C). These data demonstrate conclusively that the C-terminal tail of HPC-1/syntaxin 1A is buried in the lipid bilayer. Western blot analysis was carried out to further examine the properties of the C-terminal membrane-anchoring region. HeLa cells were transfected with the indicated plasmids, and then lysed and subjected to SDS–PAGE, followed by Western blotting with the indicated antibodies. As shown in Fig. 5G (left panel), mAb 8G5 did not react with the C-terminal tail of HPC-1/syntaxin 1A expressed in HeLa cells, despite its reactivity to HPC-1/syntaxin 1A expressed in *E. coli* (Figs. 4D and 5H). Likewise, mAb 8G5 did not react with the C-terminal tail of HPC-1/syntaxin 1A when the protein was

expressed in either PC12 cells, rat brain, or transfected COS7 cells (Fig. 5H). Similarly, HPC-1/syntaxin 1A translated in a rabbit reticulocyte lysate expression system was not able to be recognized by mAb 8G5 (data not shown).

DISCUSSION

Identification of Membrane-Anchoring Regions—Structural determination of the N-terminal domain of HPC-1/syntaxin 1A was achieved previously by crystallography, NMR, and electron spin resonance spectroscopy of recombinant proteins lacking the transmembrane domains (*5*, *6*, *8*). Previous reports have suggested important roles for the C-terminal membrane-anchoring region in both cytoplasmic and transmembrane protein-protein interactions (*14*, *16*, *17*), and in membrane fusion (*18*, *19*). These studies were carried out on the basis of the predicted transmembrane domain. To better understand the role of the C-terminal hydrophobic region of syntaxins, we investigated the intrinsic properties of the C-terminal membrane-anchoring region of HPC-1/syntaxin 1A. Since none of the transmembrane regions of the syntaxin family have been identified experimentally, we used Kyte-Doolittle hydrophobicity plot (*27*), and various computer programs to predict transmembrane segments to facilitate our experimental design. These programs included: TMHMM (*28*, *29*); TopPredII (*30*, *31*); and SOSUI (*32*). All of the programs predicted that aa I266 was the N-terminal boundary of the transmembrane segment, since HPC-1/syntaxin 1A contains a hydrophilic cluster (^{262}RRKK^{265}) that is a characteristic initiation signal of the membrane-anchoring region (*27*, *33*, *34*). In contrast, each program gave distinct results concerning the C-terminal edge of the transmembrane segment. The programs indicated that the C-terminal boundary was at positions aa I286 through aa G288. In order to determine biochemically the structure and properties of the C-terminal membrane-anchoring region, we used the substituted-cysteine-accessibility method (SCAM). A series of mutants was created from a cysteine-less mutant of HPC-1/syntaxin 1A by substitution of each of the nine C-terminal amino acids (A280 to G288) with cysteine (Fig. 2A). We expressed these constructs in cells, and determined experimentally the membrane-anchoring regions by *in vivo* labeling of the introduced cysteines in the various mutants. The advantages of using *in vivo* labeling with cysteines are that the native folding of full-length proteins is less perturbed than that of proteins with large deletions, and that the observed results are illustrative of the native structure of the protein. To avoid binary complex formation between HPC-1/syntaxin 1A and SNAP-25 (*35*), non-neuronal HeLa cells that lacked both HPC-1/syntaxin 1A and SNAP-25 (*36*) were chosen for this study. Using SCAM, we showed that the C-terminal tail of HPC-1/syntaxin 1A was anchored in the lipid bilayer of the plasma membrane, and that it did not traverse the plasma membrane (Figs. 2 and 3). Although we used BM at a high concentration (100 μM) to chemically modify the thiol groups in cysteine, HPC-1/syntaxin 1A was labeled only at the N-terminal cytosolic region (C145), *i.e.* not at C271, C272, or any of the introduced cysteines at the C-terminus (Fig. 2C). Similar results were obtained in *in vitro* labeling experiments (data not shown). We observed that the edge of HPC-1/syntaxin 1A, which was tagged with additional amino acids at the C-terminus (Fig. 3A), appeared on the exoplasmic side of the cells (Fig. 3C). This finding supports the idea that the C-terminus of the native HPC-1/syntaxin 1A is embedded in the leaflet of the lipid bilayer. Furthermore, we produced specific mAbs that recognize the C-terminal tail of HPC-1/syntaxin 1A (Fig. 4), and showed that the C-terminus was not accessible from either the cytoplasmic or exoplasmic side of the cell (Fig. 5). It was only when the cells were permeabilized that the N-terminally HA-tagged HPC-1/syntaxin 1A was accessible to the anti-HA antibody and to mAb 14D8, which recognizes the N-terminus of HPC-1/syntaxin 1A (Fig. 5, D–F). Unexpectedly, mAb 8G5 did not react with the C-terminal tail of HPC-1/syntaxin 1A on Western blotting when the protein was expressed in eukaryotic cells (Fig. 5, G and H), or in the reticulocyte lysate expression system (data not shown), despite its reactivity with HPC-1/syntaxin 1A expressed in *E. coli* (Figs. 4, C and D, and 5H). It is not clear why mAb 8G5 reacts differently with these cells. It is possible that HPC-1/syntaxin 1A is tightly associated with unidentified lipids within lipid rafts (which are absent in *E. coli*), since HPC-1/syntaxin 1A reportedly binds to detergent-insoluble lipid rafts in the mammalian cell membrane (*37*, *38*). This antibody may provide new insights into the organization of clusters of syntaxin 1A in the rafts at the plasma membrane. We are currently examining the targeting of HPC-1/syntaxin 1A to the lipid rafts and the transportation of the protein to the cell surface. In any case, we obtained no evidence supporting the notion that the C-terminal membrane-anchoring region is exposed to the extracellular space. Together with the results obtained on SCAM analysis (Figs. 2 and 3), these findings indicate that the N-terminal portion of HPC-1/syntaxin 1A is oriented towards the cytosol, and that the C-terminal tail terminates within the lipid bilayer. We conclude that the membrane-anchoring region is 23 amino acids in length, starting at I266 and ending at G288, and that the C-terminal tail is essential for anchoring of HPC-1/syntaxin 1A to the plasma membrane.

Role of the Membrane-Anchoring Region in Localization to the Plasma Membrane—Consistent with previous observations with COS-1 cells (*20*), we have shown that HPC-1/syntaxin 1A has the ability to be localized to the plasma membrane of non-neuronal cells (Fig. 1B). This property has also been seen for the endogenous protein in PC12h cells (data not shown). Single substitutions of the C-terminal amino acids of HPC-1/syntaxin 1A did not affect its intracellular localization, which indicates that the amino acid sequence of the C-terminal tail is not important in this regard (Fig. 2B). In addition, it has been suggested that mutations of most of the amino acids in the membrane-anchoring region still allow association of the protein with the membrane fraction (*17*). In contrast, deletion of the eight amino acids at the C-terminus meant that the protein was not integrated into membranes, but remained partially associated with the ER (Fig. 1C). These results are compatible with the idea that HPC-1/syntaxin 1A is initially integrated into the ER membrane, subsequently escapes from the ER, and is transported to the cell surface along a secretory pathway

(*20*). It is possible that once inserted into the ER membrane, HPC-1/syntaxin 1A is trafficked to the plasma membrane by means of a passive signal, such as the transmembrane length, since known active-type signals, such as the targeting motif, are absent. In support of this idea, it has been reported that the localization of syntaxins 3 and 4 at the plasma membrane is dependent on the length of the transmembrane region (*39*). Moreover, the membrane-anchoring region might be sufficient for targeting to the cell surface, since we have observed in an immunocytochemical study that the GFP-tagged membrane-anchoring region (*23* amino acids corresponding to I266 through G288) of HPC-1/syntaxin 1A was localized to the plasma membrane (unpublished observation). Although the transmembrane domain length plays a major role in membrane localization, active-type determinants, such as protein–protein interactions and protein-targeting motifs, may override the membrane-targeting mechanism in certain cases. This study provides a basis for the targeting of SNARE proteins harboring membrane-anchoring regions as well as C-terminal anchoring proteins to the plasma membrane.

Possible Structure and Roles of the Membrane-Anchoring Region in the Plasma Membrane of Mammalian Cells—The plasma membrane is the final destination of the HPC-1/syntaxin 1A protein, where it becomes competent to function as a t-SNARE during exocytosis. Although the exact mechanisms of SNARE-dependent membrane fusion in eukaryotes are unknown, the complex between the transmembrane region of HPC-1/syntaxin 1A and that of VAMP2 might be formed during late steps of fusion (*13*, *14*). In support of this idea, Langosch *et al.* showed that peptide mimics of transmembrane regions of these two proteins drive membrane fusion of liposomes (*14*). Interestingly, they suggested that the fusogenic activity of liposomes decreased with increasing stability of the α-helical structures of the transmembrane peptide mimics. Furthermore, increasing the ratio of phosphatidylethanolamine (PE) and phosphatidylserine (PS), that favor distribution in the cytoplasmic leaflet, stimulates peptide-induced fusion of liposomes. Taken together with the results obtained on SCAM analysis (Figs. 2 and 3) and with mAb 8G5 against the C-terminal tail (Fig. 5), it is possible to think that the location of the C-terminal tail within the plasma membrane allows tight association with the hydrophobic core of lipids or cholesterol enriched not only in the inner leaflet but also in a specialized compartment such as lipid rafts. The association of the transmembrane region with unidentified lipids and/or cholesterols, without the formation of a simple α-helix, may stabilize the overall structure of HPC-1/syntaxin 1A in the plasma membrane. The above structural requirements of the protein may regulate membrane fusion. Consistent with the above ideas, it has been reported that HPC-1/syntaxin 1A does not require an anchor capable of spanning both leaflets, but requires its sufficient hydrophobicity upon fusion of SNARE-reconstituted liposomes (*19*). The location of the C-terminal tail in the membrane leaflets may be important for the function as a t-SNARE by stabilization of the structure with the specific lipid content of the plasma membrane. Thus, further investigation of the relationships between the function and structural features of HPC-1/syntaxin 1A may provide insights into the mechanism of membrane fusion that actually occurs in intact cells.

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