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Trafficking of Green Fluorescent Protein-Tagged SNARE Proteins in HSY Cells[1]

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SNARE proteins are widely accepted to be involved in the docking and fusion process of intracellular vesicle trafficking. VAMP-2, syntaxin-4, and SNAP-23 are plausible candidate SNARE proteins for non-neuronal exocytosis. Thus, we examined the localization, protein–protein interaction, and intracellular trafficking of these proteins by expressing them as green fluorescent protein (GFP)– and FLAG-tagged fusion proteins in various cells, including HSY cells, a human parotid epithelial cell line. GFP–VAMP-2 was expressed strongly in the Golgi area and weakly on the plasma membrane. Although GFP–SNAP-23 seemed to be expressed universally in the cytosol, the GFP signal was clearly seen on the plasma membrane, when soluble GFP–SNAP-23 was removed by treatment with saponin. GFP–syntaxin-4 was undetectable on the plasma membrane but was strongly expressed on unidentified unusually large vesicles. GFP–syntaxin-4 without its transmembrane domain was still incompletely soluble and observed as aggregates. When syntaxin-4 and munc18c were coexpressed, syntaxin-4 was translocated at least in part to the plasma membrane. The protein–protein interaction between syntaxin-4 and VAMP-2 with their transmembrane domains was markedly inhibited on coexpression of munc18c. These results suggest that munc18c plays an important role in the trafficking of syntaxin-4 to its proper destination by preventing premature interactions with other proteins, including SNARE proteins.

Key words: green fluorescent protein, munc18c, SNAP-23, syntaxin-4, VAMP-2.

Many proteins on intracellular membranes known as SNARE proteins are generally accepted to be involved in membrane fusion processes during diverse intracellular vesicle trafficking (*1–6*). The best-characterized SNARE proteins are those involved in neurotransmitter release. The role of SNARE proteins in the docking and fusion processes on synaptic vesicles and presynaptic membranes is strongly supported by a large body of strong evidence. The X-ray crystal structure of the core complex of SNARE proteins composed of VAMP-2, syntaxin-1, and SNAP-25 revealed a four-helix-bundle structure of helices designated as the SNARE motif (*3*); syntaxin-1 and VAMP-2 have one SNARE motif each, and SNAP-25 has two of them. Liposomes containing recombinant VAMP-2, and ones containing recombinant syntaxin-1 and SNAP-25 could specifically and spontaneously dock and fuse, although the fusion process itself was much slower than that observed in intact neurons (*2*). Thus, the SNARE proteins associated with some regulatory proteins are believed to be the fundamental machinery for the membrane fusion.

In non-neuronal cells, most secretory vesicles have VAMP-2 as a candidate v-SNARE (SNARE proteins on trafficking vesicle membranes), but t-SNAREs (SNARE proteins on target membranes) on the plasma membrane have still not been identified or are controversial. Syntaxin-4 and SNAP-23 are recognized as the most plausible t-SNAREs (*6*). Indeed, there is strong evidence supporting the involvement of these proteins in insulin-mediated translocation of GLUT4 from intracellular vesicles to the cell surface in adipocytes (*6–11*). In exocrine cells, VAMP-2 is also found on the secretory granule membranes, and the prolonged incubation of streptolysin-O permeabilized cells with clostridial neurotoxins, which cleave VAMP-2, partially but significantly inhibited amylase release from pancreatic (*12*) and parotid (*13*) acinar cells. In previous studies (*14, 15*), however, although we clearly demonstrated that parotid acinar cells contained syntaxin-4 and SNAP-23, an antibody for VAMP-2 failed to coprecipitate either syntaxin-4 or SNAP-23 from a lysate of the acinar cells. Conversely, antibodies for syntaxin-4 and SNAP-23 were also unable to coprecipitate VAMP-2, although the anti-syntaxin-4 clearly coprecipitated NSF, munc18c, and SNAP-23 (*15*). Thus, even if syntaxin-4 and SNAP-23 are t-SNAREs, the binding of these proteins to VAMP-2 would be very weak or be hindered by other proteins in parotid aci-

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Abbreviations: GFP, green fluorescent protein; NSF, *N*-ethylmaleimide–sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VAMP, vesicle-associated membrane protein; SNAP-23, synaptosome-associated protein of 23 kDa.

nar cells. Furthermore, it has been reported that syntaxin-4 and SNAP-23 are predominantly located on the basolateral plasma membrane in pancreatic acinar cells (*16, 17*), although the possibility can not be excluded that a relatively small amount of these proteins is located on the luminal plasma membrane, a proper target membrane in exocrine cells. Thus, the role of these SNARE proteins in the exocrine glands has not yet been established.

Although *in vitro* protein–protein interactions between SNARE proteins have been extensively studied, the results obtained showed that SNARE proteins can bind promiscuously with cognate SNARE proteins. In addition, the effect of the protein–protein interaction on the localization and intracellular trafficking of SNARE proteins in living cells remains to be elucidated. GFP-tagged SNARE proteins can be observed directly on confocal microscopy in living cells. Thus, in this study we expressed GFP- and FLAG-tagged SNARE proteins in HSY salivary cells, COS-7 cells, and 3T3-L1 adipocytes to examine the localization, protein–protein interaction, and intracellular trafficking of these proteins. During these experiments we found an unexpected distribution of these proteins and obtained results suggesting that munc18c is involved in the proper targeting of syntaxin-4 to the plasma membrane by preventing premature interaction with other proteins that exhibit intrinsic affinity to syntaxin-4.

MATERIALS AND METHODS

Materials—Anti–rat syntaxin-4 (rabbit, polyclonal) was raised against a synthetic peptide (ELRQGDNISDDEDE) corresponding to the N-terminal portion of rat syntaxin-4, and was affinity-purified as described previously (*15*). Polyclonal antibodies to SNAP-23 and munc-18c were generous gifts from Dr. Yoshikazu Tamori (2nd Department of Internal Medicine, Kobe University) (*7–9*). The pEF-BOS vector was kindly provided by Dr. Shizuo Akira (Research Institute for Microbial Diseases, Osaka University). An antibody to rat VAMP-2 (polyclonal) was purchased from Wako (Osaka). The pEGFP-C3 vector and the antibodies to GFP (monoclonal and polyclonal) were from CLONTEC (Palo Alto, CA), whereas the pFLAG-CMV-5c vector and the antibody to FLAG were from Sigma (St. Louis, MO). Pefabloc SC was obtained from Merck (Darmstadt, Germany). Fluorescent dyes, Calcium Green-C18 (C-6804), and BODIPY FL ceramide (D-3521), were obtained from Molecular Probes (Eugene, OR). The enhanced chemiluminescence kit came from NEN (Boston, MA); and Block Ace from Dainippon Seiyaku (Osaka). All other chemicals used were of the highest grade commercially available.

Cell Culture and Transfection—HSY cells were cultured in Dulbecco's modified Eagle's medium with nutrient mixture F-12 Ham (DMEM/F-12) containing 10% FBS. COS-7 (JCRB) and 3T3-L1 (JCRB9014) cells were obtained from the Riken Cell Bank (Tokyo), and were cultured in DMEM containing 10% calf serum. 3T3L1 cells were allowed to differentiate into adipocytes as described previously (*7–9*). These cells were transfected with plasmids mainly by the standard method of calcium phosphate precipitation and glycerol shock, and sometimes by lipofection with Effecten TM (Qiagen) according to the instructions.

Confocal Microscopy—After transfection, cells were cultured for 20 h, and then the expression and localization of GFP-tagged proteins in the living cells were observed under an inverted confocal microscope (Leica TCS-SP system; Leica, Germany) controlled by TCS-NT4 software. Images were obtained with 488 nm excitation and an emission wavelength of 500–550 nm, and were processed with either NIH Image 1.59 or Adobe Photoshop 6.0.

Plasmid Construction—Full-length cDNAs of rat VAMP-2, SNAP-23, syntaxin-4, and mouse munc18c were amplified by RT-PCR from total RNA prepared from rat and mouse parotid acinar cells. The primer sets used were 5′-CGGAATTCCCATGTCGGCTACCGCTGCCAC-3′ and 5′-CGGAATTCTTAAGTGCTGAAGTAAACGATG-3′ for VAMP-2; 5′-AACTGCAGATGGATGATCTATCACCAGAAG-3′ and 5′-AACTGCAGTTAGCTGTCAATGAGTTTCTTTG-3′ for SNAP-23; 5′-CGGAATTCCCATGCGCGACAGGACCCATGAG-3′ and 5′-CGGAATTCTTATCCAACGGTTATGGTGATG-3′ for syntaxin-4; and 5′-GGAATTCCGATGGCGCCGCCGGTATCGGAG-3′ and 5′-GGAATTCCTCCTTAAAGGAAACTTTATCC-3′ for munc18c. The cDNAs of VAMP-2, syntaxin-4, and munc18c were subcloned into the *Eco*RI site of pEGFP-C3 (CLONTEC). The SNAP-23 cDNA was subcloned into the *Pst*I site of pEGFP-C3. To express FLAG-tagged SNARE proteins, we subcloned the same cDNAs into the *Eco*RI site of pFLAG-CMV-5c (Sigma). The cDNA of syntaxin-4 without a tag was ligated into the *Xba*I site (a blunt end site) of pEF-BOS. All sequences amplified by PCR were confirmed with an ABI 310-sequence analyzer after the constructs had been made.

Immunoprecipitation and Immunoblotting—Cells were pelleted, and then lysed on ice for 30 min with PBS containing 2% Triton X-100, 2 mM EDTA, and 1 mM Pefabloc SC. The lysate was centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatant was rotated at 4°C overnight with anti-FLAG antibodies conjugated to agarose beads (Sigma). After incubation, the beads were washed 5 times with lysis buffer, and the bound proteins were eluted with 0.5 mM FLAG peptide. The eluate was mixed with the same volume of 2× Laemmli cocktail and boiled for 5 min.

Proteins in the immunoprecipitate were resolved by SDS-PAGE on a 5–20% gradient gel (ATTO, Tokyo). Proteins in the gel were transferred to a PVDF membrane at 100 mA per mini-gel (90 × 73 × 1 mm) for 60 min in a semi-dry blotter with 0.1 M Tris–0.192 M glycine buffer containing 5% methanol. The membrane was washed twice with water, blocked with Block Ace at room temperature for 1 h or overnight at 4°C, and then incubated with properly diluted primary antibodies in PBS containing 0.05% Tween-20 (PBST) and 20% Block Ace for 1 h at room temperature or overnight at 4 °C. SNARE proteins were visualized with the NEN Chemiluminescence Reagent Plus system, and the images were captured and analyzed in an ATTO Cool Saver (ATTO).

RESULTS

Localization of GFP-SNARE Proteins in HSY and 3T3L1 Cells—We first examined the expression and localization of GFP–VAMP-2, which is the full-length VAMP-2 tagged with GFP at its N-terminus, in HSY cells. As shown in Fig. 1A-1 and -2, GFP–VAMP-2 fluorescence was strong in the Golgi area and weak on the plasma membrane, suggesting that VAMP-2 functions in vesicle transport from the Golgi complex to the plasma membrane; although HSY cells do

not contain large secretory granules like those seen in parotid acinar cells. GFP alone was expressed in the whole cells including the nucleus (data not shown).

The plasma membrane and Golgi complex of HSY cells were separately visualized with fluorescent dyes; Calcium Green-C18 for the plasma membrane and BODIPY FL ceramide for the Golgi complex. To identify the plasma membrane, we observed the cells that expressed GFP-VAMP-2 before (Fig. 2A) and after (Fig. 2B) incubation with 10 μM Calcium Green-C18 for 5 min (*18*). The plasma membranes of HSY cells were clearly visualized with Calcium Green-C18, and the GFP signal at the cell periphery mostly merged with the signal of the fluorescent dye. To visualize the Golgi complex, HSY cells were incubated with 2 μM BODIPY FL ceramide for 60 min at 37°C. As shown in Fig. 2C, the fluorescent dye strongly visualized the Golgi complex and weakly the endoplasmic reticulum. When cells that expressed GFP–VAMP-2 were stained with BODIPY FL ceramide, the signal of GFP–VAMP-2 was indistinguishable from that of the dye (data not shown). These results support that GFP–VAMP-2 is expressed in the Golgi area and on the plasma membrane.

Next we examined the localization of putative t-SNAREs, *i.e.*, GFP–SNAP-23 and GFP–syntaxin-4, in HSY cells. Although these proteins have been reported to be mainly expressed on the plasma membrane, as judged on immunocytochemistry (*7, 16, 17*), GFP–SNAP-23 was clearly observed in the cytoplasm but not seen in the nucleus or in some vacuoles (Fig. 1B-1). To examine the possibility that over-expressed GFP–SNAP-23 becomes soluble and masks GFP–SNAP-23 on the plasma membrane, we incubated HSY cells with 50 μg/ml saponin. As shown in Fig. 1B-2, the GFP signal disappeared from the cytosol and became prominent on the plasma membrane, suggesting that GFP–SNAP-23 was actually located on the plasma membrane, at least in part. In the presence of saponin, GFP–VAMP-2 was more clearly seen on the plasma membrane also (Fig. 1A-2).

Surprisingly, as can be seen in Fig. 1C-1 and -2, GFP-syntaxin-4 was observed on unidentified extraordinarily large intracellular vesicles and also on the peri-nuclear membranes. The GFP signal was not detectable on the plasma membrane even in the presence of saponin (data not shown).

We next examined the localization of GFP-SNARE proteins in 3T3-L1 adipose cells, in which these proteins function in insulin-dependent trafficking of membrane vesicles carrying Glut4 (*6–11*). As shown in Fig. 1A-3, GFP–VAMP-2 was observed on the plasma membrane in addition to the strong signal seen in the Golgi area. GFP–SNAP-23 was also mainly seen in the cytoplasm (Fig. 1B-3). On the other hand, GFP–syntaxin-4 was observed on smaller membrane vesicles in 3T3-L1 adipose cells than those in HSY and COS-7 cells, and some of these vesicles seemed to be attached to the plasma membrane of the adipose cells (Fig. 1C-3).

Interactions between Syntaxin-4, SNAP-23, and VAMP-2—To evaluate the effect of protein–protein interaction on the localization of SNARE proteins, we coexpressed VAMP-2, syntaxin-4, and SNAP-23 in various combinations in HSY and COS-7 cells. During these experiments, we observed that syntaxin-4 influenced the localization of SNAP-23 and VAMP-2. To visualize these effects, we expressed either GFP–SNAP-23 or GFP–VAMP-2 concurrently with syntaxin-4 without the GFP-tag. As shown in Fig. 3A, the GFP signal of SNAP-23 was partly translocated onto the large vesicles that had been previously seen in cells trans-

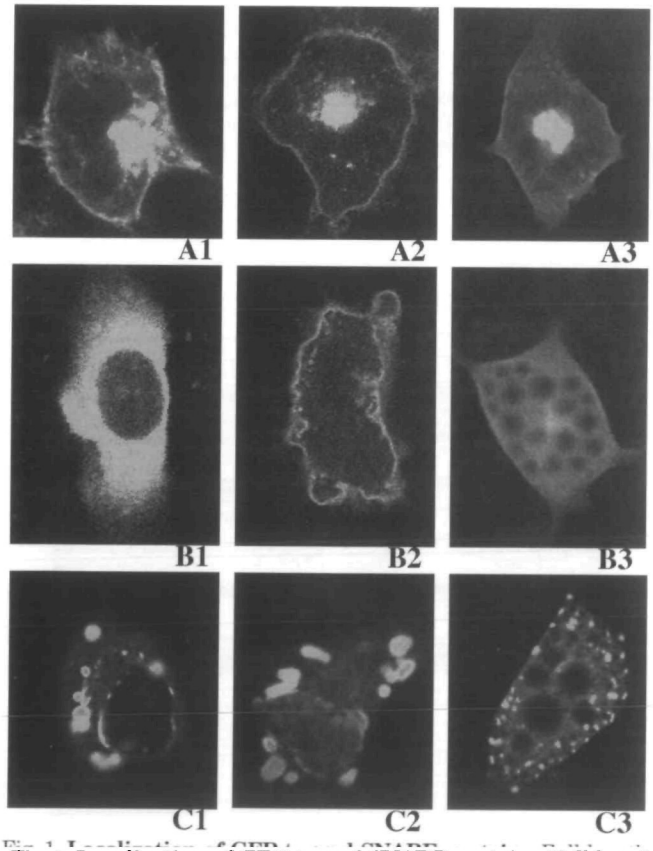

Fig. 1. **Localization of GFP-tagged SNARE proteins.** Full-length SNARE proteins tagged with GFP at their N-termini were transiently expressed in HSY, COS-7, and 3T3-L1 cells; and the localization of GFP signals in the living cells was examined by confocal microscopy at 20 h after transfection with the plasmids. GFP–VAMP-2 was expressed in HSY (A1 and 2) and 3T3-L1 (A3) cells; GFP–SNAP-23 was detected in HSY (B1 and 2) and 3T3-L1 (B3) cells; and GFP–syntaxin-4, in HSY (C1), COS-7 (C2), and 3T3-L1 (C3) cells. Some cells were examined in the presence of 50 μg/ml saponin (A2 and B2).

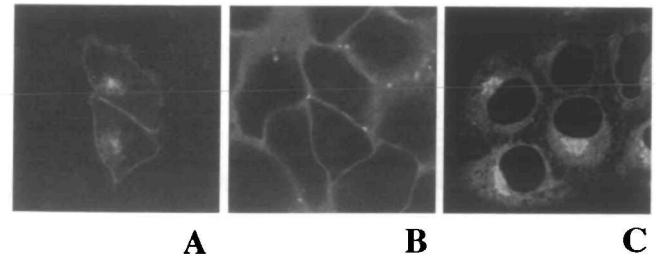

Fig. 2. **Visualization of the plasma membrane and Golgi complex with fluorescent dyes.** Confocal microscopic images of HSY cells, which expressed GFP-VAMP-2, were captured before (A) and after (B) incubation with 10 μM calcium Green-C18 for 5 min under a microscope. C, control HSY cells without coexpression of GFP–VAMP-2 were incubated with 2 μM BODIPY FL ceramide for 60 min at 37°C. Images A and C were obtained with 488 nm excitation and an emission wavelength of 500–550 nm, and B with 568 nm excitation and an emission wavelength of 580–700 nm.

fected with GFP-syntaxin-4 (Fig. 1C-1 and 2), suggesting that the destination on intracellular trafficking of SNAP-23 is determined at least in part by syntaxin-4. Similarly, the GFP signal of VAMP-2 was seen on the large vesicles in addition to being detected in the Golgi area and on perinuclear membranes, but it was not found on the plasma membrane.

For evaluation of the protein–protein interaction between these proteins, VAMP-2–FLAG and syntaxin-4–FLAG, both of which are full-length proteins tagged with the FLAG epitope (DYKDDDDK) at their C-termini, were coexpressed with other SNARE proteins in COS-7 cells; and VAMP-2–FLAG and syntaxin-4–FLAG were immunoprecipitated with anti-FLAG antibodies conjugated to agarose beads. As shown in Fig. 4 (lane 2), VAMP-2 and syntaxin-4 bound together much more strongly than VAMP-2- and SNAP-23 did (Fig. 4, lane 1). Coprecipitation of GFP–SNAP-23 with VAMP-2–FLAG increased when GFP–syntaxin-4 was coexpressed (Fig. 4, lane 4), indicating that most of the SNAP-23 indirectly binds to VAMP-2 through the binding to syntaxin-4 (*15*). The interaction between VAMP-2 and syntaxin-4 was greatly reduced (Fig. 4, lane 3) when the transmembrane domain of syntaxin-4 was truncated. Identical results were obtained when the transmembrane domain of VAMP-2 was truncated (data not shown). These results suggest that the transmembrane domain of both proteins is necessary for the strong interaction between VAMP-2 and syntaxin-4.

As can be seen in Fig. 5, A and B, GFP–syntaxin-4 lacking its transmembrane domain was expressed as small particles without connection to the plasma membrane in COS-7 and 3T3-L1 adipose cells, suggesting that the cytosolic

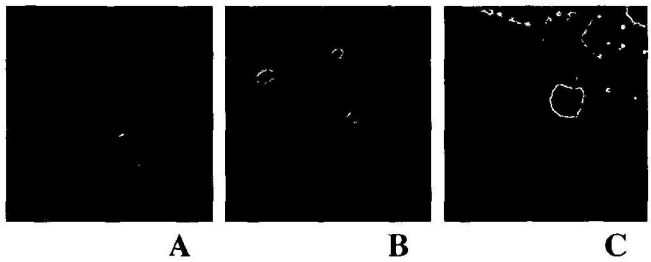

Fig. 3. **Effect of coexpression of syntaxin-4 on the localization of GFP–SNAP-23 and GFP–VAMP-2.** A, GFP–SNAP-23 and syntaxin-4 without GFP tag were coexpressed in HSY cells; B, GFP–VAMP-2 and syntaxin-4 were coexpressed in COS-7 cells; C, GFP–VAMP-2 alone was expressed in COS-7 cells.

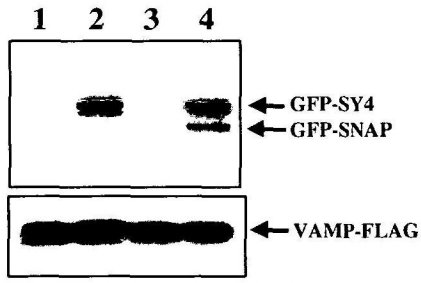

Fig. 4. **Protein–protein interaction between SNARE proteins.** Full-length VAMP-2 tagged with the FLAG epitope at its C-terminus was coexpressed with full-length GFP–SNAP-23 (lane 1), full-length GFP–syntaxin-4 (lane 2), GFP–syntaxin-4 without its transmembrane domain (lane 3), or full-length GFP–SNAP-23 and GFP–syntaxin-4 (lane 4) in COS-7 cells. At 24 h after transfection, cell lysates were prepared, and VAMP-2–FLAG was immunoprecipitated with anti-FLAG antibodies conjugated to agarose beads. The immunoprecipitates were resolved by SDS-PAGE, and proteins coprecipitated with VAMP-2–FLAG were analyzed by immunoblotting with anti-GFP.

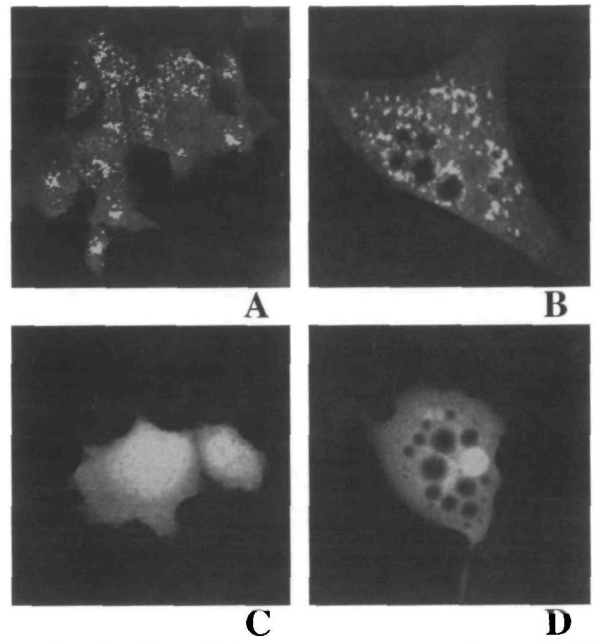

Fig. 5. **Localization of truncated GFP–syntaxin-4 and GFP–VAMP-2 without their transmembrane domains.** GFP–syntaxin-4 (A and B) and GFP–VAMP-2 (C and D), both of which lacked the transmembrane domain, were expressed in COS-7 (A), HSY (C), and 3T3L1 (B and D) cells.

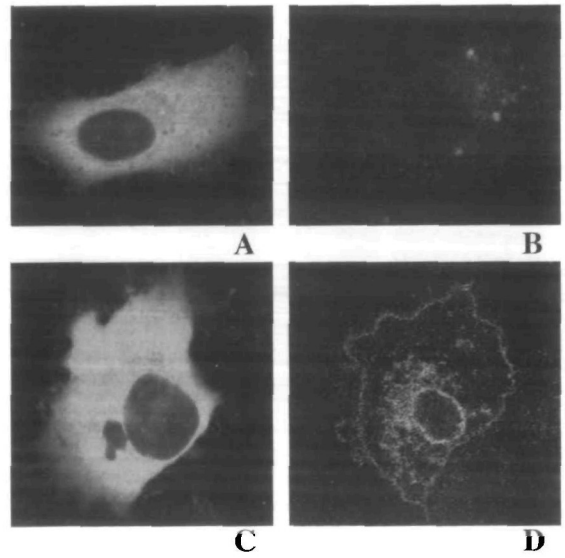

Fig. 6. **Effect of coexpression of munc18c on the localization of syntaxin-4.** Full-length munc18c tagged with GFP at its N-terminus was expressed alone (A and B) or with syntaxin-4 (C and D) in HSY cells. At 20 h after plasmid transfection, the cells were examined by confocal microscopy in the absence (A and C) or presence (B and D) of 50 μg/ml saponin.

domain of syntaxin-4 is still mostly insoluble, and that it self-aggregates or is contained in aggregates of other proteins. GFP–VAMP-2 without its transmembrane domain was expressed ubiquitously, including in the nucleus (Fig. 5, C and D).

Interaction between Munc18c and Syntaxin-4—Because munc18 strongly binds to syntaxins (*4, 5, 8, 10, 11*) and is believed to play a regulatory role in exocytosis (*4–11*), we examined the effect of coexpression of munc18c on the localization of syntaxin-4. As shown in Fig. 6A, GFP–munc-18c was expressed uniformly in the cytosol but not in the nucleus. When GFP–munc18c and syntaxin-4 were coexpressed (Fig. 6, C and D) and soluble GFP–munc18c was removed by treatment with 50 μg/ml saponin, the GFP signal was clearly observed on the plasma membrane, Golgi area, and peri-nuclear membranes (Fig. 6D). GFP-munc18c, which was expressed by itself, was undetectable on the plasma membrane even in the presence of saponin (Fig. 6B).

Immunoprecipitation analysis clearly demonstrated the interaction between GFP-munc18c and syntaxin-4–FLAG (Fig. 7, lane 1). Furthermore, the coexpression of GFP-munc18c markedly inhibited the protein–protein interaction between syntaxin-4–FLAG and GFP–VAMP-2 (lane 3), and also that between syntaxin-4–FLAG and GFP–SNAP-23 (lane 5).

DISCUSSION

VAMP-2 is widely distributed on exocytotic vesicles and is believed to act as a v-SNARE (*1–3, 12–15*). Because the cells used in this study do not have conspicuous secretory granules, GFP–VAMP-2 was mainly observed in the Golgi area of HSY, COS-7, and also 3T3-L1 adipose cells (Fig. 1A), as expected. We also detected a weak but obvious GFP signal on the plasma membrane, suggesting that vesicles containing VAMP-2 had been transported from the Golgi apparatus to the plasma membrane. Unexpectedly, however, in the protein–protein interaction experiments, VAMP-2–FLAG and GFP–syntaxin-4 bound together much more strongly than VAMP-2–Flag and GFP–SNAP-23 did (Fig. 4). In previous studies (*7–9*), no protein–protein interaction was observed between VAMP-2 and syntaxin-4 expressed in COS cells, although the transmembrane domain of both proteins was truncated. Indeed, the interaction between VAMP-2 and syntaxin-4 was hardly detected, when the transmembrane domain either one of them was truncated (Fig. 4, lane 3), suggesting that the transmembrane domain plays an important role in the interaction between the two proteins. A similar strong interaction between VAMP-2 and syntaxin-1 through their transmembrane domains was previously observed in a reconstitution study involving liposomes (*19*). The interaction between the two transmembrane domains was proposed to force the separate membranes close together, yielding membrane continuity known as a prefusion stalk. In the present study, however, the interaction between VAMP-2 and syntaxin-4 very likely occurred in the Golgi area and on the unidentified large vesicles, but not on the plasma membrane, as can be seen in Fig. 3B. The premature interaction between VAMP-2 and syntaxin-4 is unusual and inconvenient for proper trafficking of these proteins to their destinations and thus for exocytosis, if these proteins are v- and t-SNAREs.

Previous immunocytochemical studies showed that endogenous SNAP-23 is predominantly expressed on the plasma membrane (*7, 17, 20–22*). SNAP-23 and its isoform, SNAP-25, have no transmembrane domain and are anchored to the plasma membrane through palmitoyl moieties, which are linked to cysteine residues in the cysteine-rich domain in the middle of the molecule (*21–24*). A deletion mutant of SNAP-23, which lacked the cysteine-rich domain, showed a shift in the distribution of SNAP-23 from the plasma membrane to the cytoplasm (*22–24*), indicating that the cysteine-rich domain is essential for trafficking of SNAP-23 to the plasma membrane. In the present study, however, GFP–SNAP-23, which had an intact cysteine-rich domain was mainly expressed in the cytoplasm of HSY cells (Fig. 1B) and COS-7 cells (data not shown). Only after the removal of soluble SNAP-23 by treatment with saponin could GFP–SNAP-23 be seen on the plasma membrane. These results suggest two possibilities, *i.e.*, that (i) the plasma membrane has a limited capacity for anchoring of SNAP-23, and (ii) the intact cysteine-rich domain is insufficient and additional factors are necessary for the proper distribution of SNAP-23. Recently, syntaxin-1 was suggested to play a critical role in the targeting of SNAP-25 to the plasma membrane prior to the palmitoylation of SNAP-25 (*24*). In the present study, when syntaxin-4 and GFP–SNAP-23 were concurrently expressed, GFP–SNAP-23 was transported at least in part to the vesicles in which syntaxin-4 was located (Fig. 3A). Specific interaction between syntaxin-4 and SNAP-23 is widely recognized both *in vivo* and *in vitro* (*7–9, 15, 25*). Thus, syntaxin-4 might be involved in the trafficking of SNAP-23 to the plasma membrane.

Previous immunocytochemical studies have also shown that endogenous syntaxin-4 is mainly on the plasma membrane (*16*). In the present study, however, GFP–syntaxin-4 was scarcely expressed on the plasma membrane but strongly expressed on the unidentified large intracellular vesicles (Fig. 1C). This localization seems not to be due to the GFP tag, since syntaxin-4 without the tag was also very likely located on the same vesicles (Fig. 3, A and B). Similar large membrane vesicles were previously observed when the inositol trisphosphate receptor was transiently expressed in COS cells (*26*). Although the precise mechanism of the vesicle formation is unclear, the overexpression of the proteins, and the binding *via* homotypic and heterotypic interactions with proteins on adjacent membranes were suggested to be the cause of the vesicle formation. In the present study, however, GFP–syntaxin-4 was hardly detectable on the plasma membrane even in cells that expressed the protein very weakly (data not shown). In this study, the homotypic interaction of GFP–syntaxin-4 seemed to be very strong, since GFP–syntaxin-4 without its transmembrane domain was still mostly insoluble and formed aggregates (Fig. 5, A and B), although other proteins might have been involved in the aggregate formation.

The interaction between the munc18/n-sec1 and syntaxin protein families has been extensively studied [see reviews (*1, 4–6*)], but the role of munc18 in the regulation of membrane fusion remains obscure. Munc18 is a binding protein for syntaxins, and inhibits the binding of both VAMP-2 and SNAP-25 or -23 to syntaxins. Thus, it was thought to have an inhibitory effect on exocytosis (*27*). Indeed, overexpres-

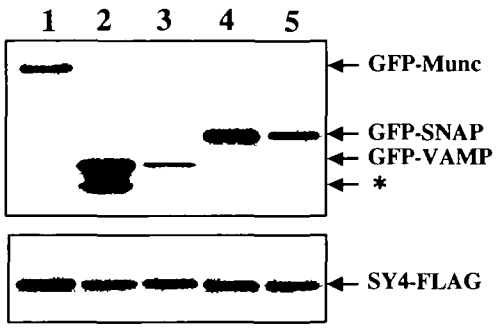

Fig. 7. **Effect of coexpression of munc18c on protein–protein interaction between syntaxin-4 and VAMP-2 or SNAP-23.** Syntaxin-4–FLAG was expressed with GFP-munc18c (lane 1), GFP–VAMP-2 (lane 2), GFP-munc18c plus GFP–VAMP-2 (lane 3), GFP–SNAP-23 (lane 4), or GFP-munc18c plus GFP–SNAP-23 (lane 5) in COS-7 cells. At 24 h after transfection, cell lysates were prepared, and syntaxin-4–FLAG was then immunoprecipitated with anti-FLAG antibodies conjugated to agarose beads. Proteins in the immunoprecipitates were resolved by SDS-PAGE, and proteins coprecipitated with syntaxin-4–FLAG were analyzed by immunoblotting with anti-GFP. *Degradation products of GFP–VAMP-2.

sion of munc18c inhibited the translocation of GLUT4 to the plasma membrane in adipocytes (*8, 11*). Munc18a, however, was demonstrated to play an es-sential role in neurotransmitter release in a study on munc18a knockout mice (*28*), suggesting that munc18 has both stimulatory and inhibitory functions in exocytosis. Previously, we demonstrated that munc18c bound strongly to syntaxin-4 in rat parotid acinar cells (*15*). In the present study, we observed that munc18c could support the translocation of syntaxin-4 to the plasma membrane in HSY cells (Fig. 6, C and D), suggesting that munc18c is involved in the trafficking of syntaxin-4 to the plasma membrane. As described above, syntaxin-4 and VAMP-2 readily interacted *via* their transmembrane domains when they were coexpressed in COS-7 cells (Fig. 4). If VAMP-2 and syntaxin-4 are v- and t-SNAREs, respectively, their premature interaction on the endoplasmic reticulum must be strictly prevented for normal exocytosis. Previously, Rowe *et al.* (*29*) reported that syntaxin-1A, but not syntaxin-3, was mistargeted to the Golgi-TGN area in a defective PC12 clone and some epitherial cell lines, which did not express rbSec1/munc18a. However, when munc18a was coexpressed, syntaxin-1A was properly transported to the plasma membrane, suggesting that munc18a plays an important role in the trafficking of syntaxin-1A. A recent study on the crystal structure of the munc18a–syntaxin 1a complex revealed that munc18a tightly held syntaxin 1a in a closed conformation, in which syntaxin hardly could interact with other proteins (*4*). Munc18c supposedly forms a similar complex with syntaxin-4 and thus would be expected to inhibit the interaction between syntaxin-4 and other proteins, including VAMP-2, as was in fact observed (Fig. 7). Taken to-gether, these findings suggest that munc18 would be essential for exocytosis through the trafficking of syntaxins to their proper destination by preventing premature interaction with other proteins, including SNARE proteins.

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REFERENCES

1. Jahn, R. and Sudhof, T.C. (1999) Membrane fusion and exocytosis. *Annu. Rev. Biochem.* **68**, 863–911
2. Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., and Rothman, J.E. (1998) SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759–772
3. Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* **395**, 347–353
4. Misura, K.M., Scheller, R.H., and Weis, W.I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404**, 355–362
5. Jahn, R. (2000) Sec1/Munc18 proteins: mediators of membrane fusion moving to center stage. *Neuron* **27**, 201–204
6. Foster, L.J. and Klip, A. (2000) Mechanism and regulation of GLUT-4 vesicle fusion in muscle and fat cells. *Am. J. Physiol. Cell. Physiol.* **279**, C877–C890
7. Araki, S., Tamori, Y., Kawanishi, M., Shinoda, H., Masugi, J., Mori, H., Niki, T., Okazawa, H., Kubota, T., and Kasuga, M. (1997) Inhibition of the binding of SNAP-23 to syntaxin 4 by Munc18c. *Biochem. Biophys. Res. Commun.* **234**, 257–262
8. Tamori, Y., Kawanishi, M., Niki, T., Shinoda, H., Araki, S., Okazawa, H., and Kasuga, M. (1998) Inhibition of insulin-induced GLUT4 translocation by Munc18c through interaction with syntaxin4 in 3T3-L1 adipocytes. *J. Biol. Chem.* **273**, 19740–19746
9. Kawanishi, M., Tamori, Y., Okazawa, H., Araki, S., Shinoda, H., and Kasuga, M. (2000) Role of SNAP23 in insulin-induced translocation of GLUT4 in 3T3-L1 adipocytes. Mediation of complex formation between syntaxin4 and VAMP2. *J. Biol. Chem.* **275**, 8240–8247
10. Tellam, J.T., Macaulay, S.L., McIntosh, S., Hewish, D.R., Ward, C.W., and James, D.E. (1997) Characterization of Munc-18c and syntaxin-4 in 3T3-L1 adipocytes. Putative role in insulin-dependent movement of GLUT-4. *J. Biol. Chem.* **272**, 6179–6186
11. Thurmond, D.C., Ceresa, B.P., Okada, S., Elmendorf, J.S., Coker, K., and Pessin, J.E. (1998) Regulation of insulin-stimulated GLUT4 translocation by Munc18c in 3T3L1 adipocytes. *J. Biol. Chem.* **273**, 33876–33883
12. Gaisano, H.Y., Sheu, L., Foskett, J.K., and Trimble, W.S. (1994) Tetanus toxin light chain cleaves a vesicle-associated membrane protein (VAMP) isoform 2 in rat pancreatic zymogen granules and inhibits enzyme secretion. *J. Biol. Chem.* **269**, 17062–17066
13. Fujita-Yoshigaki, J., Dohke, Y., Hara-Yokoyama, M., Kamata, Y., Kozaki, S., Furuyama, S., and Sugiya, H. (1996) Vesicle-associated membrane protein 2 is essential for cAMP-regulated exocytosis in rat parotid acinar cells. The inhibition of cAMP-dependent amylase release by botulinum neurotoxin B. *J. Biol. Chem.* **271**, 13130–13134
14. Takuma, T., Tagaya, M., and Ichida, T. (1997) Evidence for the putative docking/fusion complex of exocytosis in parotid acinar cells. *FEBS Lett.* **404**, 34–36
15. Takuma, T., Arakawa, T., and Tajima, Y. (2000) Interaction of SNARE proteins in rat parotid acinar cells. *Arch. Oral Biol.* **45**, 369–375
16. Gaisano, H.Y., Ghai, M., Malkus, P.N., Sheu, L., Bouquillon, A., Bennett, M.K., and Trimble, W.S. (1996) Distinct cellular locations of the syntaxin family of proteins in rat pancreatic acinar cells. *Mol. Biol. Cell.* **7**, 2019–2027
17. Gaisano, H.Y., Sheu, L., Wong, P.P., Klip, A., and Trimble, W.S. (1997) SNAP-23 is located in the basolateral plasma membrane of rat pancreatic acinar cells. *FEBS Lett.* **414**, 298–302
18. Tanimura, A. and Turner, R.J. (1996) Inositol 1,4,5-trisphosphate-dependent oscillations of luminal [Ca^{2+}] in permeabilized HSY cells. *J. Biol. Chem.* **271**, 30904–30908

19. Margittai, M., Otto, H., and Jahn, R. (1999) A stable interaction between syntaxin 1a and synaptobrevin 2 mediated by their transmembrane domains. *FEBS Lett.* **446**, 40–44
20. Wang, G., Witkin, J.W., Hao, G., Bankaitis, V.A., Scherer, P.E., and Baldini, G. (1997) Syndet is a novel SNAP-25 related protein expressed in many tissues. *J. Cell. Sci.* **110**, 505–513
21. Low, S.H., Roche, P.A., Anderson, H.A., van Ijzendoorn, S.C., Zhang, M., Mostov, K.E., and Weimbs, T. (1998) Targeting of SNAP-23 and SNAP-25 in polarized epithelial cells. *J. Biol. Chem.* **273**, 3422–3430
22. Koticha, D.K., Huddleston, S.J., Witkin, J.W., and Baldini, G. (1999) Role of the cysteine-rich domain of the t-SNARE component, SYNDET, in membrane binding and subcellular localization. *J. Biol. Chem.* **274**, 9053–9060
23. Gonzalo, S., Greentree, W.K., and Linder, M.E. (1999) SNAP-25 is targeted to the plasma membrane through a novel membrane-binding domain. *J. Biol. Chem.* **274**, 21313–21318
24. Vogel, K., Cabaniols, J.P., and Roche, P.A. (2000) Targeting of SNAP-25 to membranes is mediated by its association with the target SNARE syntaxin. *J. Biol. Chem.* **275**, 2959–2965
25. Ravichandran, V., Chawla, A., and Roche, P.A. (1996) Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J. Biol. Chem.* **271**, 13300–13303
26. Takei, K., Mignery, G.A., Mugnaini, E., Sudhof, T.C., and De Camilli, P. (1994) Inositol 1,4,5-trisphosphate receptor causes formation of ER cisternal stacks in transfected fibroblasts and in cerebellar Purkinje cells. *Neuron* **12**, 327–342
27. Fujita, Y., Sasaki, T., Fukui, K., Kotani, H., Kimura, T., Hata, Y., Sudhof, T.C., Scheller, R.H., and Takai, Y. (1996) Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C: its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *J. Biol. Chem.* **271**, 7265–7268
28. Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J., and Sudhof, T.C. (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* **287**, 864–869
29. Rowe, J., Corradi, N., Malosio, M.L., Taverna, E., Halban, P., Meldolesi, J., and Rosa, P. (1999) Blockade of membrane transport and disassembly of the Golgi complex by expression of syntaxin 1A in neurosecretion-independent cells: prevention by rbSEC1. *J. Cell. Sci.* **112**, 1865–1877