

Syntaxin 1A is delivered to the apical and basolateral domains of epithelial cells: the role of munc-18 proteins

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SUMMARY

SNARE (Soluble N-ethyl-maleimide sensitive factor Attachment protein Receptor) proteins assemble in tight core complexes, which promote fusion of carrier vesicles with target compartments. Members of this class of proteins are expressed in all eukaryotic cells and are distributed in distinct subcellular compartments. The molecular mechanisms underlying sorting of SNAREs to their physiological sites of action are still poorly understood. Here we analyzed the transport of syntaxin1A in epithelial cells. In line with previous data we found that syntaxin1A is not transported to the plasma membrane, but rather is retained intracellularly when overexpressed in MDCK and Caco-2 cells. Its delivery to the cell surface is recovered after munc-18-1 cotransfection. Furthermore, overexpression of the ubiquitous isoform of munc-18, munc-18-2, is also capable of rescuing the transport of the t-SNARE. The interaction between syntaxin 1A and munc-18 occurs in the

biosynthetic pathway and is required to promote the exit of the t-SNARE from the Golgi complex. This enabled us to investigate the targeting of syntaxin1A in polarized cells. Confocal analysis of polarized monolayers demonstrates that syntaxin1A is delivered to both the apical and basolateral domains independently of the munc-18 proteins used in the cotransfection experiments. In search of the mechanisms underlying syntaxin 1A sorting to the cell surface, we found that a portion of the protein is included in non-ionic detergent insoluble complexes. Our results indicate that the munc-18 proteins represent limiting but essential factors in the transport of syntaxin1A from the Golgi complex to the epithelial cell surface. They also suggest the presence of codominant apical and basolateral sorting signals in the syntaxin1A sequence.

Key words: SNARE, Membrane transport, Golgi complex, Munc-18

INTRODUCTION

The SNARE (Soluble N-ethyl-maleimide sensitive factor Attachment protein Receptor) family of integral membrane proteins plays a crucial role in mediating intracellular membrane fusion in eukaryotic cells (for recent reviews, see Bock and Scheller, 1999; Jahn and Südhof, 1999). Intracellular SNAREs can be divided into two categories: the v-SNAREs located on carrier vesicles, and the t-SNAREs present on target compartments. Assembly of cognate v- and t-SNARE proteins promotes the formation of extremely stable core complexes, which serve as functional receptors for cytosolic factors originally implicated in transport and vesicle fusion: soluble NSF attachment proteins (SNAPs) and the N-ethyl-maleimide sensitive factor (NSF; Söllner et al., 1993). The ATPase activity of NSF was originally postulated to be required for membrane fusion, but a number of recent studies have revealed that SNARE complexes are themselves capable of promoting membrane fusion, while the NSF activity is required for the disassembly of the complexes in order to allow subsequent rounds of v- and t-SNARE association and vesicle fusion (Bock and Scheller, 1999; Jahn and Südhof, 1999, and

references therein). Besides NSF, various other molecules are known to regulate SNARE protein interactions and activity (McMahon et al., 1995; Fujita et al., 1998; Beites et al., 1999; Lao et al., 2000). Among these, the sec-1-related proteins play a crucial role in modulating SNARE complex formation (Halachmi and Lev, 1996, and references therein). As it has been extensively demonstrated for the neuronal isoform munc-18-1 (also known as n-Sec or rbSEC1; Hata et al., 1993; Pevsner et al., 1994; Garcia et al., 1994; Garcia et al., 1995), these proteins are expected to bind with high affinity to syntaxins and regulate their association with cognate SNAREs (Hata et al., 1993; Pevsner et al., 1994; Garcia et al., 1994; Dulubova et al., 1999; Yang et al., 2000; Misura et al., 2000).

The first SNARE proteins to be identified were the v-SNAREs synaptobrevin 1 and 2 and the t-SNAREs SNAP-25 and syntaxin1A and B (syn1A, B), which were found to promote synaptic vesicle fusion with the pre-synaptic plasma membrane (Söllner et al., 1993). In the light of this finding, the presence of distinct v- and t-SNARE isoforms in different subcellular compartments was postulated, and the specific pairing of these molecules was proposed to mediate the fidelity of vesicle trafficking (Rothman, 1994). In line with this

hypothesis, many different isoforms of v- and t-SNARE proteins have been identified and localized to specific organelles (Hay and Scheller, 1997; Jahn and Südhof, 1999). In addition, the central role of SNAREs in vesicle targeting has been supported by recent data demonstrating that specific cognate SNARE pairing is required for the fusion of secretory granules with the plasma membrane (Scales et al., 2000). Furthermore, taking advantage of the identification in yeast of v- and t-SNAREs mediating vesicle fusion between the endoplasmic reticulum (ER) and the Golgi complex, vacuoles or plasma membrane, Rothman and colleagues have recently demonstrated that the compartmental specificity of intracellular membrane fusion is a property encoded by the SNARE proteins localized in each of these compartments (McNew et al., 2000).

In order to function appropriately, the SNAREs themselves must be correctly sorted and distributed to the appropriate compartments. Whereas more information is available on the mechanisms of SNARE targeting in yeast (Rayner and Pelham, 1997; Lewis et al., 2000), similar mechanisms in animal cells are only partially understood. In some cases, SNARE sorting to the correct compartment has been shown to depend on the trans-membrane domains (Rayner and Pelham, 1997); in the case of syntaxins present in the endosomal compartments, a dileucine based motif is thought to be required (Tang and Hong, 1999). Furthermore, 'accessory' proteins may modulate SNARE trafficking, as has been demonstrated for cellubrevin (Annaert et al., 1997). In this context, we have recently demonstrated that syn1A sorting from the Golgi complex and delivery to the plasma membrane required munc-18-1 (Rowe et al., 1999). Besides this finding, sorting motifs in the molecular structure of syn1A have not yet been revealed.

In order to gain further insight into the mechanisms of syn1A sorting, we have recently investigated the intracellular transport of the t-SNARE in epithelial cells. This cell type has been selected for two main reasons. First, polarized epithelial cells have been extensively used to characterize the sorting mechanisms of many plasma membrane proteins (including the syntaxins; Low et al., 1996; Delgrossi et al., 1997; Galli et al., 1998; Lafont et al., 1999; Riento et al., 1998; Riento et al., 2000) and to investigate the targeting of neuronal plasma membrane proteins, since their apical and basolateral domains are thought to parallel the axonal and dendritic compartments of neurons (Winckler and Mellman, 1999; Mostov et al., 2000). Secondly, the neuronal specific t-SNARE syn1A is also expressed in certain epithelial cells (e.g. colon epithelial cells and native intestinal epithelia), where it modulates the function of the cystic fibrosis transmembrane conductance regulator (CFTR; Naren et al., 1997; Naren et al., 1998; Naren et al., 2000). However, the cellular distribution of syn1A in these cells is not well characterized since the low amount of the endogenous protein has impaired a detailed investigation. On the other hand, after overexpression in Madin-Darby canine kidney (MDCK) cells, syn1A is retained in a degradative compartment rather than transported to the cell surface (Low et al., 1996). In the light of our previous results (Rowe et al., 1999), we decided to investigate the transport and plasma membrane targeting of syn1A after expression with munc-18 proteins in MDCK and in human colon adenocarcinoma Caco-2 cells.

The data in this study indicate that (1) the delivery of syn1A

to the cell surface of epithelial cells requires munc-18-1 and (2) the interaction between the two proteins occurs when syn1A is still in the biosynthetic pathway, at the level of the Golgi complex. Moreover, the isoform of sec-1 constitutively expressed in MDCK and Caco 2 cells, munc-18-2 (Hata and Südhof, 1995; Riento et al., 1998; Riento et al., 2000), when expressed in sufficient amounts, is also capable of restoring the transport of the t-SNARE to the plasma membrane. Finally, syn1A is delivered to both the apical and basolateral domains of polarized MDCK and Caco-2 cells.

MATERIALS AND METHODS

Expression vectors and antibodies

pCMV syn1A and pCR3-syn3 were provided by M. Bennett (Bennett et al., 1993) and T. Galli (Galli et al., 1998), respectively. The pCDNA-munc-18-1 (also named rbSec-1) was prepared as previously described (Rowe et al., 1999). The munc-18-2 cDNA was excised from pGEX-KG-munc-18-2 (provided by T. Südhof; Hata and Südhof, 1995) by *Bam*HI, *Xho*I digestion, and direct ligation into *Bam*HI, *Xho*I-digested pCDNA3. Polyclonal antibodies against munc-18-1 were raised in rabbits using a synthetic peptide encoding amino acids 463-488 of rat munc-18-1 coupled to keyhole-limpet hemocyanin, as described by Liu et al. (Liu et al., 1979). The specificity of the antiserum against human and dog tissues was tested by immunoblotting (see below). The other monoclonal and polyclonal antibodies were obtained from the following sources: anti-syn1A (HPC-1) and anti-actin antibodies, from Sigma Chemical Co. (St Louis, MO, USA); anti-caveolin-1 (N-20), from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); anti-transferrin receptor from Zymed (San Francisco, CA, USA); anti-munc-18-2, anti-syn3, anti-giantin anti-human galactosyltransferase and anti- α -subunit of the Na⁺/K⁺ ATPase were kind gifts of Drs V. Olkkonen (Riento et al., 1998), T. Galli (Galli et al., 1998), M. Renz (Linstedt et al., 1995), E. G. Berger (Berger et al., 1981) and G. Pietrini, respectively. The fluorescent and peroxidase-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA) and Sigma, respectively.

Cell culture and transfection

MDCK (strain II) and Caco-2 cells were grown in complete medium (Minimal Essential Medium (MEM) supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin) in 5% CO₂, and were transfected according to the protocol described (Rowe et al., 1999) with minor modifications. Briefly, cells from two 75 cm² flasks at 60-70% confluency were resuspended in 200 μ l ice-cold Dulbecco's modified MEM containing a total of 15 μ g cDNA and electroporated at 250 mV, 960 μ F. After a 10 minute incubation on ice, the cells were resuspended in complete MEM and centrifuged on a cushion of Ficoll (Ficoll Plus, Amersham Pharmacia Biotech Italia, Milan, Italy) to eliminate dead cells and cell debris. The live cells, at the interface between the two solutions, were washed in complete medium and plated in dishes or on glass coverslips. To investigate protein distribution in polarized monolayers, electroporated cells were plated onto Transwell filters (Costar, Corning Inc., NY, USA) and analyzed 3-7 days later.

Immunofluorescence

The cells grown on coverslips were immunostained as previously described (Rowe et al., 1999) and, after antibody labeling, mounted onto glass slides in 90% glycerol in phosphate-buffered saline (PBS) containing 1 mg/ml phenyldiamine. The cells grown on Transwell filters were similarly processed and at the final stage, the filter was cut away from the support and mounted in 1% phenyldiamine on glass

slides and covered with a coverslip. In some cases, unfixed, polarized cells were incubated at 4°C for 20 minutes with biotinylated concanavalin A, washed in ice-cold PBS and labeled with a streptavidin-fluorophore conjugate (Amersham Pharmacia Biotech). These cells were then fixed and permeabilized for antibody immunolabeling as described (Rowe et al., 1999). The images were collected using an MRC-1024 laser scanning microscope (Bio-Rad Laboratories, CA, USA) and processed using Photoshop 4 (Adobe Systems, Mountain View, CA, USA) as described (Rowe et al., 1999).

Tissue and cell extracts

Frozen human and dog tissue pieces were crushed into a fine powder on dry ice and homogenized in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.5% (w/v) Triton X-100 and protease inhibitors (0.5 mM phenylmethyl sulfonylfluoride, 2 µg/ml pepstatin and 5 µg/ml trypsin inhibitor). Confluent monolayers of transiently transfected cells were scraped from culture dishes and homogenized in 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM EDTA, 1% (w/v) Triton X-100 and protease inhibitors. After incubation on ice for 30 minutes, tissue and cell extracts were centrifuged at 800 *g* for 3 minutes in an Eppendorf centrifuge to eliminate debris and the protein concentration was determined by Bio-Rad protein assay. Samples were then analyzed by western blotting.

Detergent solubilization and sucrose flotation gradients

Detergent-insoluble complexes were isolated as previously described (Brown and Rose, 1992; Kurzchalia et al., 1992; Fiedler et al., 1993). Polarized monolayers of MDCK cells in 35 mm culture dishes, transiently cotransfected with syn1A and munc-18-1, were lysed in 300 µl of either 1% (w/v) Triton X-100 or 20 mM CHAPS (in 50 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5 mM EDTA, supplemented with protease inhibitors). After 30 minutes on ice, 150 µl of total cell lysates were centrifuged at 16,000 *g* for 5 minutes, and the remainder was centrifuged at 100,000 *g* for 1 hour in an Optima TL ultracentrifuge (Beckman Instruments Inc., Fullerton, CA, USA). The low-speed and high-speed pellets were washed once with ice-cold 1% (w/v) Triton X-100 or 20 mM CHAPS buffer and solubilized in 150 µl of SDS-sample buffer (Laemmli, 1970). Volumes of Triton or CHAPS supernatants containing 20 µg of proteins and equal volumes of the corresponding resuspended pellets were separated by SDS-PAGE. For flotation gradients, the method of Röper et al. (Röper et al., 2000) was followed. Briefly, the cells were collected in 225 µl buffer A (150 mM NaCl, 2 mM EGTA, 50 mM Tris-HCl, pH 7.5, supplemented with 2 µg/ml aprotinin), and lysed by addition of Triton X-100 to a final concentration of 1% (w/v) on ice for 30 minutes. An equal volume of buffer A containing 2.4 M sucrose was added to the lysate and placed in a centrifuge tube. The following sucrose solutions (all prepared in buffer A), were overlaid onto the sample: 1 ml 0.9 M, 0.5 ml 0.8 M, 1 ml 0.7 M and 1 ml 0.1 M. The discontinuous gradient was centrifuged at 335,000 *g* for 16 hours in an ultracentrifuge (Beckman), after which 500 µl fractions were taken. The proteins in 400 µl of each fraction were precipitated overnight at -20°C after addition of 4 volumes of ice-cold acetone containing 3 µg of haemoglobin as a carrier. The precipitates were pelleted at 8000 *g*, dried briefly and resuspended in SDS-sample buffer and analysed by SDS-PAGE and western blotting.

SDS-PAGE and western blotting

Tissue and cell samples were subjected to SDS-PAGE on either 7.5, 10 or 12% acrylamide gels and then blotted onto 0.45 µm pore size nitrocellulose membrane (Sartorius AG, Göttingen, Germany). Immunolabeling was carried out as previously described (Corradi et al., 1996). Briefly, blots were first blocked overnight in 5% not-fat milk in Tris-buffered saline (TBS), washed in labeling buffer (5% not-fat milk, 0.3% Tween 20 in TBS), and then incubated for 2 hours in the primary antibody diluted in the labeling buffer. The blots were washed and then incubated with the appropriate secondary antibodies

conjugated to peroxidase. After another series of washes, peroxidase was detected using chemiluminescent substrates (Pierce, Rockford, IL, USA). The levels of proteins in the various samples (total extracts or detergent supernatants and pellets) were quantitated by measuring the density of the bands. Autoradiograms showing the appropriate band intensities were acquired by means of an ARCUS II scanner (Agfa-Gevaert NV, Mortsel, Germany) and the density of each band was quantitated using the NIH Image program 1.61 (National Technical Information Service, Springfield, VA, USA).

RESULTS

Previous studies have shown that the HT-29 intestinal epithelial cell line expresses syn1A and munc-18-1, though at low levels (Naren et al., 1997). This result prompted us to use western blotting to investigate the expression of the two proteins in MDCK and Caco-2 cells. To analyze the presence of syn1A in total cell and tissue extracts, we used a monoclonal antibody directed against rat syn1 (anti-HPC-1) that specifically recognizes a doublet of 35 and 38 kDa corresponding to syn1A and B, respectively. These closely related isoforms have been found to have a distinct expression pattern in various regions of the nervous system and in neuroendocrine tissues (Ruiz-Montasell et al., 1996). As shown in Fig. 1A, in tissue extracts from human cerebellum and canine brain cortex, the anti-syn1 antibody strongly immunodecorated one band with an apparent molecular mass of about 38 kDa, but no immunoreactive proteins were identified in a human meningioma or in canine liver extracts. Similarly, syn1A or B were not immunodetected in either Caco-

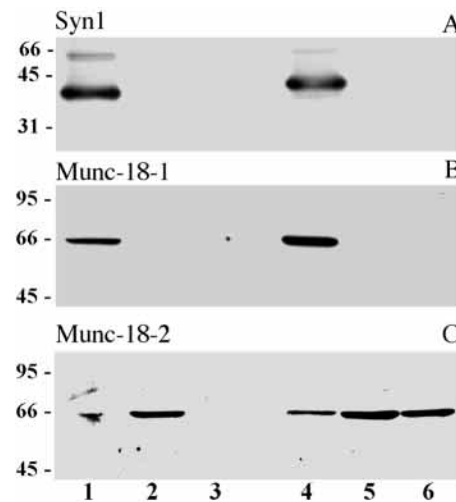


Fig. 1. Syn1A and munc-18-1 are not immunodetected in epithelial cell lines. Total tissue and cell extracts were prepared from human cerebellum (lane 1), human colon carcinoma derived Caco-2 cells (lane 2), human meningioma (lane 3), canine brain cortex (lane 4), MDCK cells (lane 5) and canine liver (lane 6). Equal amounts of protein were separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose and probed with an anti-syn1A monoclonal antibody (Syn1; A), a polyclonal antibody raised against a synthetic peptide of the rat munc-18-1 (Munc-18-1; B) sequence and an anti-munc-18-2 antiserum (Munc-18-2; C). Endogenous syn 1 and munc18-1 could be detected in the human and canine neuronal tissues, whereas the more widespread munc-18-2 protein was immunodetected in all tissues examined with the exception of human meningioma. The positions of molecular mass markers are shown.

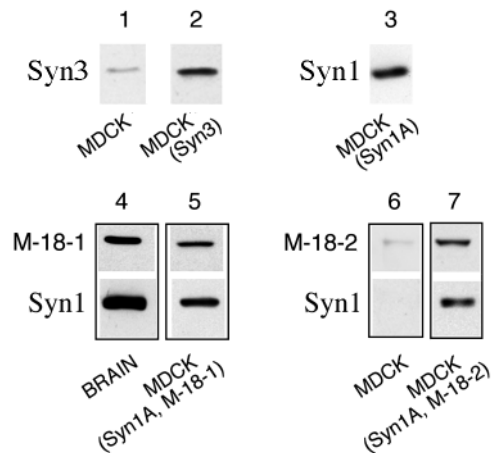
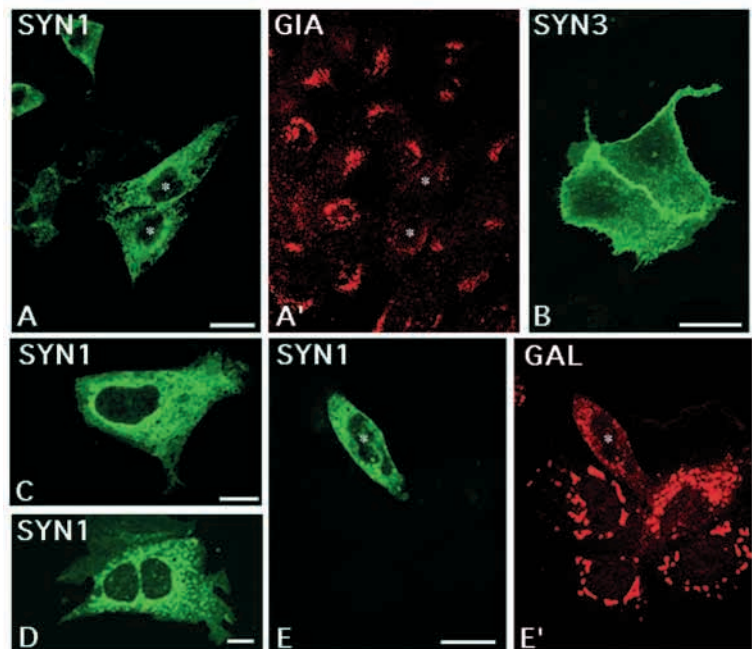


Fig. 2. Levels of expression of syntaxins and munc-18 proteins in transfected epithelial cells. Total rat brain extract (lane 4, 15 μ g of protein), and total cell extracts (40 μ g of protein) prepared from MDCK cells non-transfected (lanes 1 and 6) or transfected with either syn3 (Syn3, lane 2), syn1A (Syn1A, lane 3), syn1A and munc-18-1 (Syn1A, M-18-1, lane 5), or syn1A and munc-18-2 (Syn1A, M-18-2, lane 7), were analyzed by western blotting using antibodies against syn3 (Syn3, lanes 1, 2), syn1 (Syn1, lanes 3,4-7), munc-18-1 (M-18-1, lanes 4 and 5) or munc-18-2 (M-18-2, lanes 6 and 7). After cDNA transfections, syn1A (lane 3) or syn1A and munc-18-1 (lane 5) are immunodetected in MDCK cells. Note the increased levels of syn3 after transfection (compare lanes 1 and 2). Similarly, after cotransfection of munc-18-2 with syn1A (lane 7) the levels of munc-18-2 are also largely increased compared to untransfected MDCK cells (compare lanes 6 and 7).

2 or MDCK cells. In order to analyze the endogenous expression of sec-1 related proteins, we used a well-characterized antibody against the ubiquitous isoform of munc-18, munc18-2 (Hata and Südhof, 1995; Riento et al., 1998; Riento et al., 2000), and an antibody that we raised against a synthetic peptide encoding amino acids 463-488 of rat munc-18-1 (Fig. 1B,C). The munc-18-2 antibody revealed a protein, with an apparent molecular mass of 66 kDa, mainly expressed in epithelial cells and in dog liver (Fig. 1C). The antibody raised against rat munc-18-1 specifically recognized a band of a similar size (66 kDa) in human and dog neuronal tissues, but no immunoreactive bands were detected in the epithelial cells or liver (Fig. 1B).

Taken together these results demonstrate that Caco-2 and MDCK cells do not express detectable amounts of syn1 and munc-18-1.

Fig. 3. In transfected epithelial cells, syn1A, but not syn3, is retained intracellularly in the absence of munc18-1. Non-polarized MDCK (A,A',B) and Caco-2 cells (C,D,E,E') were fixed 24 hours after transfection with syn1A (A,A',C,D,E,E'), or syn3 (B). The cells were immunolabeled using an anti-syn1 monoclonal antibody (B,C) or polyclonal antibodies against syn3 (D). In A,A' and E,E' the cells were double immunolabeled using the monoclonal antibodies against syn1 and a polyclonal antibodies against giantin (A' GIA) and galactosyltransferase (GAL, E'). The cells were analyzed by confocal immunofluorescence microscopy. In A,A' and E,E', asterisks indicate syn 1A transfected cells showing a reticular-like distribution of the t-SNARE (in A) and Golgi complex dispersion (A'). Bars, 20 μ m (A,A',D,E,E'); 10 μ m (B,C).



Expression of syntaxin 1A and munc-18 isoforms in epithelial cells

In order to investigate the transport and targeting of syn1A, epithelial cells were transfected with the different vectors as detailed in Materials and Methods. The cellular distributions of syn1A and, as a control, of endogenous or overexpressed syn3, were investigated.

After electroporation, about 50% of the cells were found to express the exogenous proteins, as determined by immunofluorescence analysis (data not shown). In order to analyze the expression levels of the various exogenous proteins, total cell extracts from transfected or non-transfected MDCK cells and, as a control, rat brain were separated by SDS-PAGE and analyzed by immunoblotting (Fig. 2). Syn3 is endogenously expressed in wild type MDCK and Caco-2 cells (Fig. 2, lane 1; see also Low et al., 1996; Delgrossi et al., 1997; Galli et al., 1998) and its level in transfected cells increased about six times in comparison with the untransfected cells. After cDNA electroporation, a band corresponding to syn1A was clearly detected in the cell extracts (Fig. 2, lane 3). The coexpression of syn1A and munc-18-1 was also confirmed by immunoblotting (Fig. 2, lane 5) and, most importantly, the ratio between the exogenously coexpressed proteins was found to be similar to that observed for the endogenous syn 1 and munc-18-1 in rat brain extracts processed in parallel (Fig. 2, compare lanes 4 and 5). Finally, in cells transfected with syn1A and munc-18-2, the levels of the t-SNARE were similar to that detected after syn1A and munc-18-1 expression while the total amount of munc-18-2 in the transfected cell samples was significantly higher than that found in untransfected samples processed in parallel, thus confirming the coexpression of syn1A and munc-18-2 (Fig. 2, compare lanes 6 and 7).

Localization of syntaxins and munc-18 proteins in non-polarized epithelial cells

We then investigated the cellular distribution of overexpressed syn1A (Fig. 3A-C) and syn3 (Fig. 3D). After cDNA

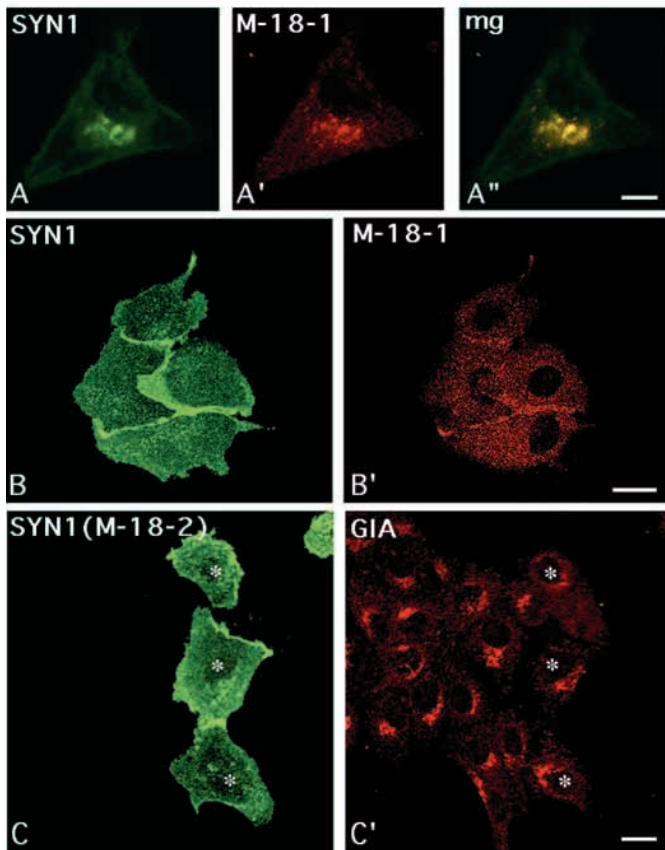


Fig. 4. Syn1A transport to the cell surface is rescued by coexpression of munc-18 proteins. Non-polarized MDCK cells were fixed 5 (A-A'') or 48 hours (B-C') after the coexpression of syn1A and munc-18-1 (A-B') or munc-18-2 (C,C'). Cells were double-immunolabeled using the anti-syn1 (SYN1) antibody together with the anti-munc-18-1 (M-18-1), or anti-giantin (GIA) polyclonal antibodies. In C,C', asterisks label cells with syn1A at the plasma membrane and no disturbance of the Golgi complex. Bars, 10 μ m.

electroporation, MDCK (Fig. 3A,A',D) and Caco-2 (Fig. 3B,C) cells were plated on coverslips, fixed at different times (3-96 hours), labeled using appropriate primary and secondary antibodies, and then analyzed by scanning laser confocal microscopy (Rowe et al., 1999). At early time points of protein expression (3-5 hours), syn1A was detected in the Golgi complex (data not shown; Rowe et al., 1999). After longer times (24-96 hours) over 90% of the positive cells showed a distribution of syn1A in a reticular-like cytoplasmic structure characteristic of the ER, and in dot-like structures presumably corresponding to autophagic vacuoles and/or lysosomes (Fig. 3A,C,D). Moreover, the juxtannuclear immunofluorescence pattern of proteins markers of the Golgi complex, giantin and galactosyltransferase, were lost in MDCK (Fig. 3A,A') and Caco-2 transfected cells (Fig. 3E,E'). In line with previous data (Rowe et al., 1999), these results indicate that syn1A, in the absence of munc-18-1, is accumulated first in the Golgi complex of MDCK and Caco-2 cells, and later in the ER due to the disassembly of the Golgi cisternae. These effects are specific for the neuronal isoform of syntaxins, since overexpressed syn3 appeared to be normally transported to the plasma membrane in transfected MDCK cells (Fig. 3B).

We then investigated the transport of syn1A after

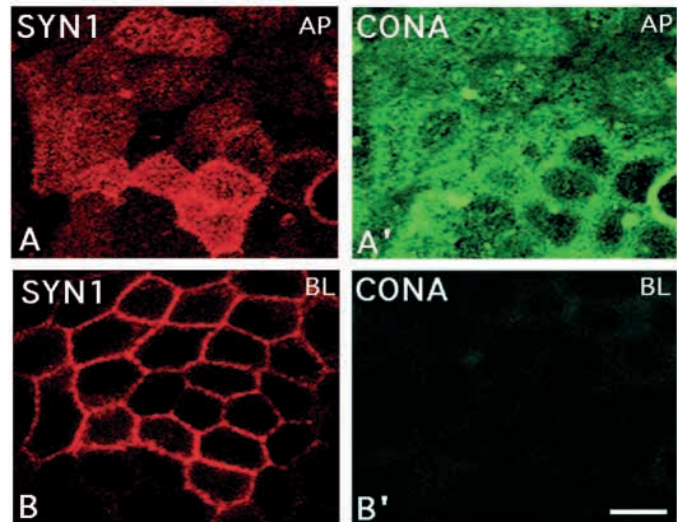


Fig. 5. Localization of syn1A at the apical and basolateral domains of polarized MDCK cells. MDCK cells cotransfected with syn1A and munc-18-1 were grown on permeable filters for 4 days. Before fixation the apical surface of cell monolayer was labeled using concanavalin A (CONA, A',B') as described in Materials and Methods. Cells expressing syn1A were detected using the anti-syn1 antibody followed by secondary antibody conjugated to rhodamine (SYN1, A,B). Consecutive horizontal optical sections through the cell monolayers were recorded by means of confocal fluorescence microscopy at 0.5 μ m intervals. Sections are shown at the level of the apical surface (AP, A,A') and the nucleus (BL, B,B'). Note the presence of syn1A at both the apical and basolateral sides of the expressing cells. Bars, 10 μ m.

coexpression with munc-18-1. Electroporated cells were fixed and analyzed by confocal microscopy at different times after plating. In line with previous results, at 3 hours the majority of the expressing cells showed syn1A immunostaining in the perinuclear region with a distribution coinciding with that of Golgi markers (data not shown and see Rowe et al., 1999). Interestingly, at 3-4 hours after transfection munc-18-1 was found to colocalize with syn1A in the perinuclear region, suggesting the presence of both proteins in the Golgi complex (Fig. 4A-A'). The localization of munc-18-1 on the Golgi cisternae occurred only in the presence of syn1A; when transfected alone in MDCK cells, munc-18-1 showed a diffuse pattern of immunostaining at all time points examined (3-18 hours; data not shown). As shown in Fig. 4B,B', 36 hours after cotransfection the t-SNARE was no longer found accumulated intracellularly but was immunodetected at the cell surface. The immunostaining pattern for munc-18-1 was widespread in the cells, as expected for a cytosolic protein, with some degree of colocalization with syn1A at the cell surface. Taken together these data strongly suggest that interaction of munc-18-1 with syn1A occurs in the exocytic pathway (most likely in the Golgi complex) and is required for syn1A transport to the plasma membrane.

Syn1A is known to associate with other munc-18 isoforms including munc-18-2 (Hata and Südhof, 1995), although it is not clear whether these proteins are functional partners of the neuronal isoform of syntaxins *in vivo*. Munc-18-2 is expressed in MDCK cells where it is known to participate with syn3 in apical membrane trafficking (Riento et al., 1998; Riento et al.,

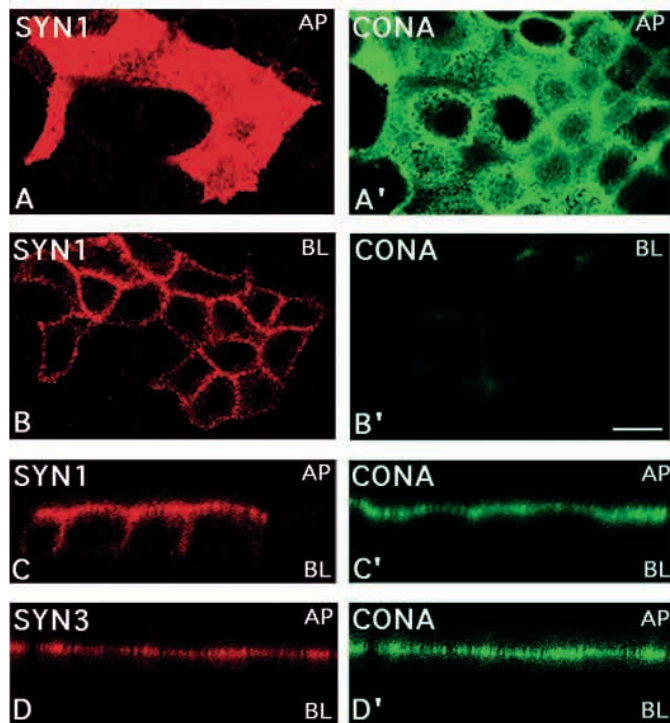


Fig. 6. Distribution of syn1A and syn3 in polarized Caco-2 cells. Caco-2 cells cotransfected with syn1A and munc-18-1 (A-C' or untransfected (D,D')) were grown on permeable filters for 7 days. Transfected cells were double-immunolabeled for syn1A (SYN1, A-C), and untransfected cells were stained for syn3 (SYN3, D). In all cases the apical cell membrane was labeled using concanavalin A (CONA, A'-D'). Consecutive horizontal optical sections through the cell monolayers were recorded as described in Fig. 5. In C-D', confocal sections in the vertical plane are shown with the apical side of the cell monolayer at the top. Note the presence of syn1A at both the apical (AP) and basolateral (BL) membranes, whereas endogenous syn3 appears exclusively at the apical side. Bars, 10 μ m.

2000). Our data, however, indicated that endogenous munc-18-2 was unable to rescue the transport of syn1A to the cell surface. We supposed that the levels of endogenous munc-18-2 were insufficient to assist in the transport of overexpressed syn1A. To test this hypothesis MDCK cells were cotransfected with syn1A and munc 18-2. After overexpression of munc-18-2, syn1A was mainly detected at the cell surface and the Golgi structure appeared to be protected, as revealed by double-labeling with giantin (see Fig. 4C,C'). These results indicate that munc-18-2 can assist syn1A intracellular transport and may be a functional partner of syn1A *in vivo*.

Sorting of syntaxin1A in polarized epithelial cells

Since syn1A transport to the cell surface is rescued by coexpression with munc-18 proteins, we next investigated the sorting of the t-SNARE in polarized monolayers. MDCK (Fig. 5, Fig. 7) or Caco2 cells (Fig. 6) were cotransfected with syn1A and munc-18-1 (Fig. 5, Fig. 6, Fig. 7) or munc-18-2 (Fig. 7), and grown for 3-5 days on permeable filters or coverslips. In order to verify that the cells were capable of forming tight impermeable junctions after munc-18 and syn1A overexpression, monolayers were incubated at 4°C with concanavalin A before fixation and in some experiments cells

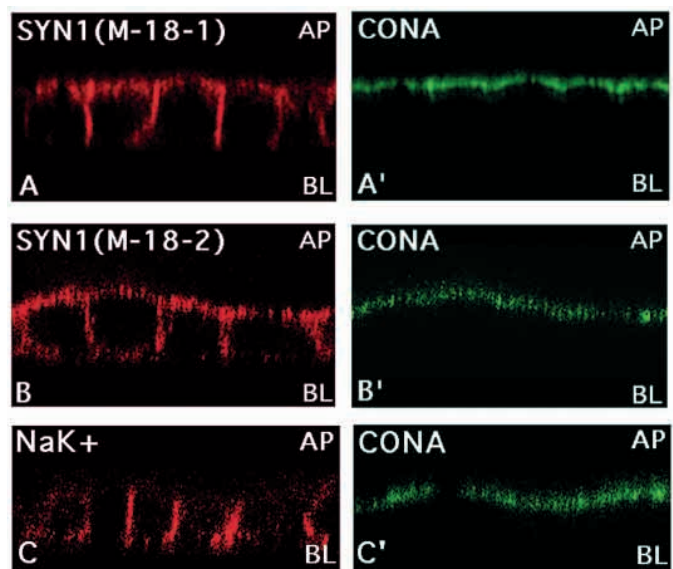


Fig. 7. Coexpression of munc-18-2 with syn1A does not alter the localization of the t-SNARE in polarized MDCK cells. Cells were cotransfected with syn1A and munc-18-1 (SYN1(M-18-1), A,A', C,C') or munc-18-2 (SYN1(M-18-2), B,B'). The polarized monolayers were grown as described in Fig. 5. The cells were immunolabeled for syn1A (A,B) or the α -subunit of the Na^+/K^+ ATPase (C) whereas the apical membrane was stained with concanavalin A (CONA, A',B',C'). Confocal sections in the vertical plane are shown with the apical surface (AP) of the cells at the top.

were labeled for the marker of the basolateral domain, Na^+/K^+ ATPase. As shown in Fig. 5, Fig. 6, Fig. 7, the staining for the lectin was restricted to the apical surface as observed in the horizontal and in the vertical sections collected in different cell monolayers, whereas the immunolabeling for the α -subunit of the Na^+/K^+ ATPase was restricted to the basolateral domain, thus suggesting that polarized cell monolayers had been formed. Under these conditions, and in the presence of munc-18-1, syn1A was found at both the basolateral and apical cell surfaces of polarized cells as observed in the horizontal sections collected at different heights throughout the cells (Fig. 5, Fig. 6A,B) and vertical sections (Fig. 6C). In contrast, the endogenous syn3 was mainly found at the apical domain of Caco-2 cells (Fig. 6D) as previously described (Delgrossi et al., 1997; Galli et al., 1998).

Since munc-18-2 is known to interact with syn3 at the apical domain of epithelial cells, we investigated whether it could influence the cell surface targeting of syn1A. MDCK cells were cotransfected with syn1A together with munc18-1 or munc-18-2 and then grown for 5 days until the formation of polarized monolayers. Immunolabeling for syn1A revealed that, in both cases, the t-SNARE is distributed to the apical and basolateral domains (Fig. 7).

In order to gain insights into the mechanisms underlying the targeting of the syn1A/munc18 complex to the plasma membrane, we analyzed whether syn1A partitions into the detergent-insoluble sphingolipid and cholesterol-enriched complexes (rafts) that are thought to function in apical sorting (Simons and Ikonen, 1997). Since the raft-associated proteins are not solubilized during treatment with detergents such as Triton X-100 and CHAPS at 4°C (Brown and Rose, 1992;

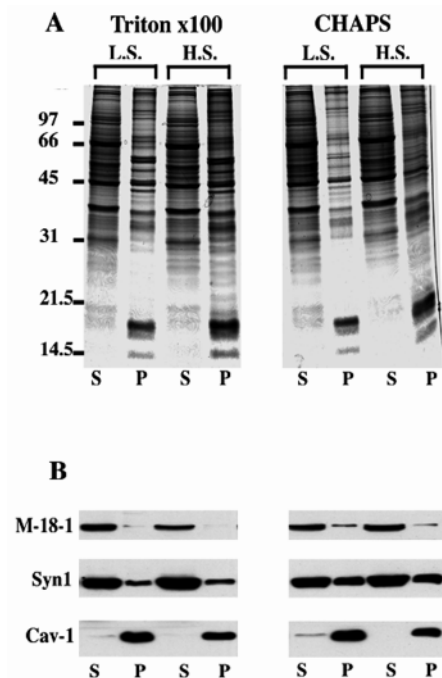


Fig. 8. Detergent insolubility. MDCK cells cotransfected with syn1A and munc-18-1 (A-B') were grown on permeable filters for 5 days and then homogenized at 4°C in buffer containing either 1% (w/v) Triton X-100 or 20 mM CHAPS. Total homogenates were subjected to centrifugation at 15,000 *g* for 5 minutes (L.S. = low speed) or 100,000 *g* for 1 hour (H.S. = high speed). The supernatants (S) and corresponding pellets (P) were analyzed by SDS-PAGE. After electrophoresis proteins in the gels were either stained with Coomassie Blue (A) or transferred to filters (B) and probed with anti-munc-18-1 (M-18-1), anti-syn1A (Syn1) and caveolin 1 (Cav-1) antibodies.

Kurzchalia et al., 1992; Fiedler et al., 1993), monolayers of MDCK cells transfected with syn1A and munc-18-1 were solubilized with buffers containing these non-ionic detergents. The insoluble proteins were isolated by means of low- and high-speed centrifugation. The proteins in the supernatants and pellets were separated by SDS-PAGE and analyzed by western blotting. As shown in Fig. 8, the pattern of proteins in the Triton- and CHAPS-insoluble fraction appeared to be qualitatively similar. The distribution of syn1A and munc-18-1 in the samples was analyzed by immunoblotting and compared to that of caveolin 1, a major component of the caveolae membrane coat, which has been shown to form oligomers insoluble in nonionic detergents (Rothberg et al., 1992; Kurzchalia et al., 1992). In line with these previous observations, caveolin-1 was immunodetected almost exclusively in the pellets. A small but significant portion of syn1A (about 10%) was found in the Triton-insoluble pellets. An even larger amount of the t-SNARE (about 30%) was sedimented in the pellets after solubilization with CHAPS. Next we analyzed the low density, Triton X-100-insoluble complexes from MDCK cells transfected with syn1A and munc-18-1 or syn1A using a flotation-equilibrium sucrose density gradient (Röper et al., 2000). Western blot analysis of the fractions probed with anti-syn1A antibodies demonstrated that, in syn1A and munc-18-1 cotransfected cells, a portion of syn1A floats in a low density fraction, which also contains

1	2	3	4	5	6	7	8	P	Fraction
0.17	0.33	0.50	0.66	0.80	0.95	0.95	1.2		Sucrose (M)

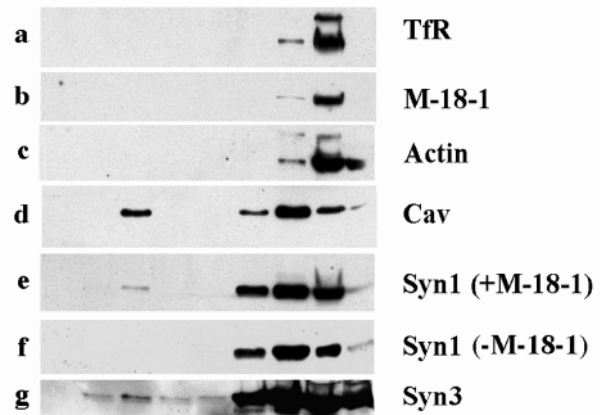


Fig. 9. Discontinuous sucrose flotation gradients. MDCK cells transfected with either syn1A and munc-18-1 (a-e) or syn 1A alone (f) and untransfected Caco-2 cells (g) were lysed at 4°C in 1% (w/v) Triton X-100 and then loaded at the bottom of a discontinuous sucrose gradient. After centrifugation proteins in the collected fractions (1-8) and the pellets (P) were separated by SDS-PAGE, blotted onto a membrane and analyzed, using specific antibodies, for the distribution of transferrin receptor (TfR), munc-18-1 (M-18-1), actin, caveolin-1 (Cav), syn1A (Syn1) and syn3 (Syn3). Note that in the presence of munc-18-1 (+M-18-1), a portion of syn1A floats in a low density fraction, which also contains caveolin-1 and syn3.

caveolin-1. On the other hand, when the t-SNARE was transfected alone, the amount of protein present in the low density fraction was largely reduced, suggesting that the correct transport of syn1A, in the presence of munc-18-1, coincides with its partial association to lipid rafts (Fig. 9). As a control we analyzed the distribution in the gradient of syn3, which has been reported to be associated to rafts (Lafont et al., 1999). Caco-2 cells, which endogenously express detectable amounts of syn3 at the apical surface (Galli et al., 1998) were treated as described for MDCK cells and the Triton-insoluble complexes analyzed using similar flotation equilibrium sucrose density gradients. As shown in Fig. 9, a sample of syn3 was immunodetected in the lighter fraction of the gradient. In contrast to caveolin syn1A and syn3, transferrin receptor, actin and munc-18-1 were all found exclusively in non-raft, high density fractions.

DISCUSSION

The results of the present study show that syn1A delivery to the plasma membrane of epithelial cells requires munc-18 proteins. In MDCK and Caco-2 cells syn1A appearance at the cell surface is largely inhibited in the absence of munc-18-1. Initially the t-SNARE is accumulated in the Golgi complex and seems to be responsible for the subsequent disassembly of this organelle followed by retention in the ER of newly synthesized proteins, as previously demonstrated (Rowe et al., 1999). Coexpression of munc-18-1 rescues the transport of syn1A to the cell surface. In addition, here we demonstrate that the ubiquitously expressed form of the munc-18 family, munc-18-

2, is also capable of restoring the cell surface transport of syn1A. Originally, it was reported that munc-18-2 binds to syn1A by yeast two-hybrid screening and *in vitro* assays (Hata and Südhof, 1995), but the functional significance of this interaction was unclear. Our data strongly suggest that munc-18-2 may interact with syn1A and play a role similar to that of munc-18-1 in the intracellular transport of syn1A. This function may be relevant in epithelial cells that express low levels of syn1A at the cell surface with the CFTR (Naren et al., 1998; Naren et al., 2000). Since the association of syn1A with the CFTR closely depends on munc-18-1, (which prevents the binding of the t-SNARE to the channel) our finding opens the possibility that munc-18-2 may also be involved in the regulation of the CFTR channel activity. Further analysis of the CFTR activity in the presence of syn1A and munc-18-2 is needed to support this hypothesis.

A further important aspect raised by our data is the nature of the molecular mechanisms by which munc-18 proteins function in the transport of syn1A from the Golgi to the plasma membrane. Recent work has demonstrated that syn1A can assume two different conformations: a so-called 'closed' structure, in which the alpha-helical domain at the N-terminus associates with a similar structure present at the C terminus, and an open conformation, in which the association between these two domains is abolished (Dulubova et al., 1999; Yang et al., 2000; Misura et al., 2000). The switch between these two molecular structures is required to expose the SNARE motif and allow the interaction between syn1A and cognate SNAREs. Since munc-18-1 binds to the closed structure of syn1A, it may function as a chaperone-like protein interacting with syn1A not only at the plasma membrane, but also in the biosynthetic pathway. Our data showing the colocalization of munc-18-1 with syn1A at the level of the Golgi complex (although we cannot exclude the possibility that the complex may be formed earlier, in the ER) strongly supports this hypothesis. Munc-18-1 may therefore allow the exit of syn1A from the Golgi by keeping the t-SNARE in the 'closed' conformation, and thus protecting unstructured syn1A regions and/or abolishing syn1A interaction with resident proteins of the Golgi complex.

In line with previous results (Rowe et al., 1999), the mistargeting of the neuronal t-SNARE in the Golgi complex of transfected epithelial cells induces inhibition of intracellular trafficking and the disassembly of the Golgi cisternae, as indicated by the dispersion of the Golgi-marker giantin in the cytoplasm. The mechanisms involved in this traffic disturbance are unknown. We speculate that, in the absence of munc-18 proteins, the neuronal t-SNARE acts by disturbing the function of other SNARE proteins involved in the Golgi vesicular traffic. If this occurs, presumably it does not imply a direct binding of syn1A with other syntaxins of the Golgi complex such as syn5 or 6, since anti-syn1A antibodies were unable to coimmunoprecipitate these syntaxin isoforms from MDCK cell expressing syn1A (J. Rowe and P. Rosa, unpublished results).

A further aim of this study was to investigate the plasma membrane targeting of syn1A in polarized epithelial cells in order to understand whether its distribution correlates with that of the apically sorted CFTR (Anderson et al., 1992). In addition we expected to obtain information on the general mechanisms of plasma membrane targeting of syn1A in neurons, since the axonal and dendritic domains are thought to parallel the apical

and basolateral compartments of polarized epithelial cells (Winckler and Mellman, 1999, and references therein). Here, we demonstrate that syn1A, at the steady state, is distributed to both the apical and the basolateral domains. We do not think that this unpolarized distribution of syn1A is simply due to protein overexpression since under similar conditions (i.e. in overexpressing MDCK cells), syn3 and syn4 maintain their polarized distribution, respectively, at the apical and basolateral plasma membrane (Low et al., 1996). Our data also suggest that specific munc-18 proteins are not involved in the syn1A targeting since the coexpression of syn1A with munc-18-2 (which is usually localized at the apical membrane with syn3; Riento et al., 2000), did not modify the distribution of the t-SNARE. Although we cannot exclude the absence of specific sorting signals in the syn1A sequence, its distribution in polarized epithelial cells suggests the presence of codominant apical and basolateral determinants. Syn1A partitioning in non ionic-detergent-insoluble complexes may indicate the involvement of lipids rafts in its apical delivery. A similar mechanism has been proposed for syn3 sorting to the apical domain of MDCK cells (Lafont et al., 1999). However, the precise nature of the molecular interaction of syn3 and syn1A with the rafts is unknown and further investigations are necessary for determining whether the trans-membrane domain of syn1A is involved in the targeting of the protein to the apical domain as suggested for other proteins (Scheiffele et al., 1997; Harder et al., 1998). Unlike syn3, syn1A is also found at the basolateral membrane, thus suggesting that it may also possess a basolateral sorting motif(s). Previous work has demonstrated that sorting to the basolateral domain depends on di-leucine- or tyrosine-based motifs located in the cytoplasmic domain of the proteins (Mostov et al., 2000, and references therein). The cytosolic domain of syn1A does not contain any di-leucine motifs but does contain five tyrosine residues. Interestingly, one of these residues is not present in the apically sorted syn3 but it is conserved in the cytosolic domain of the basolaterally sorted syn4. Alternatively, or in addition, scaffold proteins might be required for the plasma membrane localization of the t-SNARE. In epithelial cells PDZ-containing proteins are capable of forming highly ordered complexes involved in protein plasma membrane retention. Similar proteins have been shown to exist in neurons where they play a central role in the organization of pre- and post-synaptic domains (Fanning and Anderson, 1999). Interestingly, the PDZ containing proteins Mint 1 and 2, originally identified as binding partners of munc-18, were localized in the presynaptic region and thought to be responsible for the presynaptic localization of the SNARE machinery (Butz et al., 1998). Mint-orthologues have also been identified in epithelial cells of *C. elegans* where, together with other PDZ proteins, they define the basolateral localization of plasma membrane proteins (Kaech et al., 1998; Rongo et al., 1998).

Although further studies are needed to clarify the mechanisms of syn1A targeting, our results show that the distribution of syn1A in MDCK and Caco-2 cells mimics the unpolarized distribution of the protein in neurons. Indeed syn1A is located not only in the axons but also in the soma and dendrites (Garcia et al., 1995; MacMahon et al., 1995). This finding suggests that neurons and epithelial cells may sort the neuronal isoform of syntaxins using a similar machinery. The comprehension of syn1A sorting mechanisms in epithelial cells

may provide a good starting point to understand how the analogous process occurs in neurons.

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