Blockade of membrane transport and disassembly of the Golgi complex by expression of syntaxin 1A in neurosecretion-incompetent cells: prevention by rbSEC1

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Abstract

The t-SNAREs syntaxin1A and SNAP-25, i.e. the members of the complex involved in regulated exocytosis at synapses and neurosecretory cells, are delivered to their physiological site, the plasma membrane, when transfected into neurosecretion-competent cells, such as PC12 and AtT20. In contrast, when transfection is made into cells incompetent for neurosecretion, such as those of a defective PC12 clone and the NRK fibroblasts, which have no endogenous expression of these t-SNAREs, syntaxin1A (but neither two other syntaxin family members nor SNAP-25) remains stuck in the Golgi-TGN area with profound consequences to the cell: blockade of both membrane (SNAP-25, GAT-1) and secretory (chromogranin B) protein transport to the cell surface; progressive disassembly of the Golgi complex and TGN; ultimate disappearance of the latter structures, with intermixing of their markers (mannosidase II; TGN-38) with those of the endoplasmic reticulum (calreticulin) and with syntaxin1A itself. When, however, syntaxin 1A is transfected together with rbSec1, a protein known to participate in neurosecretory exocytosis via its dynamic [...]
INTRODUCTION

Studies carried out during the last several years have revealed that the extraordinary fidelity of the fusion events taking place within eukaryotic cells to sustain the multiple pathways of membrane traffic depends on the interactions occurring among membrane proteins of the vesicular and target compartments. These proteins, the so called SNAREs, act as receptors for the soluble attachment proteins (SNAPs) and NEM-sensitive fusion factor (NSF; Söllner et al., 1993; Rothman, 1994; Rothman and Warren, 1994; Calakos et al., 1994; Bennett, 1995; Hay and Scheller, 1997; Jahn and Hanson, 1998). In mammalian cells, the first SNAREs to be identified were those of regulated exocytosis at synapses and neurosecretory cells, the vesicle (v) SNAREs, synaptobrevin (VAMP) 1 and 2, and the plasma membrane (target, t) SNAREs, SNAP-25 and syntaxin 1A (syn1A) and B (Söllner et al., 1993). These SNAREs are also known as the substrates of the toxins that block transmitter release, the chlostridial tetanus and botulinum neurotoxins (Schiavo et al., 1994; Ludger and Galli, 1998, and references therein). Additional SNAREs have later been identified and characterized by various degrees of homology with respect to the first (Hay and Scheller, 1997; Patel et al., 1998; Weimbs et al., 1998, and references therein). These SNAREs regulate traffic between various membrane compartments, including that from the ER to Golgi, between Golgi cisternae and from Golgi to lysosomes/endosomes (Dascher et al., 1994; Bock et al., 1996, 1997; Advani et al., 1998; Hay et al., 1998). At each of these sites the SNAREs are known to interact not only with SNAP and NSF but also with other proteins located in the fusing membranes and in the surrounding cytosol (Stiithof, 1995; Lin and Scheller, 1997; Rothman and Söllner, 1997). Among these, the proteins of the SEC-1 family (Aalto et al., 1992) have been shown to regulate

SUMMARY

The t-SNAREs syntaxin1A and SNAP-25, i.e. the members of the complex involved in regulated exocytosis at synapses and neurosecretory cells, are delivered to their physiological site, the plasma membrane, when transfected into neurosecretion-competent cells, such as PC12 and AtT20. In contrast, when transfection is made into cells incompetent for neurosecretion, such as those of a defective PC12 clone and the NRK fibroblasts, which have no endogenous expression of these t-SNAREs, syntaxin1A (but neither two other syntaxin family members nor SNAP-25) remains stuck in the Golgi-TGN area with profound consequences to the cell: blockade of both membrane (SNAP-25, GAT-1) and secretory (chromogranin B) protein transport to the cell surface; progressive disassembly of the Golgi complex and TGN; ultimate disappearance of the latter structures, with intermixing of their markers (mannosidase II; TGN-38) with those of the endoplasmic reticulum (caltrectulin) and with syntaxin1A itself. When, however, syntaxin 1A is transfected together with rbSec1, a protein known to participate in neurosecretory exocytosis via its dynamic interaction with the t-SNARE, neither the blockade nor the alterations of the Golgi complex take place. Our results demonstrate that syntaxin1A, in addition to its role in exocytosis at the cell surface, possesses a specific potential to interfere with intracellular membrane transport and that its interaction with rbSec1 is instrumental to its physiological function not only at the plasma membrane but also within the cell. At the latter site, the rbSec1-induced conversion of syntaxin1A into a form that can be transported and protects the cell from the development of severe structural and membrane traffic alterations.

Key words: SNARE, Membrane transport, Golgi complex, rbSec1/munc18-1

INTRODUCTION

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the interaction of syntaxins with their partners (Halachmi and Lev, 1996). In particular, the neuronal isoform, rbSec1 (Garcia et al., 1994, 1995) (also named Munc18-1, Hata et al., 1993; nSec1, Pevsner et al., 1994a; or mSec1, Hodel et al., 1994) is able to interact with syn1A in a highly regulated manner and to inhibit the formation of the SNARE complex by preventing its binding to SNAP-25 and synaptobrevin (Pevsner et al., 1994b).

An obvious prerequisite to the involvement of various SNAREs in fusion events is the specificity of their intracellular distribution. So far, however, the mechanisms of their targeting and/or trafficking, whether depending directly on their amino acid sequence, as occurs for the ER-resident t-SNARE of the yeast, Ufe1p (Rayner and Pelham, 1997) or on their interaction with sorting/accessory proteins, as in the case of cellubrevin export from the ER (Annaert et al., 1997), largely remain to be investigated. Also unexplored is the possibility that the SNAREs, when delivered to sites different from those of their specificity, become involved in local fusion events, as could be expected from the considerable homology existing among the family members (Weimbs et al., 1998, and references therein).

The SNAREs of regulated neurosecretion appear to be good candidates to investigate these problems. Unlike many others, that are widespread or ubiquitous, these SNAREs are expressed only by the cell types competent for transmitter release, i.e. neurons and neurosecretory cells where they control the exocytic fusion of synaptic and dense-core vesicles (SVs and DCVs). Moreover, the molecular aspects of the latter process are largely known (Südhof, 1995; Lin and Scheller, 1997; Ludger and Galli, 1998; Robinson and Martin, 1998), with ensuing opportunities for mechanistic studies in the field.

The present work has been carried out along these lines by expressing neuronal isoforms of SNAREs and accessory proteins in cell types that differ in terms of competence for neurosecretion. The competent cells employed were those of the neurosecretory lines, PC12 and AtT20; the incompetent cells were not only fibroblasts (NRK) but also those of a PC12 clone isolated in our laboratory (PC12-27) that, although maintaining multiple aspects of the parent line, lacks all the proteins specifically involved in regulated exocytosis (Clementi et al., 1992; Corrada et al., 1996). Among the SNAREs, the study was particularly focused on syn1A because previous expression studies in neurosecretion-incompetent cells had already documented its missorting to lysosomes (in MDCK cells, Low et al., 1996) and the slowing down of the constitutive release of a co-transfected peptide hormone, growth hormone (in COS cells, Bittner et al., 1996).

MATERIALS AND METHODS

Antibodies

Polyclonal and monoclonal antibodies recognizing the rat and the human chromogranin B (CgB) were raised as described previously (Rosa et al., 1985 and 1992, respectively). Other monoclonal and polyclonal antibodies were obtained from the following sources: monoclonal anti-FLAG M5 and anti-syn1A (HPC-1) from Sigma (Chemical Co., St Louis, MO, USA); anti-Sso2p from S. Keränen (VTT Biotechnology and Food Research, Espoo, Finland); polyclonal anti-GAT1 R24 from G. Pietrini (University of Milan, Italy); polyclonal anti-rbSec1, SNAP-25 and cellubrevin from P. De Camilli (Yale University, New Haven, CT, USA); polyclonal anti-syntaxin 3 from T. Galli (Institute Curie, Paris, France), anti-calreticulin from H. Söling (University of Gottingen, Germany), anti-mannosidase II from M. G. Farquhar (UCSD, La Jolla, CA, USA) and anti-TGN38 from G. Banting (University of Bristol, UK). Immunofluorescent and peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and Sigma, respectively.

Expression vector construction

The pFLAG2/syn1A expression vector was constructed by excision of the syn1A cDNA from the vector syntaxin 1A-3 in pGEX-KG (Bennett et al., 1993). Briefly, the EcoRI digested fragment containing the syn1A coding sequences was ligated into the HindIII site of the pFLAG-CMV2 vector (Eastman Kodak Company, New Haven, CT) following cohesive end fill-in of the vector and the insert, and a peptide containing the FLAG epitope (MDYKDDDDK) was thus inserted at the 5′ extremity in frame to amino acids 4 to 288 of syn1A (M95734, GenBank). The pCDNA3/Sso2p expression vector was constructed by excision of the Sso2p cDNA from the pSP72/Sso2p (Jäntti et al., 1994) by BamHI digestion and direct ligation into BamHI digested pCDNA3 (Invitrogen Corporation, 9351 NV Leek, The Netherlands). The pCDNA3/rbSec1 construct was prepared by HindIII and XbaI excision of the rbSec1 cDNA from the pRSV/rbSec1 vector (Garcia et al., 1994) and direct ligation into HindIII, XbaI digested pCDNA3 vector. The production of the expression vectors pCB6/GAT-1 (Pietrini et al., 1994) pCR3Syn3 (Galli et al., 1998) pCDNA3/SNAP-25 (Sadoul et al., 1995) and pREP4-CgB (Corrada et al., 1996) has been described previously.

Cell culture

Wild-type (clone 251) and mutant (clone 27) PC12 cells were maintained in DME medium containing 100 i.u./ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and supplemented with 10% horse serum, 5% fetal calf serum, at 37°C, 10% CO2. NRK and AtT20 cells were grown in the above DME medium supplemented with 10% fetal calf serum.

Transfections

Electroporation was carried out using the method described by Armstrong et al. (1996) with minor modifications. Briefly, 107 cells from a 60-80% confluent culture were trypsinized and resuspended in 400 μl DME medium supplemented with 2 mM glutamine together with 25 μg DNA for a single transfection, or a total of 30 μg for a cotransfection (1:1 ratio for syn1A:SNAP-25 cotransfection and 1:2 ratio for all other cotransfections). After incubation on ice for 10 minutes in a Bio-Rad Gene Pulser cuvette (0.4 cm electrode gap) the cells were electroporated with one shock at 250 mV, 960 μF, using a Bio-Rad Pulse Controller (Bio-Rad Laboratories GmbH, Munich, Germany), then incubated on ice for 10 minutes, resuspended in serum-containing medium and plated on poly-L-lysine-coated coverslips. In some experiments the number and volume of the cell aliquots, as well as the amounts of cDNA employed, were reduced by 50%. NRK cells were transfected using the Lipofectin system (Life Technologies Italia, Milan, Italy). A total of 2×105 cells were plated onto poly-L-lysine-coated coverslips, washed in serum free medium 24 hours later, and then incubated for up to 5 hours with a pre-equilibrated solution of 10 μl lipofectin and 1 μg of cDNA to a final volume of 1 ml in OptiMEM serum free medium. After extensive washing with the latter, the cells were returned to normal culture conditions.

Microinjection

PC12-251, PC12-27 and NRK cells, plated onto poly-L-lysine-coated coverslips, were microinjected as described previously (Rosa et al., 1989) using an Eppendorf micromanipulator 5171 connected to a Leitz inverted microscope. The cDNAs, solubilized in water, were used at concentrations ranging from 1.0 to 0.125 μg/ml.
**Immunofluorescence**

Between 2 and 48 hours after transfection, cells were fixed for 30 minutes in 3% formaldehyde. In some experiments cells were incubated for 2-4 hours in 10 µg/ml cycloheximide before fixation. Immunofluorescence was carried out as described by Rosa et al. (1989). The fixed cells were permeabilized with Triton X-100 and then incubated with the primary antibodies for 2 hours. After washing, the cells were incubated with an appropriate secondary antibody conjugated to LRSC or FITC, washed again, mounted with Mowiol on glass coverslips and visualized under oil immersion using a Zeiss Axiosplan immunofluorescence microscope. The cells to be labeled with the lectin concanavalin A (Con A, E.Y. Laboratories, San Matteo, CA, USA) were incubated in avidin and biotin solutions, treated for 30 minutes with biotinylated Con A (3 µg/ml in 10 mM Hepes, 0.15 M NaCl, 0.1 mM CaCl2, 0.1 mM Mn2SO4), washed in PBS, and labeled with a streptavidin-fluorophore conjugate (Amersham Life Science, Buckinghamshire, UK). The cells were then permeabilized and labeled with antibodies as described above.

For confocal microscopy the cells, fixed and stained as described above, were mounted in 90% glycerol/20 mM Tris-HCl, pH 8.5, containing 1 mg/ml of phenylene diamine. Images were collected in the MRC-1024 laser scanning microscope (Bio-Rad) with ×40 or ×60 objective lenses. The digitized images were analyzed using the Bio-Rad computer software. For comparison of double-stained patterns, images from the FITC and TRITC channels were acquired independently from the same area of sample and then superimposed. Images were processed using Photoshop 4 (Adobe Systems, Mountain View, CA, USA) and printed using an Epson Stylus color printer.

**Image processing and quantitative analysis**

The FLAG-syn1A expression levels were quantitated by measuring the fluorescence intensity in both the wild-type PC12-251 and the regulated secretion-incompetent PC12-27 cells transfected with identical amount of FLAG-syn1A vector. All samples were immunostained with a monoclonal antibody against syn1A (HPC-1) followed by anti-mouse IgG conjugated to FITC. The fluorescence signals, acquired at the confocal microscope with identical settings, were analyzed using the analysis programme Image 1.61 (National Technical Information Service, Springfield, VA/USA). The fluorescence intensity was measured in cells with boundaries marked manually. The number of cells measured per preparation varied from 70 to 150. The results were expressed as fold of fluorescence intensity increase versus that measured in parallel in non-transfected, wild-type PC12-251 cells.

**SDS-PAGE and western blotting**

Confluent cultures of PC12-251, PC12-27 and NRK cells, homogenized in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.3% Tween-20, were freeze-thawed once and then centrifuged to pellet insoluble material. Volumes of the supernatant containing 35 to 40 µg of total protein were subjected to SDS-PAGE on either 7.5 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). Blots were first blocked overnight in 5% non-fat milk in tris-buffered saline (TBS), washed in labeling buffer (5% non-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 (diluted 1:8,000) conjugated to peroxidase. After another series of washes, peroxidase was detected using chemiluminescent substrates (Pierce, Rockford, IL or Amersham Life Science).

**RESULTS**

Results of previous (Corradi et al., 1996; Burgoyne and Morgan, 1998) as well as of the present studies (Fig. 1) show that regulated secretion-competent cells, PC12 and ArT20, express considerable levels of all the SNAREs involved in the regulated exocytosis (syn1A, SNAP-25, VAMP-1 and 2) and of their accessory and modulatory proteins, such as synaptotagmin I, rab 3 and rbSec1, which in contrast are inappreciable in incompetent cells, such as PC12-27 and NRK cells (Fig. 1 and Corradi et al., 1996). The levels of other proteins of the SNARE families, including syntaxin 3, SNAP-23 and cellubrevin, which are known to be involved in constitutive secretion, endocytosis and membrane traffic (De Camilli and Takei, 1996; Hay and Scheller, 1997; Galli et al., 1998), were found to be similar in both neurosecretion-competent and -incompetent cells (Fig. 1). The latter cells therefore, do not appear widely defective in SNARE-mediated functions but simply devoid of neurosecretory regulated exocytosis. For simplicity, the SNAREs involved in the latter process will be indicated from hereon as the SNAREs.

**Expression and transport of transfected t-SNAREs, syn1A and SNAP-25, in neurosecretion-competent cells**

In order to establish the levels of SNAREs after cDNA transfection compared to those of the endogenous proteins, parallel cell samples were probed using anti-syn1A and anti-SNAP-25 antibodies. As the syn1A cDNA employed in the present work was inserted into the pFLAG/CMV2 vector, the distribution of the corresponding protein could be established using a monoclonal anti-FLAG antibody. Preliminary experiments revealed that, under the experimental conditions employed, transfection efficiency was 60±11.7% (n=3) in both wild-type and secretion-incompetent PC12. In wild-type cells, the ratio between transfected and endogenous syn1A remained almost always below 10, with about half of the cells expressing the transfected protein at levels of 5-fold or below. In PC12-27, the expression was in the same range as in transfected wild-type cells (Fig. 2). Similar results were obtained with SNAP-25 (not shown).

Parallel experiments carried out in two types of neurosecretion-competent cells, PC12 and ArT20 (Fig. 3, and data not shown), excluded any blocking or mistargeting of the t-
SNARE proteins occurring as a consequence of their overexpression or, in the case of syn1A, of the inclusion of the FLAG epitope. As revealed by immunofluorescence, using Con A as a marker for the plasma membrane, and by confocal microscopy (Fig. 3, and data not shown), 24 or 48 hours after transfection most of the t-SNAREs were in fact at the cell surface, with a distribution largely coinciding with that of endogenous syn1A and SNAP-25 (Fig. 3, compare A with B and C with D, respectively). Additional, small syn1A-immunolabeled puncta, concentrated primarily in a perinuclear region, most likely corresponded to the fraction of the protein in transit through the Golgi complex, since they could be chased to the surface by a 4 hour incubation with cycloheximide (not shown). We conclude that, in the cells where the t-SNAREs are endogenously expressed, the strategy developed to investigate the expression of syn1A and SNAP-25 does not induce any major unspecific damage. This approach was therefore employed to investigate the transport of the t-SNARE in neurosecretion-incompetent cells, that are naturally devoid of these proteins.

Fig. 2. Quantitation of syn1A expression in transfected PC12 cells. Wild-type (PC12wt) and neurosecretion-incompetent (PC12-27) cells were fixed before or 24 hours after transfection with the FLAG-syn1A vector. Cells were then immunolabeled using the monoclonal anti-syn1A followed by FITC-conjugated secondary antibodies. Images were collected under identical settings with a confocal microscope. Fluorescence intensity was assayed as described in Materials and Methods and expressed as fold increase with respect to the intensity measured in non-transfected, wild-type PC12 cells. Data are averages from a total of 70 (wild-type) and 150 (PC12-27) transfected cells prepared in two independent experiments.

Fig. 3. Distribution of syn1A and SNAP-25 in wild-type PC12 cells. Non-transfected (A,C), and syn1A- (B) and SNAP25 (D)-transfected wild-type PC12 cells, probed with antibodies against syn1A (HPC-1, 1:200), or SNAP-25 (1:250) followed by fluorochrome-conjugated secondary antibodies, were visualized by confocal immunofluorescence microscopy. A and C show the preferential localization of endogenous syn1A (SYN-ed) and SNAP-25 (SN-ed), respectively, at the plasma membrane. A similar distribution is shown also for the transfected FLAG-syn1A (SYN-ex, B) and SNAP-25 (SN-ex, D). Asterisks in B and D mark non-transfected cells. Selected images from three independent experiments for each construct are shown. Bars, 10 μm.
In neurosecretion-incompetent cells, syn1A, but not SNAP-25, is retained intracellularly and induces disassembly of the Golgi complex with blockade of protein trafficking to the cell surface

To obtain information about the fate of the t-SNAREs when expressed into incompetent cells and about the functional consequences of this expression, PC12-27 and NRK cells were examined from 2 up to 24 hours after cDNA transfection. In some experiments, in order to obtain a more accurate timing, especially at the early time-points of the protein expression, cDNA microinjection was also employed. At 2-3 hours, the majority of syn1A-positive cells clearly showed the protein almost exclusively accumulated in the Golgi complex (Fig. 4A-A'). Beginning however at 4-6 hours and at later time-points, over 90% of the positive cells showed a distribution of syn1A into reticular-like structures identified as the ER because of the large coincidence of its staining pattern with that obtained for the luminal ER protein, calreticulin (Fig. 4B-B'; Pezzati et al., 1997). In contrast, no detectable appearance of the t-SNARE was observed at the cell surface as revealed by double immunofluorescence using Con A (Fig. 4C-D'). These results were independent of the level of syn1A expression since they were observed in both low and high expressing cells. In the latter cells, however, syn1A labeling appeared not only in the...
Fig. 5. Expression of syn1A in regulated secretion-incompetent cells causes disassembly of the Golgi complex. PC12-27 cells were transfected for FLAG-syn1A. Fixed cells were double immunolabeled with anti-FLAG (A,C, SYN) together with either anti-TGN38 (A' and C', TGN) or anti-mannosidase II (1:200; B', MN) antibodies. (B-B' and C-C') Double labeled fields visualized by confocal fluorescence microscopy; (B'' and C'') the superimposed images (mg = merge). Six hours after transfection (A,A'), an extensive fragmentation of the Golgi complex can be observed in the syn1A-transfected cells (compare in A' the transfected cell labeled with an asterisk to the surrounding, non-transfected cells, arrowheads). Twenty-four hours after transfection (B and C lines) the mannosidase II (B'') and TGN38 (C'') immunostainings exhibit a diffuse, reticular distribution largely coinciding with that of transfected syn1A (yellow or orange staining in B'' and C''). Notice the classical structure of the Golgi complex in the cells not expressing syn1A (A',B',C', arrowheads) and the presence of syn1A-rich vacuoles in a fraction of those transfected for the SNARE (open arrow in C). Selected images from five independent experiments are shown. Bars, 10 µm.
Fig. 6. Syn1A expression in regulated secretion-incompetent cells inhibits transport of GAT-1. PC12-27 cells were either transfected only with the cDNA coding for GAT-1 (A,B) or cotransfected for GAT-1 (C) and FLAG-syn1A (C′). Eighteen hours after transfection the cells were fixed and immunolabeled either with anti-GAT (1:200; A and B) alone or with anti-GAT-1 (C) together with anti-FLAG (C′). Note in A and B the localization of GAT-1 not only within but also at the cell surface; and in C and C′ the diffuse reticular colocalisation of syn1A and GAT-1 within a cell expressing both proteins. Selected images from four independent experiments are shown. GAT: GAT-1. Bars, 10 µm.

ER but also in large intracellular structures (Fig. 4D), presumably corresponding to lysosomes and autophagic vacuoles. In addition, these observations were independent of the presence of the FLAG epitope since wild-type syn1A was also detected in a reticular-like structure when expressed in the neurosecretory incompetent cells (Fig. 4E).

Beginning also at 4-6 hours, immunolabeling of the cells for TGN and Golgi markers, TGN38 and mannosidase II (Luzio et al., 1990; Velasco et al., 1993), documented in the syn1A-transfected cells an initial fragmentation of the Golgi complex (Fig. 5A′). At later time-points, in 70% of the syn1A expressing cells the immunolabeling for the Golgi markers appeared no longer discrete, but re-distributed into reticular-like structures (Fig. 5B′ and C′). Likewise the t-SNARE was arranged in a delicate, ER-type network occupying the entire cytoplasm, with a distribution coinciding with that of the ER marker, calreticulin. Confocal microscopy demonstrated a clear overlapping of syn1A immunostaining with those of mannosidase II and TGN38 (Fig. 5B′ and C′). Taken together these results indicate that a disassembly of the Golgi structure had taken place, with internixing of the Golgi with the ER. These observations were independent of the transfection procedure employed since similar results were obtained after both electroporation and microinjection.

In order to study whether the structural alterations induced by the mistargeted syn1A were accompanied by membrane trafficking lesions, transfection experiments were carried out in PC12-27 cells with cDNAs coding for either the GABA transporter (GAT-1, a plasma membrane spanning protein; Pietrini et al., 1994) or human CgB (CgB, a secretory protein of the granin family normally packaged into the DCVs of neuroendocrine cells; Rosa and Gerdes, 1994). When expressed alone, a large proportion of GAT-1 reached the cell surface of PC12-27 cells (Fig. 6A-B) while CgB-immunolabeling was concentrated in the Golgi complex (Fig. 7, compare A and A′). Since the latter staining was abolished after 1 hour of treatment with cycloheximide (Fig. 7C-C′) we conclude that in PC12-27 cells CgB is not trapped inside the cell but rather transported to the cell surface along the constitutive secretory pathway, in line with previous data (Corradi et al., 1996). However, when either GAT-1 or CgB were co-expressed with syn1A, both proteins largely colocalized with the t-SNARE in the widespread ER-type reticular network described above (Figs. 6C-C′ and 7B-B′, and data not shown). In addition, a 4-6 hour blockade of protein synthesis with cycloheximide was unable to significantly modify the intracellular staining for CgB, documenting that its release was markedly delayed (Fig. 7, compare C′ and D′). We therefore conclude that the disassembly of the Golgi complex resulting from the mistargeting of syn1A severely affects the trafficking towards the cell surface of both membrane and secretory proteins.

In contrast to the results obtained with syn1A, when SNAP-25 was transfected into a clone derived from PC12-27, stably transfected with CgB (clone 7, Corradi et al., 1996), no major structural modifications or effects on protein trafficking were revealed (Fig. 8B-B′). This t-SNARE, which is not transmembrane but is bound to membranes by palmitoylation (Gonzalo and Linder, 1998), was shown to accumulate neither in the ER nor in the Golgi complex, but to proceed freely to its major physiological location, the plasmalemma, even in PC12-27 cells, as revealed by confocal microscopy (Fig. 8).
shown, but also in the -incompetent cells) the two non-neuronal syntaxin isoforms, when transfected alone, are correctly transported to the plasma membrane.

**rbSec1 restores syn1A transport to the plasma membrane**

Altogether the data presented so far demonstrate that transport of syn1A from the Golgi complex to the plasma membrane, an essential step for exocytosis competence in wild-type neurosecretory cells, does not take place when the protein is expressed in neurosecretion-incompetent cells. A mechanism that might account for this specific blockade could be the lack of the recognition system for the t-SNARE-specific targeting sequence. Alternatively, transport of the SNARE could require its binding to sorting accessory proteins expressed specifically in neurosecretion-competent cells. Recent studies have documented that indeed the binding potential of syn1A is quite wide, including not only the other SNAREs but also additional proteins that are believed to play key roles in the development of the exocytic process (Südhof, 1995; Lin and Scheller, 1997; Ludger and Galli, 1998). In an initial series of experiments we first explored whether in neurosecretion-incompetent cells co-expression of the other t-SNARE, SNAP-25 was capable of releasing the syn1A-induced block. The results obtained demonstrated however that
not only the trapping of syn1A in the Golgi-ER area was unchanged but also the delivery of SNAP-25 to the plasma membrane was affected since double immunofluorescence revealed some colocalization of SNAP-25 with the intracellularly retained syn1A (Fig. 10A-B’). In light of this result indicating that SNAP-25 is not involved in the correct targeting of syn1A, the attention was focussed on the regulatory protein rbSec1 (Hata et al., 1993; Pevsner et al., 1994a,b; Hata and Südhof, 1995; Tellam et al., 1995; Dresbach et al., 1998).

In both PC12-27 and NRK cells cotransfected with syn1A and rbSec1 the picture that developed was profoundly different from that of single syn1A transfections. In fact, the majority of cotransfected cells exhibited neither the syn1A trapping at the ER nor the disassembly of the TGN and Golgi complex, whose immunolabeling for TGN38 and other markers remained indistinguishable from that of control cells (Fig. 11B-B’). In 100% of the cells expressing both proteins, syn1A was detected at the plasma membrane, accompanied at this level by...
**Fig. 10.** Syn1A and SNAP-25 co-expression in neurosecretion-incompetent cells. The cells were co-transfected for both SNAP-25 and FLAG-syn1A. Twenty-four hours later, the cells were fixed and stained using the monoclonal antibody against FLAG (A,B, SYN) and the antiserum against SNAP-25 (A’,B’, SN). Cells were visualized by confocal microscopy. B” is the superimposed image (mg, merge). In two cells expressing both proteins note the widespread localization of syn1A and SNAP-25, largely coinciding with each other except in the large vacuoles, prominent especially for syn1A. Selected images from four independent experiments are shown. Bars, 10 μm.

**Fig. 11.** rbSec1 restores the transport of syn1A to the plasma membrane. PC12-27 (A-A”) and NRK (B-B”) cells were cotransfected with the cDNAs coding for FLAG-syn1A and rbSec1. Twenty-four hours after cotransfection, the cells were fixed and immunolabeled using the anti-FLAG (A and B, SYN) and the anti-rbSec1 (1:400, A’, SEC) or anti-TGN38 (B’, TGN) antibodies. In A-A”, the cells were visualized by confocal immunofluorescence microscopy, and A” is the superimposed image (mg = merge). In the cells co-expressing rbSec1 notice the preferential distribution of syn1A to the plasma membrane (A). RbSec1 is distributed throughout the cytoplasm, and present in smaller amounts at the cell surface, where it can colocalize with syn1A (A”). In NRK cells cotransfected with syn1A and rbSec1 some distribution of the first protein to the plasmalemma is also evident. In the latter cells, the structure of the TGN, as revealed by the marker, TGN38, appears perfectly preserved (B”, arrowheads). Selected images from five (A-A”) or two (B-B”) independent experiments are shown. Bars, 10 μm.
appreciable amounts of rbsec1A, while the latter protein was distributed also throughout the cytoplasm, as expected for a cytosolic protein (Fig. 11A-A”). Colocalization of syn1A and rbSec1 does not necessarily correspond to a direct binding of the two proteins at the cell surface. Thus, co-expression with the regulatory protein seems to permit, even in neurosecretion-incompetent cells, the syn1A intracellular transport pathway typical of the neurosecretion-competent cells.

DISCUSSION

The widely accepted general view about the various SNARE systems and their roles within the cells (Rothman and Warren, 1994; Hay and Scheller, 1997) implies the localization of each member at a specific site, to ensure their participation in a single type of membrane fusion. The possibility that mistargeting of SNAREs induces traffic disturbances remained to be investigated. The present results in neurosecretion-incompetent cells demonstrate that syn1A, one of the t-SNAREs specifically involved in the regulation of transmitter release, when mistargeted to the Golgi area induces a block of membrane traffic with ensuing disassembly of the Golgi complex and ER trapping of the proteins destined to be transported along intracellular membrane pathways. Based on the knowledge developed recently, especially by the study of drugs that affect transport along the ER-Golgi pathway, such as brefeldin A (Lippincott-Schwartz et al., 1998; and references therein), these changes do not appear to be independent from each other but coordinately linked. They might in fact be due to a slow, and probably partial, inhibition of membrane transport in the forward direction with maintenance of the retrograde transport, resulting in the ultimate exhaustion of the Golgi compartment. Although not investigated in detail in the present work, structural alterations of the type we have observed are expected to slow down the flux of constitutive secretion and could thus account for the delay of growth hormone release observed previously by others in transfected COS cells and attributed to the development of a post-Golgi compartment that so far remains unidentified (Bittner et al., 1996).

The effects described above appear specific for syn1A. In fact, single transfection of the other t-SNARE involved in transmitter release, SNAP-25, did not induce any alteration in the cells since the protein remained targeted to its major physiological localization site, the plasma membrane. The difference between the two SNAREs, however, is not surprising because their intracellular life is quite different. While syn1A is a typical type-2 membrane protein, SNAP-25 is not. After synthesis on free polysomes, SNAP-25 resides in the cytosol for ~20 minutes and then undergoes palmitoylation and is redistributed to membranes (Gonzalo and Linder, 1998). Its insertion, therefore, might occur too late to cause the effects on membrane traffic that we observe for syn1A. On the other hand, when the Golgi complex was disassembled by syn1A, the SNAP-25 traffic through the cell was blocked as well. In this respect, SNAP-25 appears to resemble the other two trafficking proteins, addressed to the plasmalemma (GAT-1) and exocytic release (CgB) that we have investigated. Alternatively, the syn1A trapped inside the cell could serve as a receptor for SNAP-25, thus causing its intracellular retention.

Experiments such as ours, in which exogenous proteins are transfected into non-expressing cells, should always be considered with caution because of the possibility of artifacts. In our work this possibility was seriously considered beginning with the choice of the investigated cell models. Among them, two clones of PC12 were employed, one wild-type, and thus typically competent for neurosecretion; the other (PC12-27) defective for the latter function and devoid of all the specific proteins, but maintaining many other aspects of the PC12 phenotype (see Corradi et al., 1996). Diversity between these two types of cells was thus restricted especially to the neurosecretion process. Moreover, most of the results we obtained exhibited a high degree of specificity, as shown by various lines of evidence. Firstly, the disassembly of the Golgi complex and ER trapping of syn1A and other plasma membrane/exocytosed proteins took place only in neurosecretion-incompetent cells, no matter whether the cDNAs were transfected or microinjected, whereas in the neurosecretion-competent cells the same proteins always found their way without appreciable traffic alterations when transfected under identical conditions. In addition, the changes of membrane traffic in incompetent cells appeared already at low/moderate levels of syn1A expression (between 2- to 4-fold increase compared to the endogenous levels in wild-type PC12). Only the appearance of large vacuoles (similar to those reported previously in another cell type, MDCK; Low et al., 1996) required high expression levels. Secondly, the structural alterations in the transfected cells failed to appear in response to the expression of other syntaxins, syntaxin3 and yeast Sso2p, that were able to reach their location site at the plasma membrane. Whether the different fate of these SNAREs was due to the molecular differences among the syntaxins or to the endogenous expression in neurosecretion-incompetent cells of accessory proteins appropriate for the intracellular transport of syntaxin3 and Sso2p has not been established. Thirdly, the specificity of the syn1A blocking effects is confirmed also by another series of experiments in which overexpression of a GAT-1 chimeric protein, resulting in its intracellular retention, failed to cause any inhibition of protein trafficking along the secretory pathway (Bittner et al., 1996). Finaly, and most importantly, the syn1A-induced block was prevented by the co-expression of its specific binding protein, rbSec1. Since the latter protein is endogenous to neurosecretory cells, its interaction with syn1A could explain the lack of any blockade observed in those cells even when the t-SNARE was overexpressed at considerable levels.

The traffic disturbances observed in neurosecretion-incompetent cells after syn1A transfection open interesting mechanistic problems. At the moment we do not know whether the observed blockade occurs because, in the absence of rbSec1 the t-SNARE acts by disturbing the functioning of other syntaxin isoforms known to operate in the Golgi area, such as syntaxin 5 and 6 (Rabouille et al., 1998; Bock et al., 1997); by binding to ubiquitous isoform(s) of rbSec1 incompetent for transport activation, for which syn1A has also high affinity (Hata and Südhof, 1995; Tellam et al., 1995); or by different mechanisms (see, for example, Monier et al., 1998; Lippincott-Schwartz et al., 1998). What appears likely is that the syn1A-rbSec1 complex, in addition to its well known role in the regulation of the exocytic process at the plasma membrane, is needed also within the cell to make transport of the SNARE possible through sites where membrane fusions are controlled.
by other syntaxin isoforms. Whether the synaptic deficiencies previously reported in Caenorhabditis elegans and Drosophila expressing mutants of rbSec1 (Gengyo-Ando et al., 1993, 1996; Harrison et al., 1994) are due also to the intracellular role of the protein remains to be established. The colocalization of rbSec1 and syn1A that we observed at the cell surface, argues for a substantial interaction of the two proteins at the plasma membrane. However, it has been reported that in the rat brain binding of rbSec1 to the plasmsa membrane is not mediated by syn1 (Garcia et al., 1995). Subcellular fractionation experiments and/or co-immunoprecipitations on extracts from cotransfected cells are needed to investigate the ratio of rbSec binding to syn1A in neurosecretory incompetent cells.

A final important issue concerns the intracellular pathway by which the t-SNAREs of regulated exocytosis reach the plasma membrane. At synapses and neurosecretory cells the two proteins are known to be distributed also to the membrane of SV and DCV, as well as to that of their post-exocytic recycling vesicles (Schulze et al., 1994; Walch-Solimena et al., 1995). A hypothesis was therefore that transport of the t-SNAREs from the TGN could take place via the above exocytic vesicles. In neurosecretion-incompetent cells, however, those vesicles are not present and yet SNAP-25 and (in the presence of rbSec1) also syn1A can reach the plasmalemma. A pathway other than that of SV and DCV appears therefore to exist, at least in neurosecretion-incompetent cells.

In conclusion, the present studies have revealed that syn1A (but not other t-SNAREs, including SNAP-25 and at least two members of the syntaxin family) needs the co-expression of rbSec1 to travel through its intracellular membrane pathway and ultimately reach the plasmalemma. Otherwise, syn1A is trapped in the Golgi complex and induces the disassembly of the latter followed by its redistribution to the ER, with blockade of the transport of membrane and secretory proteins, which become unable to reach their sites of destination. Coordinated activity of rbSec1 and syn1A, therefore, does not take place only at the cell surface, where the two proteins interact in the regulation of transmitter release, but also during their intracellular transport. Our results open the possibility that mechanisms of intracellular transport regulation similar to those first revealed here for syn1A exist also for other SNAREs, contributing to their specific localization and thus to the fidelity of the membrane fusion events within the cell.

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