Ectopic expression of syntaxin 1 in the ER redirects TI-VAMP- and cellubrevin-containing vesicles

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Accepted 10 March 2003
Journal of Cell Science 116, 2805-2816 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00467

Summary

SNARE proteins are key mediators of membrane fusion. Their function in ensuring compartmental specificity of membrane fusion has been suggested by in vitro studies but not demonstrated in vivo. We show here that ectopic expression of the plasma membrane t-SNARE heavy chain syntaxin 1 in the endoplasmic reticulum induces the redistribution of its cognate vesicular SNAREs, TI-VAMP and cellubrevin, and its light chain t-SNARE SNAP-23. These effects were prevented by co-expressing nSec1. Expression of syntaxin 1 alone impaired the cell surface expression of TI-VAMP and cellubrevin but not the recycling of transferrin receptor. TI-VAMP, cellubrevin and SNAP-23 associated in vivo with exogenous syntaxin 1. Redistribution of TI-VAMP in the ER of syntaxin-1-expressing cells was microtubule dependent and impaired the trafficking of CD63, a cargo of TI-VAMP-containing vesicles. We conclude that the destination of v-SNAREs is driven by their specific interaction with cognate t-SNAREs. Our in vivo data provide strong support for the theory that highly specific v-SNARE–t-SNARE interactions control compartmental specificity of membrane fusion.

Supplementary data available online

Key words: Membrane fusion, SNARE proteins, TI-VAMP, Cellubrevin, Syntaxin

Introduction

Soluble N-ethylmaleimide sensitive fusion protein attachment protein receptors (SNAREs) play a fundamental role in membrane fusion in the secretory and degradative pathways of eukaryotic cells. SNAREs were originally classified into two categories: vesicular SNAREs (v-SNAREs), localized in the donor membrane, and target SNAREs (t-SNAREs), localized in the acceptor compartment (Chen and Scheller, 2001; Galli and Haucke, 2001; Rothman, 2002). SNAREs are small proteins composed mainly of a C-terminal membrane anchoring domain and an α-helical domain (the SNARE motif) that mediates membrane fusion. In some cases this domain is preceded by a regulatory N-terminal extension (Filippini et al., 2001). The SNARE motif mediates pairing of SNAREs, leading to a parallel α-helical bundle, made of four chains, called the SNARE complex (Poirier et al., 1998; Sutton et al., 1998). The structure of the synaptic SNARE complex showed that the interaction between these proteins is mediated by hydrophobic interactions, except at the central region (ionic 'zero' layer) where an arginine residue from the v-SNARE synaptobrevin 2 (also called vesicle-associated membrane protein 2, VAMP2) forms hydrogen bonds with three glutamines, each from a distinct SNARE motif (one from syntaxin 1 and two from SNAP-25) (Sutton et al., 1998). Accordingly, residues in the ionic layer are very well conserved and are crucial for SNARE complex formation and/or stability (Katz and Brennwald, 2000; Ossig et al., 2000; Scales et al., 2001). These observations led a new classification of SNAREs into R- and Q-SNAREs, which correspond in general to v- and t-SNAREs, respectively (Fasshauer et al., 1998). The t-SNARE is now referred to as the cis-complex of target SNAREs, composed of one heavy chain and two light chains, each one providing a helix to the four-helix bundle of the core SNARE complex (Fukuda et al., 2000). The correct topology of SNAREs – one v-SNARE on a vesicle interacting with its cognate three-component t-SNARE on the target membrane – is crucial for accurate SNARE complex formation (Parlati et al., 2000).

Membrane trafficking is a highly dynamic process. The molecular machinery mediating membrane fusion is continuously cycling between distinct intracellular compartments. This is especially true for v-SNAREs. For example, exocytic SNAREs located on vesicles that have fused with the plasma membrane need to be rapidly internalized from the cell surface in order to participate in other rounds of fusion. Moreover, one v-SNARE might participate in several fusion steps, as illustrated by cellubrevin, which mediates exocytosis and endosome-to-TGN trafficking (Galli et al., 1994; Mallard et al., 2002). Similarly, the yeast v-SNAREs Snc1 and Snc2 are involved in exocytosis and in retrograde transport to endosomes and to the Golgi complex (Gurunathan et al., 2000; Paumet et al., 2001).

Despite this continuous trafficking, SNAREs are localized at steady state in specific intracellular compartments, and the specificity of membrane fusion is ensured. Identifying the mechanism responsible for compartmental specificity is a major issue in this field. The emerging picture is that there are complementary layers of regulation that are essential for

the establishment and maintenance of organelle compartmentalization in eukaryotic cells. Among these regulators there are members of the Rab GTPase family and their effectors (Pfeffer., 2001; Siniossoglou and Pelham, 2001), tethering factors (Shorter et al., 2002) and the SNAREs themselves (McNew et al., 2000; Scales et al., 2000). Studies using an in vitro fusion assay suggested that compartmental specificity could be achieved to a large extent by the inherent specificity of cognate interactions between SNAREs (McNew et al., 2000).

In this study, we sought to test the hypothesis that the steady state subcellular localization and destination of v-SNAREs, and therefore the specificity of membrane fusion, depend on the ability of v-SNAREs to interact with their cognate t-SNAREs and thus would ultimately depend on the localization of t-SNAREs. If this was true, interfering with the targeting of a t-SNARE should in turn affect the distribution of its specific v-SNARE partners without altering the localization of its non-cognate SNAREs. To test this hypothesis, we took advantage of the fact that expression of the plasma membrane t-SNARE syntaxin 1 in non-neuronal cells leads to the accumulation of this protein in the Golgi apparatus (at short time points after transfection), followed by its redistribution to the ER (after longer times of transfection). Whereas co-transfection of syntaxin 1 and nSec1/Munc18-1 restores normal plasma membrane targeting of syntaxin 1 (Perez-Branguli et al., 2002; Rowe et al., 2001; Rowe et al., 1999). Our data show that ectopic expression of syntaxin 1 in the ER redirected its cognate v-SNAREs without affecting non-cognate ones. Therefore our results support the proposal that the localization of a v-SNARE may be linked to the distribution of its cognate t-SNAREs and provide in vivo evidence for SNARE-mediated specificity of membrane fusion.

Materials and Methods

Antibodies and clones

Mouse monoclonal antibody clone 158.2 directed against TI-VAMP will be described elsewhere (Muzerelle et al., 2003). Mouse monoclonal antibodies anti-transferrin receptor (68.4, from I. Trowbridge, Salk Institute, La Jolla, CA, USA), green fluorescent protein (GFP; clone 7.1 and 13.1, Roche, Indianapolis, IN), syntaxin 1 (HPC-1, from C. Barnstable, Yale University, New Haven, CT), Vti1b (clone 7), syntaxin 4 (clone 49), syntaxin 6 (clone 30) (from Transduction Labs, Lexington, KY), Na^+/K^+ ATPase a-1 (Upstate Biotechnology, Waltham, MA) and CD63 [Clone AD1 (Furuno et al., 1996)] have been described previously. Monoclonal biotinylated anti-CD63 antibody was from Ancell (Bayport, MN). Monoclonal antibody anti-α-tubulin was from Amersham Biosciences (Piscataway, NJ). Rabbit polyclonal antibodies anti-syntaxin 1 and anti-calreticulin were from Synaptic Systems (Göttingen, Germany) and Affinity Bioreagents (Golden, CO), respectively. Rabbit polyclonal antibody anti-syntaxin 7 was kindly provided by W. Wong (Institute of Molecular and Cell Biology, Singapore). Rabbit serums anti-TI-VAMP (TG18), SNAP-23 [TG7 (Galli et al., 1998)], cellubrevin [TG2 (Galli et al., 1998)], VAMP8 [TG15 (Paumet et al., 2000)] and VAMP4 [TG19/20 (Mallard et al., 2002)] were purified by affinity chromatography.

The human cDNA of endobrevin, originally cloned from CaCo2 cells, was obtained from ATCC (EST176564) (Paumet et al., 2000). The N-terminal GFP fusion protein GFP-TIVAMP has been described previously (Martinez-Arca et al., 2000). For the N-terminal GFP fusion proteins GFP-cellubrevin (GFP-Cb) and GFP-endobrevin (GFP-Eb) the cDNAs of cellubrevin (Galli et al., 1998) and endobrevin were cloned into the pEGFP-C3 vector (Clontech, Palo Alto, CA). For the C-terminal GFP-fusion constructs TIVAMP-GFP and Cb-GFP, we generated a superecliptic variant of the ecliptic pHLuorin (G. Miesenbock, Sloan Kettering Memorial Hospital, NY) containing two mutations (F64L and S65T) that lead to enhanced fluorescence (Sankaranarayanan and Ryan, 2000). The cDNAs of rat syntaxin 1A and nSec1 have been described previously (Bennett et al., 1992; Garcia et al., 1994). The cDNA of rat syntaxin 7 was from R. Jahn [Max-Planck Institute, Göttingen, FRG (Antonin et al., 2000)].

Cell culture and transfection

HeLa cells were cultured and transfected as described previously (Martinez-Arca et al., 2000) or with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. MDCK cells were grown in DMEM with 10% FCS. Stable MDCK clones expressing GFP-TIVAMP, GFP-Cb or GFP-Eb were produced by electroporation and selected with G418 (0.4 mg/ml). MDCK clones were transfected with Lipofectamine 2000. For the nocodazole treatment MDCK cells plated the day before were treated with 5 μM nocodazole for 1 hour at 37°C before transfection. The drug was left in the medium until fixation of the cells.

Immunoprecipitation

HeLa cells were lysed 24 hours after transfection in TSE (50 mM Tris pH 8.0, 10 mM EDTA, 150 mM NaCl) plus 1% Triton X-100 with protease inhibitors for 1 hour at 4°C under continuous shaking. The supernatant resulting from centrifugation at 20,000 ***g*** for 30 minutes, adjusted to a protein concentration of 1 mg/ml, immunoprecipitated at 4°C overnight, followed by the addition of 50 μl of magnetic beads (Dynabeads, Dynal, Compiègne, France). After 3 hours at 4°C the magnetic beads were washed four times with TSE plus 1% Triton X-100 and eluted with sample buffer. Proteins were resolved by SDS-PAGE and detected by western blotting with the ECL system (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Rockford, IL).

Antibody uptake and transferrin recycling

HeLa cells transfected with Cb-GFP or TIVAMP-GFP plus syntaxin 1 were incubated in the presence of 5 μg/ml anti-GFP monoclonal antibody in culture medium for 1 hour at 37°C, washed extensively with PBS, fixed with 4% PFA and processed for immunofluorescence. For the transferrin recycling assay, cells were starved for 30 minutes in DMEM/15 mM HEPES pH 7.5, incubated in the same medium with biotinylated human transferrin (SIGMA, St Louis, MI) for 1 hour at 37°C, washed extensively and either fixed immediately (transferrin uptake) or left at 37°C for 1 hour before fixation (transferrin release). For the detection of the CD63 molecules at the plasma membrane, cells were incubated for 1 hour at 4°C with the monoclonal anti-CD63 AD1 antibody (dilution 1/50) in DMEM/15 mM HEPES pH 7.5, washed in PBS and fixed.

Immunocytochemistry and confocal microscopy

Cells were fixed with 4% PFA and processed for immunofluorescence as described previously (Coco et al., 1999). For the immunofluorescence with anti-SNAP-23 and with anti-Na^+/K^+ ATPase, cells were fixed/permeabilized with cold methanol as described elsewhere (Faigle et al., 2000). Secondary antibodies (Molecular Probes, Eugene, OR) were coupled to Cy3 and Alexa 488 for double labeling and to Cy3 and Cy5 for triple labeling with GFP-fused proteins. Confocal laser scanning microscopy was performed using a SP2 confocal microscope (Leica, Mannheim, FRG). Images

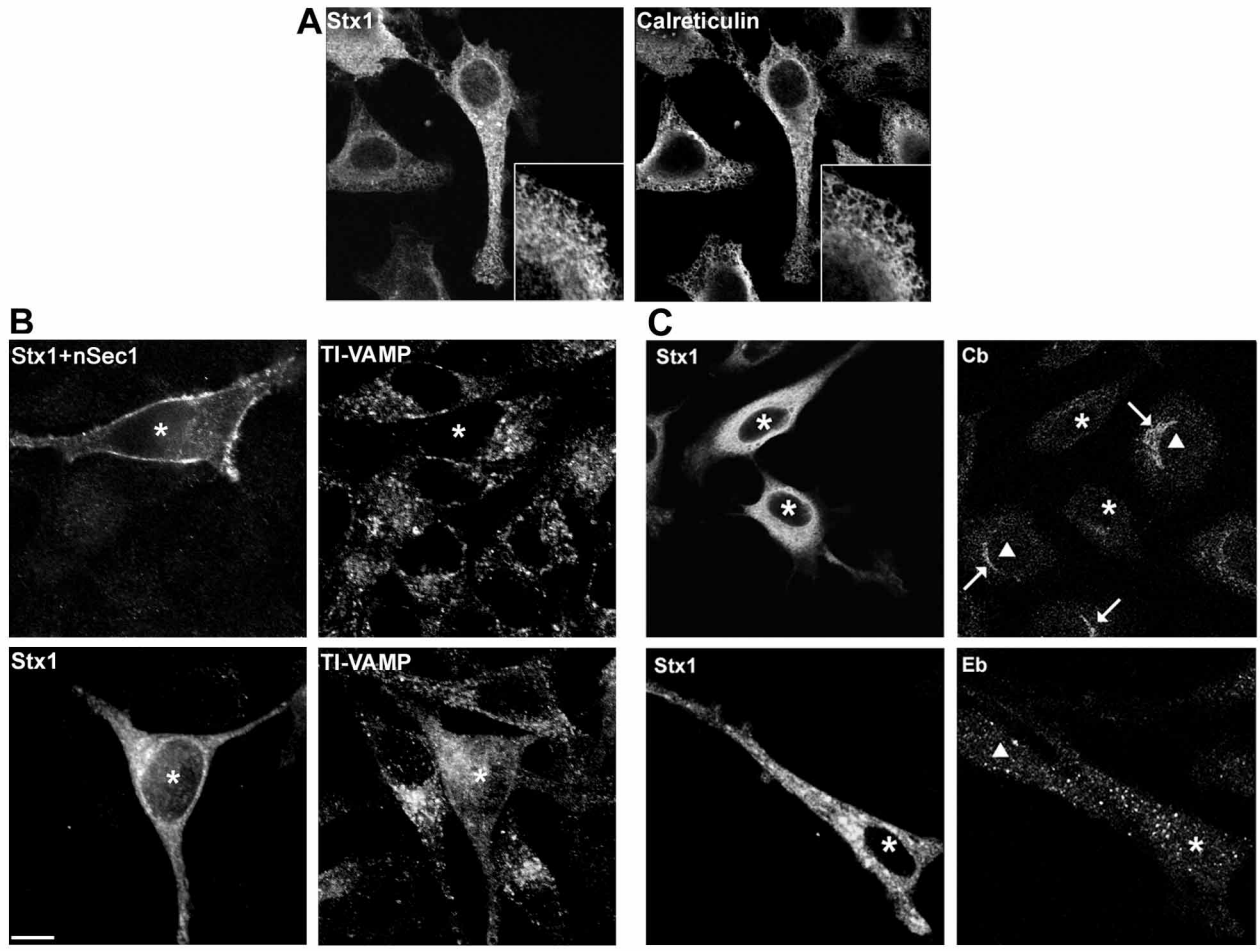

Fig. 1. Cognate v-SNAREs were mistargeted in HeLa cells expressing syntaxin 1. (A) HeLa cells transfected with syntaxin 1 were fixed and double stained for syntaxin 1 and for the endogenous ER marker calreticulin. Insets show confocal images acquired at high magnification of a syntaxin-1-expressing cell. Note the colocalization between syntaxin 1 and calreticulin. (B,C) HeLa cells transfected with syntaxin 1 or co-transfected with syntaxin 1 and nSec1 as indicated were fixed and double stained for syntaxin 1 and endogenous TI-VAMP, cellubrevin (Cb) or endobrevin (Eb). Transfected cells are marked with an asterisk. A non-transfected cell in C (low panel) is indicated with a triangle. Note that TI-VAMP vesicular staining was lost in cells expressing syntaxin 1 alone but not in those co-expressing syntaxin 1 and nSec1. In syntaxin-1-expressing cells the perinuclear enrichment characteristic of cellubrevin (arrows) was also lost. By contrast, endobrevin vesicular staining was unaffected. Bar, 6 μm. Bar in the inset, 2.5 μm.

were acquired by sequential excitation with 488 nm, 543 nm and 633 nm laser beams. Low magnification images were obtained using a 63× lens (zoom 2-3), the image size being set to 1024×1024 pixels. The high magnification images were obtained using a 100× lens (zoom 10-12), the image size being set to 256×256 pixels. Images were assembled using Adobe Photoshop (Adobe Systems, San Jose, CA).

Results

Mislocalization of syntaxin 1 induces a parallel mislocalization of cognate but not of non-cognate SNAREs

Our working hypothesis was that the intracellular distribution of v-SNAREs is regulated by their ability to participate in SNARE complexes with cognate t-SNAREs. To test this hypothesis, we transfected syntaxin 1 in HeLa cells. Intracellular retention of exogenous syntaxin 1 also occurred in these cells, as previously shown in other non-neuronal cells (Rowe et al., 2001; Rowe et al., 1999), because when syntaxin 1 was transfected alone it accumulated in the ER from 6 hours after transfection onwards, extensively colocalizing with the ER marker calreticulin (Fig. 1A). However, syntaxin 1 was transported to the plasma membrane in cells co-transfected with nSec1 (Fig. 1B, Fig. 4, Fig. 6, Fig. 8). The v-SNAREs cellubrevin and tetanus-neurotoxin-insensitive vesicle-associated membrane protein (Galli et al., 1998) [TI-VAMP, also called VAMP7 (Advani et al., 1998)] and synaptobrevin-like gene 1 [SybL1 (D'Esposito et al., 1996)] interact specifically with syntaxin 1 (Chilcote et al., 1995; Martinez-Arca et al., 2000), so we reasoned that if our hypothesis was correct, then the intracellular distribution of cellubrevin and TI-VAMP would be altered in HeLa cells expressing syntaxin 1. By contrast, v-SNAREs of the endocytic pathway, like endobrevin/VAMP8, which has not been shown to interact specifically with syntaxin 1 in vivo, should not be affected.

This was, in fact, the case. In cells co-transfected with syntaxin 1 and nSec1, endogenous TI-VAMP displayed a typical vesicular pattern, the same as in non-transfected

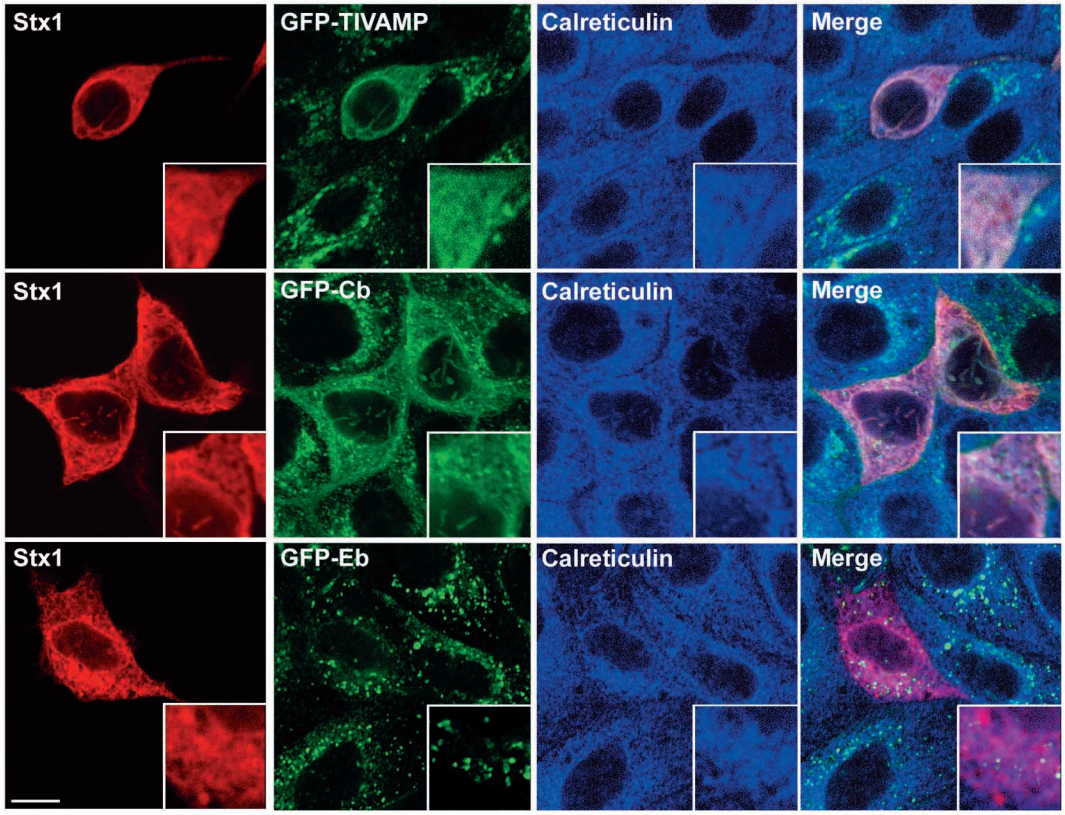

Fig. 2. Colocalization of GFP-TIVAMP and GFP-cellubrevin but not of GFP-endobrevin with syntaxin 1 in the ER of syntaxin-1-expressing cells. MDCK cells stably expressing GFP-TIVAMP, GFP-cellubrevin (GFP-Cb) or GFP-endobrevin (GFP-Eb) were fixed 24 hours after transfection with syntaxin 1. Cells were stained for syntaxin 1 (red) and calreticulin (blue). Insets show confocal images acquired at high magnification of the same transfected cells shown in the lower magnification panels. GFP-TIVAMP and GFP-Cb lost their characteristic vesicular pattern and instead colocalized with calreticulin in syntaxin-1-expressing cells, as shown by the triple colocalization in the merge panels (light pink). GFP-Eb pattern was not affected by syntaxin 1 expression, as indicated by the colocalization of syntaxin 1 and calreticulin in syntaxin-1-expressing MDCK cells, but not of GFP-Eb, in the merge panel (magenta/purple). Bar in low magnification panels, 7 μm; bar in high magnification insets, 4 μm.

cells, whereas in cells transfected with syntaxin 1 alone the vesicular pattern was changed to a diffuse reticular staining reminiscent of the ER (Fig. 1B). In the same way, endogenous cellubrevin displayed a vesicular pattern with a strong concentration in the perinuclear region in non-transfected cells or in cells co-transfected with syntaxin 1 and nSec1, whereas in syntaxin-1-expressing cells cellubrevin was widespread throughout the cytoplasm, and its perinuclear enrichment was lost (Fig. 1C and data not shown). Notably, this drastic change in distribution upon syntaxin 1 expression in HeLa cells was not seen for non-cognate v-SNAREs of syntaxin 1, such as endobrevin (Fig. 1C).

A detailed study of the compartment to which endogenous TI-VAMP and cellubrevin were redistributed in syntaxin-1-transfected HeLa cells was hampered both proteins being expressed at only a low level, and it is only their normal localization to vesicular structures that facilitates their detection. However, when the vesicular distribution was lost in syntaxin-1-expressing cells, the pattern of both v-SNAREs became diffuse, as expected for membrane proteins redistributing from discrete punctate structures to a much larger surface such as the ER. Therefore, to further analyze whether or not TI-VAMP and cellubrevin colocalized with syntaxin 1 in the ER, we used stable MDCK cell lines expressing GFP-tagged versions of TI-VAMP, cellubrevin and endobrevin. Expression of syntaxin 1 in MDCK cells also results in its intracellular retention, whereas co-transfection with nSec1 restores its delivery to the surface (Rowe et al., 2001). As shown in Fig. 2, retention of syntaxin 1 in the ER of MDCK cells induced a drastic alteration in the distribution of GFP-TIVAMP and GFP-cellubrevin (GFP-Cb) compared with neighboring cells that did not express syntaxin 1. Moreover, triple labeling with calreticulin showed triple colocalization of syntaxin 1 and GFP-TIVAMP or GFP-Cb with calreticulin (light pink in the merge panels of Fig. 2). In contrast, GFP-endobrevin (GFP-Eb) was not affected. In the case of GFP-TIVAMP, vesicular staining was completely replaced by a reticular pattern (compare the inset in Fig. 2, which shows a cell expressing syntaxin 1 displaying GFP-TIVAMP reticular distribution, with a non-transfected cell showing GFP-TIVAMP vesicles). In the case of GFP-Cb, the reticular pattern induced by expression of syntaxin 1 co-existed with some residual vesicular staining. Interestingly, in these conditions the GFP-Cb vesicles appeared perfectly aligned with the ER network whereas GFP-Eb vesicles did not (compare insets for GFP-Cb and for GFP-Eb in Fig. 2).

The redistribution of TI-VAMP and cellubrevin was not the result of syntaxin 1 overexpression but rather of the retention of syntaxin 1 in the ER, because cells co-transfected with syntaxin 1 and nSec1 expressed comparable amounts of syntaxin 1 (Fig. 1B, Fig. 3B), and yet, in this case, TI-VAMP and cellubrevin had a normal localization (Fig. 1B and not shown). We also analyzed the effect of overexpressing the endosomal syntaxin 7, which forms, together with syntaxin 8 and Vti1b, an endosomal SNARE complex with endobrevin (Antonin et al., 2000) or TI-VAMP (Bogdanovic et al., 2002;

Wade et al., 2001). However, in MDCK cells, exogenous syntaxin 7 was distributed to endosomes, as previously reported for the endogenous protein (Mullock et al., 2000) and colocalizes partially with TI-VAMP and endobrevin but not with cellubrevin (Supplementary Fig. 1 available at jcs.biologists.org/supplemental). The lack of mislocalization of syntaxin 7 upon overexpression, which is most probably caused by MDCK cells endogenously expressing this SNARE and the machinery for its correct localization and function (including its putative SM protein) did not allow further study of its role in cognate v-SNARE distribution.

Cognate v-SNAREs are retained in the ER of syntaxin-1-expressing cells through their specific interaction with syntaxin 1

The mislocalization of TI-VAMP and cellubrevin in syntaxin-1-expressing cells suggested that both v-SNAREs might be retained in the ER through a direct and specific interaction with syntaxin 1. Therefore, we searched for syntaxin-1-containing SNARE complexes. SNAREs are able to form promiscuous interactions in vitro (Fasshauer et al., 1999; Yang et al., 1999), although this is not the case in vivo or in vitro in conditions where SNAREs are inserted in a membrane rather than in solution (McNew et al., 2000; Parlati et al., 2000; Scales et al., 2000). Nevertheless, we first sought to clarify this point and verified that in our experimental conditions SNARE complexes did not form in the detergent extract of transfected HeLa cells. To test this, HeLa cells were either co-transfected with GFP-Cb plus syntaxin 1 or transfected with each construct alone. Lysates from co-transfected cells and a 1:1 mixture of lysates from cells expressing each protein alone were immunoprecipitated with antibodies against GFP or syntaxin 1. As shown in Fig. 3A, we could only detect co-immunoprecipitation of GFP-Cb and syntaxin 1 when the cells were co-transfected, indicating that the complexes were formed inside the cell and not during the extraction procedure.

We then obtained cells expressing either syntaxin 1 alone (retaining syntaxin 1 at the ER, Fig. 1) or together with nSec1 (expressing syntaxin 1 normally at the plasma membrane, see Fig. 1B) and searched for endogenous SNAREs co-immunoprecipitating with syntaxin 1. nSec1 was efficiently expressed by the co-transfected cells but it did not co-immunoprecipitate with anti-syntaxin 1 antibodies owing to the instability of the nSec1–syntaxin 1 complex (Garcia et al., 1995). As expected, SNAREs that do not normally interact with syntaxin 1, such as VAMP4, endobrevin and Vti1b, were not found in the anti-syntaxin 1 immunoprecipitates under any condition (Fig. 3B). By contrast, the plasma membrane SNARE synaptosomal associated protein of 23 kDa (SNAP-23) was efficiently recovered, supporting the specificity of the interactions. As expected, SNAP-23 co-immunoprecipitated with syntaxin 1 when syntaxin 1 was correctly sorted to the plasma membrane, although to a lesser extent than when syntaxin 1 was retained in the ER. By contrast, TI-VAMP and cellubrevin could only be detected in anti-syntaxin 1 immunoprecipitates when this t-SNARE was mistargeted to the ER. This is most probably because when syntaxin 1 is co-expressed with nSec1 the latter negatively regulates the availability of the former to participate in ternary SNARE complexes (Perez-Branguli et al., 2002; Yang et al., 2000). To detect co-immunoprecipitation of cognate v-SNAREs, and because SNARE complexes are short-lived (Peng and Gallwitz, 2002), it would be necessary to pre-treat the cells with N-ethylmaleimide (NEM) (Galli et al., 1998).

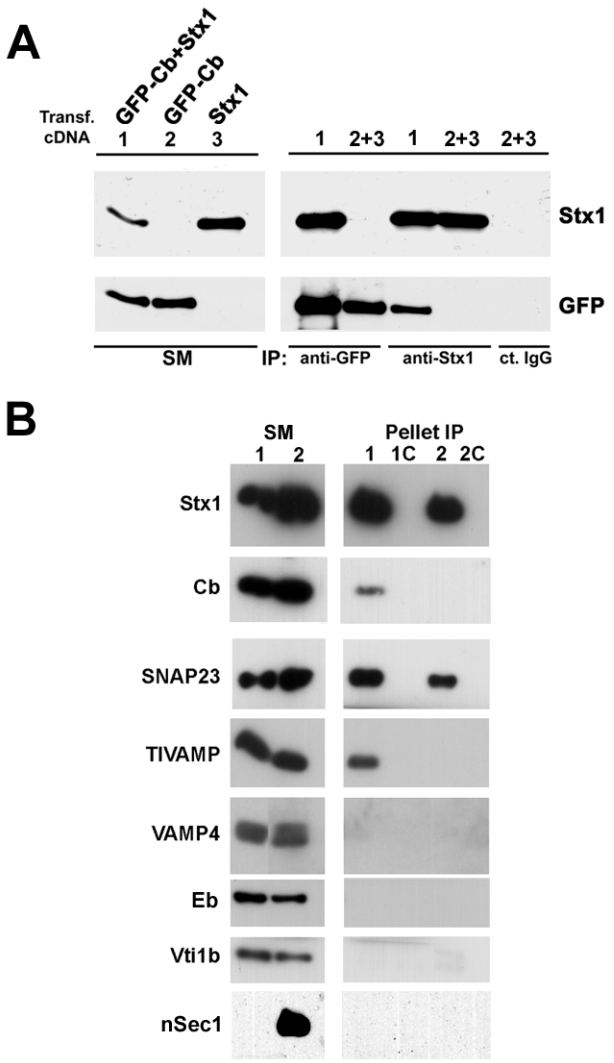

Fig. 3. TI-VAMP and cellubrevin interacted specifically in the ER with ectopic syntaxin 1. (A) HeLa cells were either co-transfected with GFP-Cb and Stx1 (1), or transfected with each cDNA alone (2,3), as indicated. 24 hours after transfection, cells were lysed and the extract from co-transfected cells was immediately immunoprecipitated (1), whereas extracts from cells transfected with only GFP-Cb or Stx1 were mixed and then immunoprecipitated (2+3) with the antibodies indicated. (B) HeLa cells were transfected with syntaxin 1 (1) or co-transfected with syntaxin 1 and nSec1 (2). 24 hours after transfection, cells were lysed and processed for immunoprecipitation with either anti-syntaxin 1 mouse monoclonal antibody (1 and 2) or with control mouse immunoglobulins (1C and 2C). Proteins were resolved by SDS-PAGE, and western blots were probed with the antibodies indicated. Note that cellubrevin, SNAP-23 and TI-VAMP but not VAMP4, endobrevin or Vti1b were recovered in the syntaxin 1 immunoprecipitate. SM, starting material.

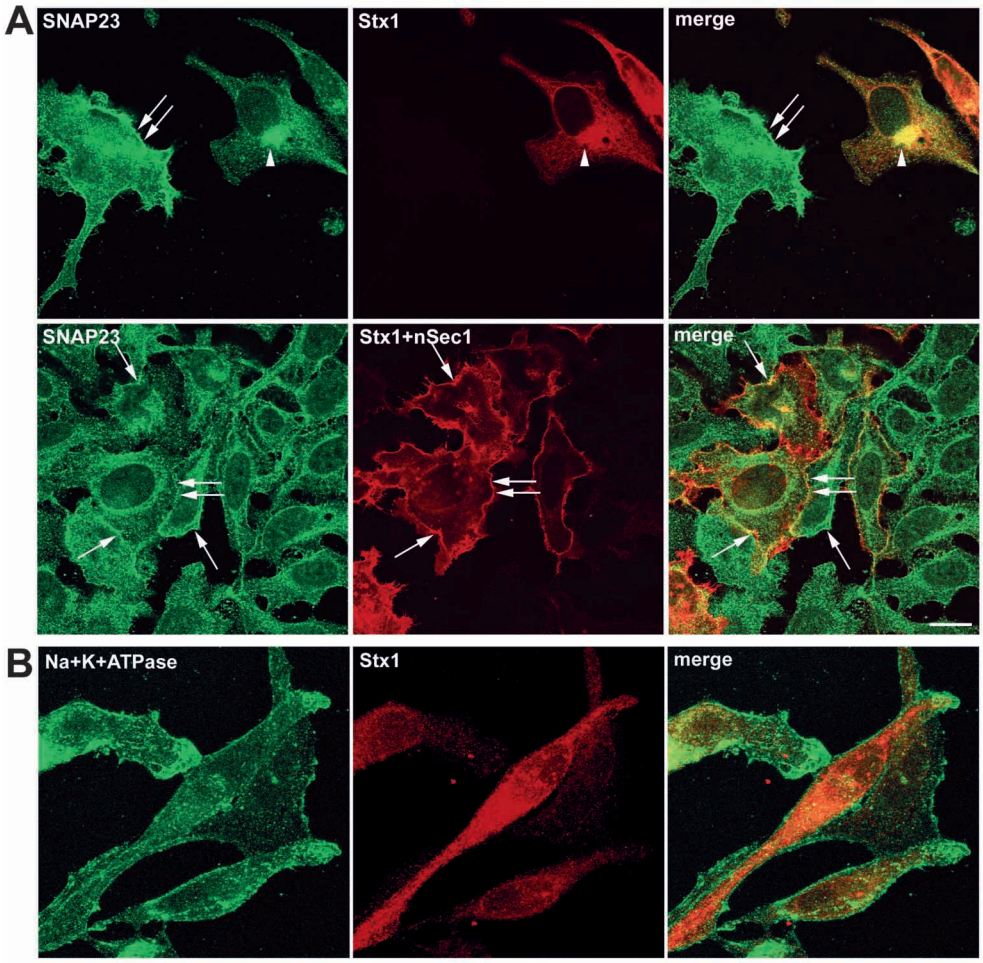

Fig. 4. SNAP-23 was redistributed to the ER in cells expressing syntaxin 1. HeLa cells transfected with syntaxin 1 or co-transfected with syntaxin 1 and nSec1 as indicated were fixed and double stained for syntaxin 1 and endogenous SNAP-23 (A) or the plasma membrane marker Na^+/K^+ ATPase (B). Note the colocalization of SNAP-23 and ectopic syntaxin 1 (arrowhead) and the absence of SNAP-23 from the plasma membrane in cells expressing syntaxin 1 alone and the plasma membrane staining of SNAP-23 in non-transfected cells or in cells co-transfected with syntaxin 1 plus nSec1 (arrows). By contrast, endogenous Na^+/K^+ ATPase is not relocalized from the plasma membrane in syntaxin-1-expressing cells. Bar, 7 μm.

Mislocalization of syntaxin 1 induces the relocalization of its cognate light chain SNAP-23

SNARE complex formation is controlled both by the strict specificity of the recognition between v- and t-SNAREs and by the correct topology of this interaction: namely one v-SNARE in one membrane and a t-SNARE complex formed by one syntaxin and two light chains in the other membrane (Parlati et al., 2000). Our biochemical data regarding SNAP-23 suggested that when syntaxin 1 is retained in the ER, the membrane of this compartment fulfilled the requirements to be a target membrane for TI-VAMP and cellubrevin vesicles (i.e. the presence of syntaxin 1 and the two light chains provided by SNAP-23). To confirm this point, we analyzed the intracellular distribution of SNAP-23 in syntaxin-1-expressing cells. As shown in Fig. 4A, in non-transfected cells or in cells co-transfected with syntaxin 1 plus nSec1, SNAP-23 displayed the expected cell surface staining (arrows, Fig. 4A) and the intracellular vimentin-associated labeling previously described (Faigle et al., 2000) (data not shown); by contrast, in cells expressing syntaxin 1 this plasma membrane pattern was completely lost, and a significant pool of SNAP-23 colocalized with syntaxin 1 (arrowhead, Fig. 4A). As a control, we analyzed the distribution of the plasma membrane protein Na^+/K^+ ATPase, which does not interact with SNAREs. As expected, the surface localization of Na^+/K^+ ATPase was not affected by the expression of syntaxin 1 (Fig. 4B). Altogether, these data suggest that the ectopic expression of syntaxin 1 in the ER induced the mislocalization of its cognate light chain by a direct interaction.

Transport to the plasma membrane of TI-VAMP and cellubrevin is impaired in syntaxin-1-expressing cells

The mislocalization of endogenous TI-VAMP and cellubrevin upon ectopic expression of syntaxin 1, together with their specific interaction with syntaxin 1 in the ER, suggested that under these conditions both proteins might not be able to reach the cell surface. To further analyze this point, we designed GFP fusion proteins of TI-VAMP and cellubrevin with the GFP tag fused to the C-terminus, so that we could monitor their appearance at the plasma membrane and endocytosis by incubating living cells with antibodies directed against GFP and measuring antibody uptake. Cells transfected with TIVAMP-GFP or Cb-GFP alone displayed typical plasma membrane and vesicular staining patterns and efficiently bound the anti-GFP antibody in the culture medium (Fig. 5, upper panels), suggesting that TIVAMP-GFP and Cb-GFP reached the plasma membrane, as is the case for the endogenous cellubrevin (Galli et al., 1994). In contrast, when TIVAMP-GFP or Cb-GFP was co-transfected with syntaxin 1, both proteins were retained in the ER and failed to bind to the extracellular anti-GFP antibody (Fig. 5, lower panels), suggesting that transport to the plasma membrane and endocytosis was abolished.

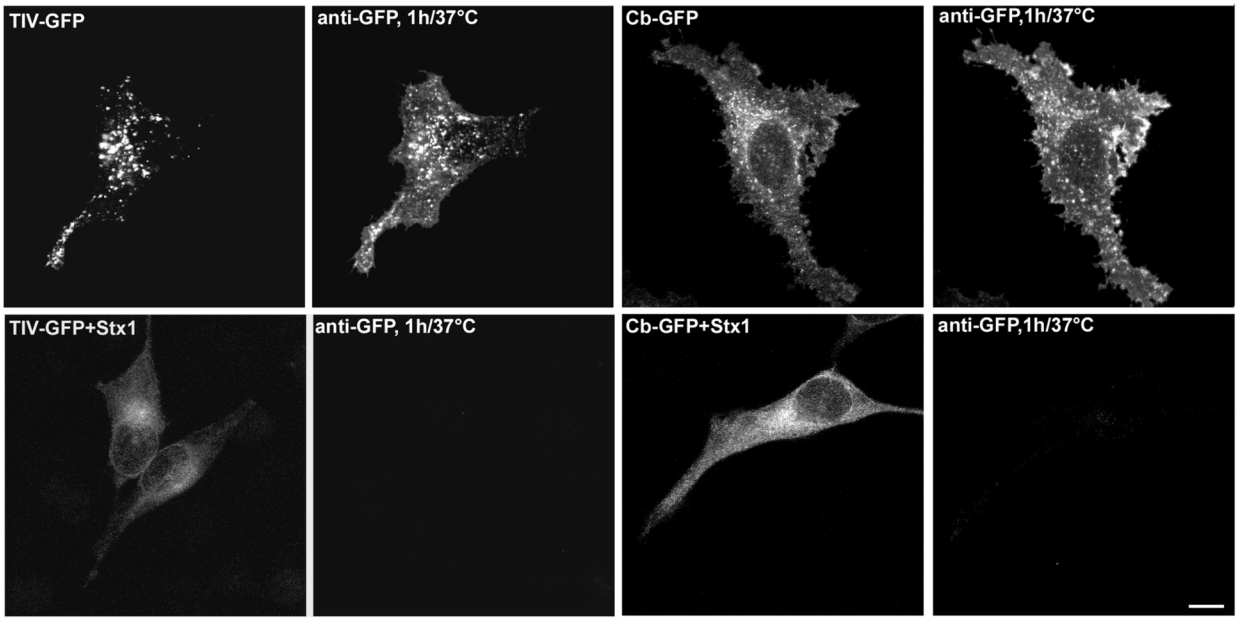

Fig. 5. Inhibition of TIVAMP-GFP and Cb-GFP transport to the plasma membrane in cells expressing syntaxin 1. HeLa cells were transfected with either TIVAMP-GFP or Cb-GFP alone (upper panels) or co-transfected with syntaxin 1 (lower panels), as indicated. 24 hours after transfection cells were incubated with anti-GFP antibodies for 1 hour at 37°C and processed for immunofluorescence. Note that when expressed alone, TIVAMP-GFP and Cb-GFP displayed their typical staining patterns and efficiently took up the anti-GFP antibody from the medium. However, when co-expressed together with syntaxin 1, TIVAMP-GFP and Cb-GFP were both retained in the ER and their expression at the plasma membrane and endocytosis was inhibited. Bar, 6 μm.

However, it is important to note that expression of syntaxin 1 did not induce pleiotropic effects on intracellular trafficking pathways because we have found that transferrin uptake and release were not affected in HeLa cells expressing either syntaxin 1 or syntaxin 1 plus nSec1 (Fig. 6), confirming the specificity of this experimental model.

Intracellular redistribution of TI-VAMP in syntaxin-1-expressing cells is microtubule dependent

The appearance of TI-VAMP and cellubrevin in the ER of syntaxin-1-expressing cells could be due either to redistribution from their normal intracellular compartment or to sequestration of the newly synthesized molecules. The latter possibility is unlikely because it would mean that the half-lives of TI-VAMP and cellubrevin are short enough to allow detection of only the newly synthesized pool after short times (8 hours) of syntaxin 1 transfection, and this is not the case (S.M.-A., V.P.-G., P.A., D.L. et al., unpublished). On the other hand, if the first hypothesis is true, inhibition of vesicle-mediated translocation on microtubules should prevent the redistribution of syntaxin 1 cognate v-SNAREs to the ER because the treatment with nocodazole abolishes microtubule-dependent endosomal movement (Matteoni and Kreis, 1987). Thus, if relocalization of v-SNAREs to the ER in our syntaxin 1 overexpression assay was the result of redistribution from endosomes, then nocodazole treatment should inhibit it. To directly test this point we have used the clone of MDCK cells that stably expresses GFP-TIVAMP. Treatment of these cells with 5 μM nocodazole for 1 hour completely disrupted the microtubules, as seen by staining with an anti-α-tubulin antibody (Fig. 7A). As expected, the GFP-TIVAMP pattern was slightly modified upon nocodazole treatment, the GFP-TIVAMP-positive vesicles being more scattered throughout the cytoplasm and less concentrated at the perinuclear area, probably because of an alteration in the microtubule-organizing center. Interestingly, when the cells were transfected with syntaxin 1 after the microtubules were disrupted by a 1 hour pre-treatment with nocodazole, GFP-TIVAMP redistribution to the ER was abolished (Fig. 7B) despite the retention of syntaxin 1 in this compartment. By contrast, in cells that were not treated with nocodazole, GFP-TIVAMP was mistargeted to the ER of syntaxin-1-expressing cells only 8 hours after transfection (Fig. 7B). These results indicate that the relocalization of TI-VAMP to the ER in syntaxin-1-expressing cells was not simply because of sequestration of newly synthesized molecules but resulted from the redistribution of TI-VAMP from a pre-existing endosomal compartment. These data also imply that TI-VAMP vesicles are able to move bi-directionally along microtubules, in agreement with our observations by time-lapse videomicroscopy in GFP-TIVAMP-transfected HeLa cells (S.M.-A. and T.G., unpublished).

Expression of syntaxin 1 in MDCK cells redirects TI-VAMP vesicles to the ER

The results obtained after the nocodazole treatment suggested that upon expression of syntaxin 1, TI-VAMP-containing vesicles fuse with the ER membrane. If this is true, then we would expect that the cargo of these vesicles would also be mislocalized in syntaxin-1-expressing cells. We have previously found that TI-VAMP defines a new endosomal compartment in neurons and PC12 cells and colocalizes with the tetraspanin

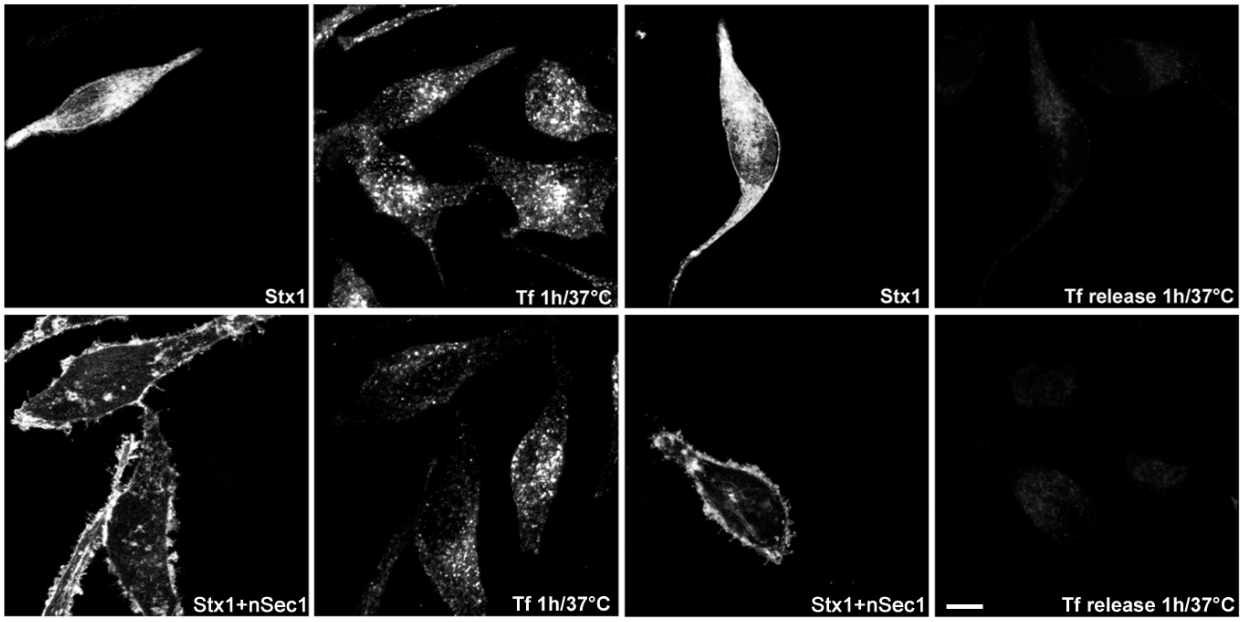

Fig. 6. Ectopic expression of syntaxin 1 did not affect transferrin recycling. HeLa cells were transfected with syntaxin 1 (upper panels) or co-transfected with syntaxin 1 and nSec1 (lower panels). 24 hours later cells were allowed to internalize transferrin for 1 hour at 37°C, washed and either fixed immediately (uptake) or incubated for a further 1 hour at 37°C before fixation (release). Notice that both the uptake and the release of transferrin were comparable in both kinds of transfected cells and indistinguishable from non-transfected cells, independently of the intracellular distribution of syntaxin 1. Bar, 5 μm.

protein CD63 (Berditchevski, 2001; Coco et al., 1999). We found the same result in HeLa cells, as shown by the extensive colocalization of TI-VAMP and CD63 (Fig. 8A, upper panels). Therefore, we investigated the fate of CD63 molecules in cells expressing and retaining syntaxin 1 in the ER. As shown in Fig. 8A (lower panels), the classic vesicular pattern of CD63 was partially lost in cells transfected with syntaxin 1. In these conditions, CD63-positive vesicles were less abundant, more heterogenous in size and shape and, importantly, the nuclear envelope and fine reticular structures characteristic of the ER were labeled with the anti-CD63 antibody (arrow in Fig. 8A). Furthermore, high magnification confocal images showed partial colocalization of CD63 with the syntaxin 1 retained in the ER (inset in Fig. 8A). This change in distribution is not the result of a block in CD63 export from the ER, because the same result was obtained when cells expressing syntaxin 1 were incubated with cycloheximide for 3 hours prior to fixation (data not shown). Furthermore, we have checked that CD63 does not colocalize with calreticulin in untransfected cells or cells expressing syntaxin 1 plus nSec1 (S.M.-A., V.P.G., P.A., D.L. et al., unpublished). In normal conditions, the tetraspanin protein CD63 cycles through the cell surface (Kobayashi et al., 2000). Therefore, to confirm the effect of the ER retention of syntaxin 1 on CD63 distribution, we performed an antibody binding assay at 4°C to detect CD63 molecules at the plasma membrane. As expected, in non-transfected cells or in cells transfected with syntaxin 1 plus nSec1, the anti-CD63 antibody was efficiently bound, as a result of the presence of CD63 at the cell surface (Fig. 8B, upper panels). Strikingly, in cells expressing syntaxin 1 there was no detectable binding of the antibody (Fig. 8B, lower panels), supporting the redistribution of CD63 in these conditions. Moreover, when the anti-CD63 antibody was incubated with living cells at 37°C for 90 minutes, it was efficiently internalized in non-transfected cells or in cells transfected with syntaxin 1 plus nSec1, whereas no internalized antibody was detected in cells transfected with syntaxin 1 alone (S.M.-A., V.P.G., P.A., D.L. et al., unpublished).

Discussion

In the present study, we sought to analyze in vivo the link between the intracellular distribution and destination of v-SNAREs and the compartmental specificity of fusion. Our results show that in the absence of nSec1 the t-SNARE heavy chain syntaxin 1, normally found at the neuronal plasma membrane, is localized to the ER and induces an active redistribution of its cognate light chain SNAP-23 and cognate v-SNAREs, cellubrevin and TI-VAMP but does not alter the distribution of non-cognate v-SNAREs, such as endobrevin.

The redistribution of TI-VAMP and cellubrevin to the ER in fibroblasts expressing syntaxin 1 points to a direct relationship between SNARE complex formation and v-SNARE distribution and destination for the following reasons. (1) It was due to syntaxin 1 mistargeting and not simply to syntaxin 1 overexpression because syntaxin 1 was expressed at the same level in the presence and absence of nSec1 and yet no effect on cognate v-SNARE distribution was seen in cells co-transfected with syntaxin 1 and nSec1. (2) It was not the result of the retention of the newly synthesized v-SNARE proteins but of an active microtubule-dependent redistribution of the pre-existing v-SNARE molecules. (3) It recapitulated the specificity found in v-/t-SNARE interactions because only syntaxin 1 cognate v-SNAREs were affected by its mistargeting, and we detected a bona fide interaction between the syntaxin 1 retained in the ER and the redistributed TI-VAMP and cellubrevin by co-immunoprecipitation. (4) The

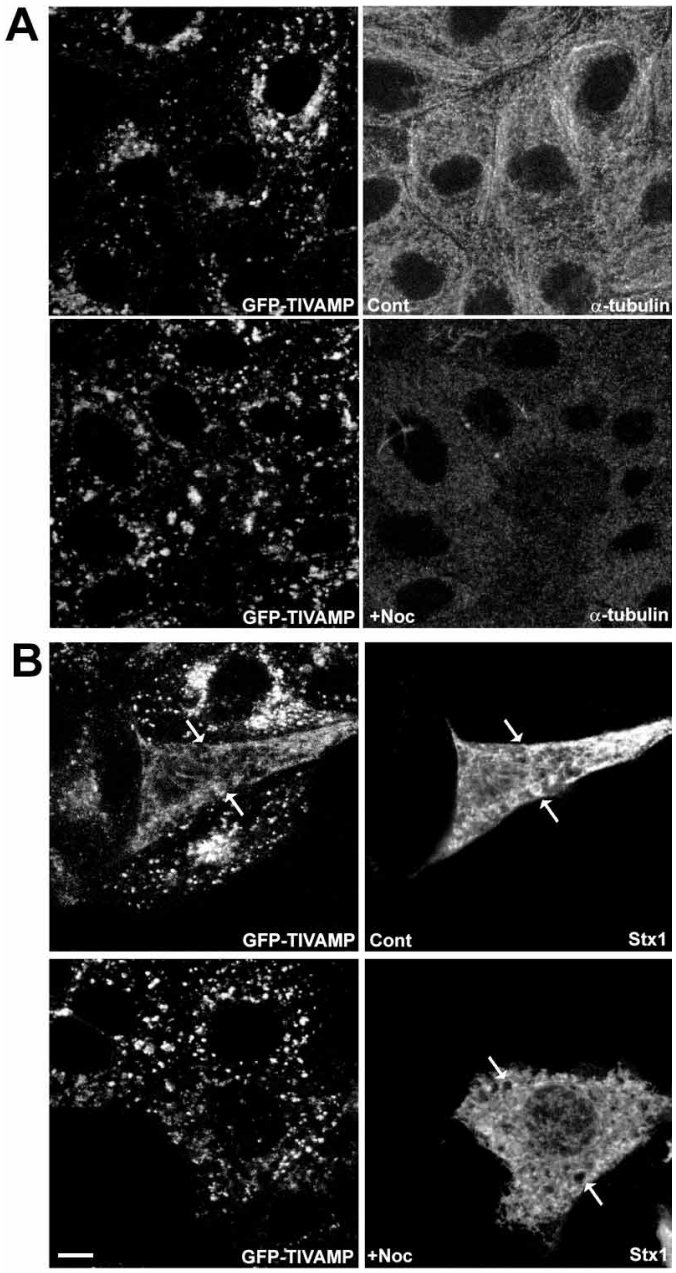

Fig. 7. TI-VAMP relocalization in syntaxin-1-expressing cells was microtubule dependent. (A) MDCK cells stably expressing GFP-TIVAMP incubated with or without 5 μM nocodazole for 1 hour were fixed and stained for α-tubulin. Note that in the presence of nocodazol, microtubules were completely disrupted. (B) MDCK cells stably expressing GFP-TIVAMP incubated with or without 5 μM nocodazole for 1 hour prior to transfection with syntaxin 1 were fixed and stained for syntaxin 1 8 hours after transfection. In control conditions (Cont), the expression of syntaxin 1 and its retention in the ER induced a relocalization of GFP-TIVAMP towards syntaxin-1-positives structures in the ER (arrows). By contrast, in the absence of functional microtubules (+Noc) the localization of GFP-TIVAMP in transfected cells was indistinguishable from non-transfected cells. Bar, 4 μm.

effect of the expression of syntaxin 1 on the redistribution of CD63 suggests that the vesicles containing TI-VAMP and CD63 docked and fused with the syntaxin-1-containing ER membrane. At steady state, the effect of the expression of syntaxin 1 in the ER on CD63 localization was only partial (Fig. 8A). This could be due to the fact that TI-VAMP and CD63 may not necessarily be transported in the same vesicles all along their trafficking pathway. In contrast, the inhibition of the expression of CD63 at the plasma membrane was very strong (Fig. 8B), thus demonstrating that the trafficking of CD63 was strongly impaired. The expression of syntaxin 1 in non-neuronal cells was also shown to induce a relocalization of Golgi markers (Rowe et al., 2001). However, the redistribution of TI-VAMP and cellubrevin observed in syntaxin-1-expressing cells (this study) was not merely the result of the disassembly of the Golgi because Brefeldin A, a drug that induces the collapse of the Golgi complex (Fujiwara et al., 1988), has no effect on the localization of TI-VAMP (Advani et al., 1999).

It is noteworthy that, despite the mislocalization of cellubrevin and TI-VAMP, cells ectopically expressing syntaxin 1 are still capable of internalizing and recycling transferrin. This indicates that the mislocalization of syntaxin 1 to the ER does not have pleiotropic effects on all membrane fusion steps. This result is rather unexpected because it suggests that a v-SNARE other than cellubrevin participates in transferrin recycling. In this regard, it is important to note that endobrevin colocalizes with internalized transferrin (Wong et al., 1998) (S.M.-A. and T.G., unpublished) as does cellubrevin (Galli et al., 1994), which suggests that cellubrevin and endobrevin may have overlapping functions. The participation of endobrevin in this transport pathway could explain why the treatment of cells with tetanus toxin only inhibited one third of the total release of apo-transferrin (Galli et al., 1994) and the lack of a major phenotype in cellubrevin-knockout mice (Yang et al., 2001). Irrespective of the molecular mechanism underlying transferrin recycling in syntaxin-1-transfected cells, these results support the specificity of the v-/t-SNARE interaction in vivo and rule out the possibility that the observed effect on TI-VAMP and cellubrevin could be because of a general defect in intracellular trafficking.

nSec1 belongs to the Sec/Munc18 (SM) family of proteins (Rizo and Sudhof, 2002) and is essential for synaptic vesicle exocytosis (Verhage et al., 2000). It binds tightly to the closed conformation of syntaxin 1 (Dulubova et al., 1999) and competes with SNARE complex formation (Yang et al., 2000). As discussed by Rowe et al., nSec1 may act as a chaperone-like protein, allowing syntaxin 1 to proceed through the secretory pathway by keeping it in a ‘closed’ conformation, unable to interact with its partner SNAREs (Rowe et al., 2001), and preventing the formation of non-productive SNARE complexes. Our data suggest that when syntaxin 1 is overexpressed in the absence of nSec1, it may display an ‘open’ conformation (Dulubova et al., 1999) that is unable to exit the ER but able to interact with its cognate partners, resulting in the final redistribution of its cognate v-SNAREs to the ER. These results point to the importance of the balance between syntaxin 1 and nSec1 for the correct functionality of both proteins, as has been shown to be the case in other systems such as chromaffin cells and *Drosophila* (Voets et al., 2001; Wu et al., 1998). The effect of syntaxin 1 expression reported here may also explain the blockade of membrane transport observed in these conditions (Rowe et al., 1999), because functional exocytic v-SNAREs would be sequestered in the ER. Moreover, several studies have

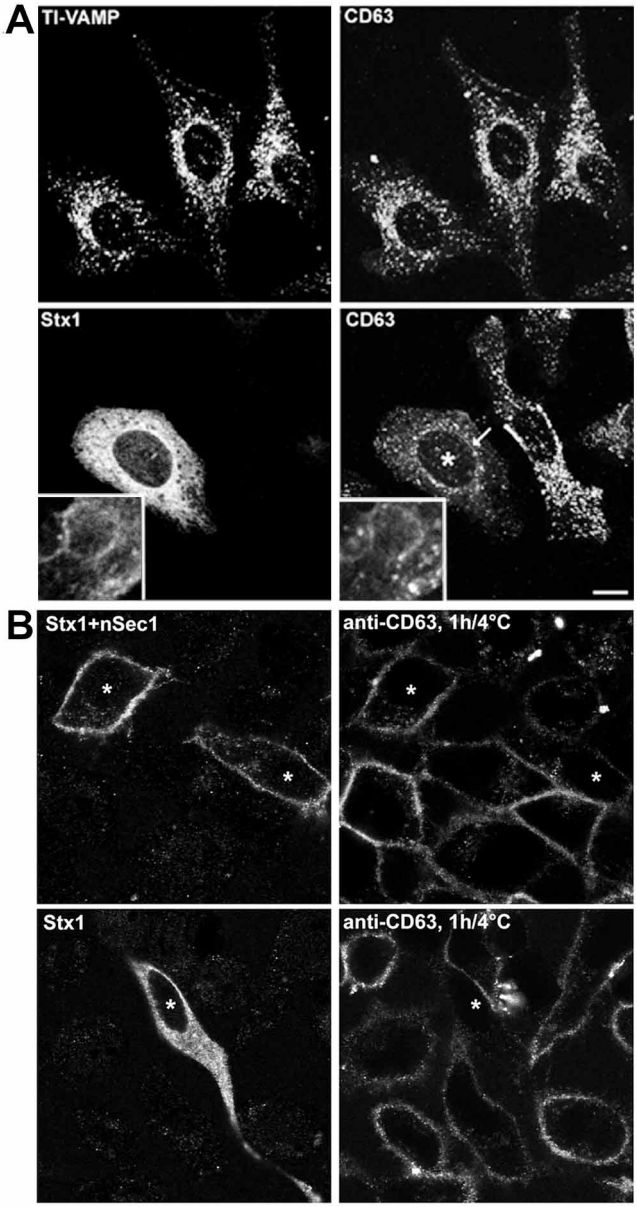

Fig. 8. Ectopic expression of syntaxin 1 altered the distribution and trafficking of TI-VAMP vesicles' cargo CD63. (A) (Upper panels) Non-transfected HeLa cells double stained for endogenous TI-VAMP and CD63. (Lower panels) HeLa cells transfected with syntaxin 1 were fixed and stained for syntaxin 1 and CD63 24 hours after transfection. Note the almost complete colocalization between TI-VAMP and CD63 in non-transfected cells. In cells transfected with syntaxin 1 (asterisk) the CD63 typical punctate staining was lost and instead the nuclear envelope (arrow) was labeled. Insets show confocal images acquired at high magnification of a syntaxin-1-expressing cell. (B) HeLa cells transfected with syntaxin 1 or with syntaxin 1 plus nSec1 were incubated for 1 hour at 4°C with anti-CD63 antibodies prior to fixation and processing for immunofluorescence with anti-syntaxin 1 antibodies. Note the plasma membrane binding of the anti-CD63 antibody in cells co-transfected with syntaxin 1 plus nSec1, which is indistinguishable from non-transfected cells. By contrast, cells expressing and retaining syntaxin 1 in the ER did not bind to anti-CD63 antibodies. Bar, 6 μm. Bar in the inset, 2 μm.

revealed the presence of SNARE proteins in lipid rafts and the importance of maintaining these structures for normal SNARE function (Chamberlain et al., 2001; Lafont et al., 1999; Lang et al., 2001). In MDCK cells co-transfected with syntaxin 1 and nSec1 there is a fraction of syntaxin 1 associated with lipid rafts that disappears when syntaxin 1 is expressed alone, resulting in a shift in syntaxin 1 distribution from the plasma membrane to intracellular structures (Rowe et al., 2001). Thus, one possibility that may explain our results is that the 'open' conformation displayed by syntaxin 1 in the absence of nSec1 is not able to enter special lipid microdomains in the ER. In these conditions syntaxin 1 would be highly active in SNARE complex formation and would trap its cognate v-SNAREs in the ER. Regarding this point, there is most probably a competition for v-SNARE between the 'open' syntaxin 1 and endogenous plasma membrane syntaxins (2, 3, 4). The overexpression of syntaxin 1 together with the fact that endogenous syntaxins are probably in 'closed' conformations because of their interaction with the corresponding SM proteins (Dulubova et al., 2003), displaces the equilibrium towards an interaction with syntaxin 1. Moreover, the lack of mislocalization of syntaxin 7 upon overexpression in MDCK cells (Supplementary Fig. 1 available at jcs.biologists.org/supplemental), probably resulting from the expression in these cells of the SM protein regulating syntaxin 7, strengthens the link between SM-protein-regulated SNARE complex formation and v-SNARE distribution.

Recent data have shown that, in an in vitro liposome fusion assay, it is possible to favor the fusion of liposomes containing the yeast v-SNARE Bet1p over those containing Sft1p by increasing the proportion of a t-SNARE complex containing Sed5p, Bos1p and Sec22p versus one containing Sed5p, Gos1p and Ykt6p (Parlati et al., 2002). An in vivo extension of this observation would imply that increasing the local concentration of a t-SNARE should increase fusion of cognate v-SNAREs with that compartment. We found that SNAP-23 partially colocalized and formed a complex with syntaxin 1 in cells ectopically expressing syntaxin 1 in the ER. Interestingly, more SNAP-23 was co-immunoprecipitated from extracts of cells expressing only syntaxin 1 than from those expressing syntaxin 1 and nSec1. Therefore, in these conditions, the ER membrane fulfilled the essential conditions to become a target membrane for TI-VAMP and cellubrevin vesicles, namely the presence of a topologically adequate t-SNARE complex (Parlati et al., 2000) composed of syntaxin 1 and SNAP-23. Our observation that the trafficking of CD63, a cargo of TI-VAMP, was strongly affected and that this protein relocalized partially to the syntaxin-1-positive compartment suggests that the presence of the syntaxin 1/SNAP-23 complex on the ER membrane triggered docking and fusion of TI-VAMP-containing vesicles.

Altogether, our data strongly suggest that the intracellular distribution and destination of v-SNAREs are governed, at least in part, by their specific interaction with cognate t-SNAREs. Interestingly, previous work on Snc1 agrees with this hypothesis. The yeast exocytic v-SNARE Snc1p, in common with mammalian exocytic v-SNAREs, is continuously cycling and participates in SNARE complexes with plasma membrane and endosomal t-SNAREs (Holthuis et al., 1998). In mutant cells lacking Tlg1p or Tlg2p, its endosomal partner t-SNAREs,

the steady-state distribution of Snc1p was affected (Lewis et al., 2000). Moreover, Snc1p is also redirected to a haze of transport vesicles in a mutant yeast strain in which Tlg1p and Tlg2p accumulated on the same structures (Siniossoglou and Pelham, 2001). Exocytic v-SNAREs, such as Snc1, synaptobrevin 2, cellubrevin and TI-VAMP, are continuously cycling between the plasma membrane and endosomes. Our results suggest that when a t-SNARE is constitutively active (i.e. syntaxin 1 in the absence of nSec1 in this study) then more v-/t-SNARE complexes form and the v-SNAREs localize, to a great extent, to the membrane where the cognate t-SNARE is expressed. In contrast, in normal conditions (i.e. co-expression of syntaxin 1 and nSec1 in this study or wild-type fibroblasts) the level of SNARE complexes formed (and therefore recovered in detergent extracts) is low, and TI-VAMP and cellubrevin localize to endosomal vesicles. This suggests that the steady-state subcellular localization of v-SNAREs is the result of equilibrium between two states: one corresponding to v-SNAREs on the donor vesicles and the other to v-SNAREs in the target membrane. The lack of nSec1 displaced this equilibrium towards the second state.

Our findings have important implications for how compartmental specificity is achieved during membrane fusion. Indeed, we have shown that the ectopic expression of syntaxin 1 induces an illegitimate rerouteing of vesicles, the fusion of which is mediated by the cognate v-SNAREs of syntaxin 1, TI-VAMP and cellubrevin. These results show that the highly controlled and specific v-/t-SNARE interaction is essential to define the destination of membrane carriers in vivo in mammalian cells. In conclusion, our results suggest that the exquisite regulation of the v-/t-SNARE interaction that ensures compartmental specificity of membrane fusion is also one of the factors accounting for the accuracy of the dynamic intracellular distribution and destination of v-SNAREs.

We thank R. Rudge and L. Johannes for critical reading of the manuscript, G. Miesenbock and J. Rothman for generous gift of pHLuorin and R. Jahn for the syntaxin 7 cDNA. S.M.-A. is a recipient of a post-doctoral fellowship from the Spanish Ministerio de Educación y Cultura. V.P.G. is a recipient of a post-doctoral fellowship from Association pour la Recherche sur le Cancer (ARC). P.A. is a recipient of a doctoral fellowship from Societé de Secours des Amis des Sciences. This work was supported in part by grants from Human Frontier Science Program (RGY0027/2001-B101), European Community (QLG3-CT-2001-02430_Retrograde Signaling), Association pour la Recherche sur le Cancer (ARC N°5873) and Ministère de la Recherche (ACI-JC5254) to T.G.

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