

A Novel Tetanus Neurotoxin-insensitive Vesicle-associated Membrane Protein in SNARE Complexes of the Apical Plasma Membrane of Epithelial Cells

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The importance of soluble *N*-ethyl maleimide (NEM)-sensitive fusion protein (NSF) attachment protein (SNAP) receptors (SNAREs) in synaptic vesicle exocytosis is well established because it has been demonstrated that clostridial neurotoxins (NTs) proteolyze the vesicle SNAREs (*v*-SNAREs) vesicle-associated membrane protein (VAMP)/brevins and their partners, the target SNAREs (*t*-SNAREs) syntaxin 1 and SNAP25. Yet, several exocytotic events, including apical exocytosis in epithelial cells, are insensitive to numerous clostridial NTs, suggesting the presence of SNARE-independent mechanisms of exocytosis. In this study we found that syntaxin 3, SNAP23, and a newly identified VAMP/brevin, tetanus neurotoxin (TeNT)-insensitive VAMP (TI-VAMP), are insensitive to clostridial NTs. In epithelial cells, TI-VAMP-containing vesicles were concentrated in the apical domain, and the protein was detected at the apical plasma membrane by immunogold labeling on ultrathin cryosections. Syntaxin 3 and SNAP23 were codistributed at the apical plasma membrane where they formed NEM-dependent SNARE complexes with TI-VAMP and cellubrevin. We suggest that TI-VAMP, SNAP23, and syntaxin 3 can participate in exocytotic processes at the apical plasma membrane of epithelial cells and, more generally, domain-specific exocytosis in clostridial NT-resistant pathways.

INTRODUCTION

The involvement of *N*-ethyl maleimide (NEM)-sensitive fusion protein (NSF), soluble NSF attachment proteins (SNAPs), and their receptors on membranes (so-called SNAREs) (Söllner *et al.*, 1993a,b) in most membrane fusion events has been extensively docu-

mented in yeast and mammalian cells (for review, see Johannes and Galli, 1998). In particular, the neuronal vesicle SNAREs (*v*-SNAREs), vesicle-associated membrane protein (VAMP)/synaptobrevin 1 and 2 and their plasma membrane targets (*t*-SNAREs) SNAP25 and syntaxin 1 have been shown to be involved in calcium-dependent synaptic vesicle exocytosis (Schiavo *et al.*, 1992; Blasi *et al.*, 1993a,b). Interestingly, nonneuronal cells possess homologues of these neuronal SNAREs. These include the *v*-SNARE cellubrevin (McMahon *et al.*, 1993) and the *t*-SNAREs syntaxins 2–7 (Bennett *et al.*, 1993; Bock *et al.*, 1996; Wang *et al.*, 1997) and SNAP23 (Ravichandran *et al.*, 1996; Wong *et al.*, 1997). An understanding of the role of the brevins syntaxin 1 and SNAP25 in exocytosis has been greatly aided by the use of clostridial neurotoxins

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¹ Abbreviations used: BoNTs, botulinum NTs; HA, influenza hemagglutinin; NEM, *N*-ethyl maleimide; NSF, NEM-sensitive fusion protein; NTs, neurotoxins; SNARE, SNAP receptor; SNAPs, soluble NSF attachment proteins; *t*-SNARE, target SNARE; TeNT, tetanus NT; TI-VAMP, tetanus neurotoxin-insensitive VAMP; VAMP, vesicle-associated membrane protein; *v*-SNARE, vesicle SNARE.

(NTs), i.e., tetanus NT (TeNT) and botulinum NT (BoNT), which specifically proteolyze these proteins (for review, see Niemann *et al.*, 1994; Schiavo *et al.*, 1994). These toxins also have effects on several exocytotic pathways in nonneuronal cells. For instance, TeNT, which cleaves cellubrevin, inhibits transferrin receptor exocytosis in fibroblasts (Galli *et al.*, 1994). Strikingly, several exocytotic events are at least partially insensitive to some NTs (for review, see Johannes and Galli, 1998). For example, although TeNT and BoNT F can inhibit transport of vesicular stomatitis virus (VSV) G protein to the basolateral plasma membrane in Madin–Darby canine kidney (MDCK) cells, apical transport of influenza hemagglutinin (HA) is insensitive to both NTs (Ikonen *et al.*, 1995). This led the authors to propose a mechanism of membrane fusion at the apical plasma membrane of epithelial cells independent of v- and t-SNAREs, although they had found HA transport to be sensitive to NEM (Simons and Ikonen, 1997). On the contrary, several groups have localized syntaxin isoforms at the apical plasma membrane of epithelial cells (Gaisano *et al.*, 1996; Low *et al.*, 1996; Mandon *et al.*, 1996; Delgrossi *et al.*, 1997) and proposed a role for these SNAREs in apical docking and fusion (Weimbs *et al.*, 1997). We have searched for a TeNT-insensitive v-SNARE that could be a partner of apical t-SNAREs in epithelial cells. Recently, D'Esposito *et al.* (1996) identified SYBL1, a synaptobrevin-like gene in Xq28 pseudoautosomal region, which undergoes X inactivation. We find that SYBL1p is TeNT insensitive and propose to name it TeNT-insensitive VAMP (TI-VAMP). Hence, we have tested the localization and biochemical properties of TI-VAMP in CaCo-2 epithelial cells.

MATERIALS AND METHODS

Antibodies

Antibodies against syntaxin 3 (TG1), SNAP23 (TG7), and TI-VAMP (TG11) were raised in rabbit against the cytoplasmic domain of rat syntaxin 3 fused to glutathione *S*-transferase (GST) (Calakos *et al.*, 1994), recombinant human SNAP23, and the recombinant cytoplasmic domain of human TI-VAMP fused to GST, respectively, and purified on the corresponding recombinant proteins. Antibodies against human cellubrevin (TG2) were obtained in rabbit against the N-terminal peptide MSTGPTAATGSNC of cellubrevin and affinity purified on the cytoplasmic domains of human cellubrevin fused to GST. All of these antibodies reacted specifically with their corresponding antigen by Western blotting and immunoprecipitation (see Results). Monoclonal antibodies against E-cadherin/uvomorulin (DECMA), sec8 (clone 14), ZO-1, and transferrin receptor (H68.4) were from Sigma (St. Louis, MO), Transduction Laboratory (Lexington, KY), Dr. M. Mooseker (Yale University, New Haven, CT), and Dr. I. Trowbridge (Salk Institute, La Jolla, CA), respectively. Secondary Cy-2 or Texas Red-labeled goat anti-rat, anti-mouse, and anti-rabbit immunoglobulins were from Jackson ImmunoResearch (West Grove, PA).

cDNA Constructs and Recombinant Proteins

Full-length cDNA of the human isoforms of cellubrevin, syntaxin 3, SNAP23, and TI-VAMP were obtained by reverse transcription-PCR on CaCo-2 mRNA using standard procedures and the following sets of oligonucleotides: 5'-ATGCTACAGGTCCAAGTCTG-3' (hcb5') with 5'-GCTGGTCTTCATGAAGAGACA-3' (hcb3'), 5'-ATGAAGG-ACCGTCTGGAGCAG-3' (hstx3-5') with 5'-TTAATTCAGCCCAACGGAAAG-3' (hstx3-3'), 5'-TGGGCTTCAGGATGAAGGA-3' (hstx3-5'ups) with 5'-GCTAGATTGTTAGCTGAGTCA-3' (hstx3-3'dos), 5'-ATGGATAATCTGTCATCAGAAGAA-3' (SNAP23-5') with 5'-TTAGCTGTCAATGAGTTTCTT-3' (SNAP23-3'), and 5'-AGACTG-AAGCCATGGCGGATT-3' (TI-VAMP 5') with 5'-CTATTTCITCACACAGCTTGGC-3' (TI-VAMP3'), respectively. In the case of human syntaxin 3, all the clones that we obtained either with the oligonucleotides hstx3-5' and hstx3-3' or with hstx3-5'ups and hstx3-3'dos, which correspond to regions upstream of the starting ATG and downstream of the stop codon, had the following modifications compared with the human syntaxin 3 cDNA sequence deposited in GenBank (accession number U32315): position 519, CACGTC → CAGCTC, which translates into HV → QL; and position 816, AGC → ACG, which translates into S → T. The rat sequence is QL and T at the same positions. In the case of human SNAP23, all our clones had the following modification compared with the sequence deposited in GenBank (accession number U55936): 207GAA → GAG, which is conservative; and 462 GTC → GCC, which translates into V → A, as seen in SNAP23A (GenBank accession number Y09567). No modification was observed in our human clones of cellubrevin, identical to synaptobrevin 3 (GenBank accession number U64520), and in TI-VAMP (GenBank accession number M90418). Fragments of human cellubrevin predicted to contain only the first coiled coil domain (amino acids 1–52, hCB1) and both coiled coil domains (amino acids 1–71, hCB2) and of human TI-VAMP predicted to contain both coiled coils (amino acids 1–179) were obtained by cloning the corresponding cDNA into pGEX vectors (Pharmacia, Saclay, France). SNAP25 and syntaxin 1a clones were described previously (Hayashi *et al.*, 1994).

Immunofluorescence on Cells Grown on Filters

CaCo-2 cells were seeded at confluency (3.10^5 cells/cm²) on Transwell-Clear filters (Costar, Cambridge, MA) and grown 7–10 d to full polarization (transepithelial resistance, $800 \Omega \cdot \text{cm}^2$). The cells were washed in PBS supplemented with 0.1 mM CaCl₂, 0.1 mM MgCl₂ (PBS+), fixed with methanol at –20°C for 3 min, and rehydrated in PBS+. The filter was then cut and incubated over a drop of PBS containing the primary antibodies, washed three times with PBS, incubated with Cy2-conjugated goat anti-rabbit antibody and Texas Red-conjugated goat anti-mouse or anti-rat antibody, washed three times, and mounted in 90% glycerol in PBS. Confocal laser scanning microscopy was performed using a Leica (Nussloch, Germany) TCS microscope. The images were assembled without modification using Adobe (Mountain View, CA) Photoshop.

Immunogold Labeling on Ultrathin Cryosections

CaCo-2 cells were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature and processed for ultracryomicrotomy as described elsewhere (Raposo *et al.*, 1997). Ultrathin cryosections were collected using a mixture of 2.3 M sucrose and methylcellulose (vol/vol) and were immunogold labeled with antibodies against syntaxin 3, SNAP23, or TI-VAMP and protein A-gold conjugates (PAG 10, Department of Cell Biology, Utrecht University, Utrecht, The Netherlands) (Raposo *et al.*, 1997).

No labeling was observed with protein A-gold alone or nonimmune serum and protein A-gold.

In Vitro Assays

The effect of light chains of the clostridial NTs was tested according to the procedure of Hayashi *et al.* (1994). Briefly, the cDNAs of syntaxin 3, cellubrevin, and TI-VAMP cloned in pCR3 (Invitrogen, NV Leek, The Netherlands), of SNAP23 cloned in pET15b (Novagen, R & D Systems, Abingdon, UK), and of SNAP25 and syntaxin 1a (Hayashi *et al.*, 1994) were translated *in vitro* using T7 polymerase and rabbit reticulocyte lysate in the presence of [³⁵S]methionine. The proteins were incubated with the light chains of TeNT (2 μ M) and BoNT A (0.8 μ M), B (2 μ M), C (0.9 μ M), D (2 μ M), E (0.4 μ M), F (2 μ M), and G (2 μ M) in 150 mM potassium glutamate, 10 mM HEPES-KOH, pH 7.2, for 1 h at 37°C. The cleavage was analyzed after the products were separated on 15% SDS-PAGE gels followed by visualization of bands by autoradiography.

For binding assays, full-length human syntaxin 3 and SNAP23 were translated *in vitro* using the Quick T7 kit (Promega, Lyon, France) according to the manufacturer's procedure. The resulting extract (8 μ l) was incubated with glutathione beads coated with GST, GST-hCB1, GST-hCB2, or GST-TI-VAMP (1 μ M final concentration) in 100 μ l of binding buffer (4 mM HEPES-NaOH, pH 7.4, 100 mM NaCl, 3.5 mM CaCl₂, 3.5 mM MgCl₂, 1 mM EDTA, 0.1% NP-40) for 12 h at 4°C as described previously (Hayashi *et al.*, 1994). The beads were collected by centrifugation and washed six times with washing buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2.5 mM MgCl₂, 0.1% NP-40), and the final bead fraction was eluted with gel sample buffer consisting of 60 mM Tris, pH 6.8, 2% (wt/vol) SDS, 10% (wt/vol) glycerol, 0.07% bromophenol blue, and 5% β -mercaptoethanol. The resulting samples were either kept at room temperature for 30 min or boiled for 10 min and analyzed using SDS-PAGE (Schagger and von Jagow, 1987) followed by visualization of bands by autoradiography.

Immunoprecipitation from CaCo-2 Cells

CaCo-2 cells grown on 25-mm-diameter wells to confluency were washed twice briefly in PBS+ and were treated in PBS+ with 1 mM NEM or 1 mM NEM plus 2 mM DTT for 15 min on ice. In the first condition, NEM was quenched by 2 mM DTT for 15 min on ice. The cells were then washed in PBS+, further incubated in culture medium for 30 min at 37°C, lysed in solubilization buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 10 mM EDTA, 1 mM PMSE, 1 mM benzamidine, 1 μ g/ml pepstatin, 1 μ g/ml antipain, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1% Triton X-100), and centrifuged at 200,000 \times g for 15 min. The resulting supernatant was incubated overnight at 4°C with rotation with anti-syntaxin 3, anti-GST, anti-cellubrevin, or anti-TI-VAMP immunobeads, which had previously been prepared as described (Chilcote *et al.*, 1995) by covalent coupling of corresponding affinity-purified antibodies. The beads were then pelleted for 1 min at 1000 \times g and washed with solubilization buffer containing 0.5% Triton X-100. Finally, the beads were eluted with 100 mM glycine, pH 2.7, 0.5% Triton X-100, and the corresponding eluate was neutralized with Tris, pH 8, supplemented with gel sample buffer, boiled for 5 min, and run on SDS-PAGE gels (Schagger and von Jagow, 1987).

RESULTS

Best-fit comparison (Genetics Computer Group [GCG], Madison, WI) of human TI-VAMP with the human brevins shows that the two putative coiled coils of this protein are 64 and 57%, respectively, similar to corresponding domains in the VAMP/brevins (Figure 1A). Overall, we found that the similarity is

59% with synaptobrevin 2 and 56% with SNC1p, a v-SNARE implicated in Golgi to the plasma membrane transport in yeast (Protopopov *et al.*, 1993). The similarity between TI-VAMP and ERS-24, GOS28, human ykt6, or rsec22 v-SNAREs found in the endoplasmic reticulum and/or in the Golgi is <50% (46, 40, 49, and 43%, respectively). Two sets of four hydrophobic residues present in hepta repeats that overlap with the two coiled coils are found both in this new molecule and in the VAMP/brevins (Figure 1A). We found that TI-VAMP, translated *in vitro*, is insensitive to TeNT and to BoNTs B, D, F, and G, which all proteolyze cellubrevin (Figure 2A), and has structural properties of v-SNAREs (see below). Moreover, it is noteworthy that the first 90 amino acids of TI-VAMP define a domain that is not found in any other v-SNARE. In this domain, we have identified a region of 40 amino acids that has 47.5% similarity and 27.5% identity with the first annexin repeat of annexin XIII (Figure 1B) which forms part of an important calcium- and phospholipid-binding site (Raynal and Pollard, 1994; Smith and Moss, 1994). We also showed that, similar to the VAMP/brevins, TI-VAMP is a type II membrane protein, because the protein inserted into microsomes is sensitive to proteinase K (our unpublished results). We have generated in rabbit an antibody directed against the putative cytoplasmic domain of TI-VAMP (amino acids 1–179) fused to GST (serum referred to as TG11). This antibody reacts specifically with its corresponding antigen, recognizes a band of the expected size (25 kDa) in CaCo-2 cell extract by Western blotting, and specifically immunoprecipitates TI-VAMP (Figure 1C).

Potential partners of TI-VAMP, SNAP23, and syntaxin 3 are produced in most cell types, including epithelial cells (see Figure 7; our unpublished observations) and are also insensitive to NTs (Figure 2, B and C). *In vitro*-translated SNAP23 is not cleaved by BoNTs A, C1, or E, whereas these toxins very efficiently cleaved SNAP25 (Niemann *et al.*, 1994; Schiavo *et al.*, 1994; Osen-Sand *et al.*, 1996) (Figure 2B), a neuronal isoform of SNAP23. We have reinvestigated the effect of BoNT C1 on syntaxin 3. We found that contrary to syntaxin 1a, human syntaxin 3 is insensitive to BoNT C1 at a concentration as high as 0.9 μ M (Figure 2C), a 10-fold excess over the concentration required for maximal cleavage of syntaxin 1a. A study with increasing concentrations of BoNT C1 (ranging from 1 nM up to 3.8 μ M) showed that rat syntaxin 3 is not cleaved by this toxin even at the highest concentration tested (our unpublished observations).

Because it was shown that apical transport of HA is insensitive to clostridial NTs (Ikonen *et al.*, 1995), we investigated the subcellular distribution of clostridial NT-insensitive SNAREs in the human epithelial cell line CaCo-2, a well-documented model of polarized intestinal cells (Weimbs *et al.*, 1997). By confocal mi-

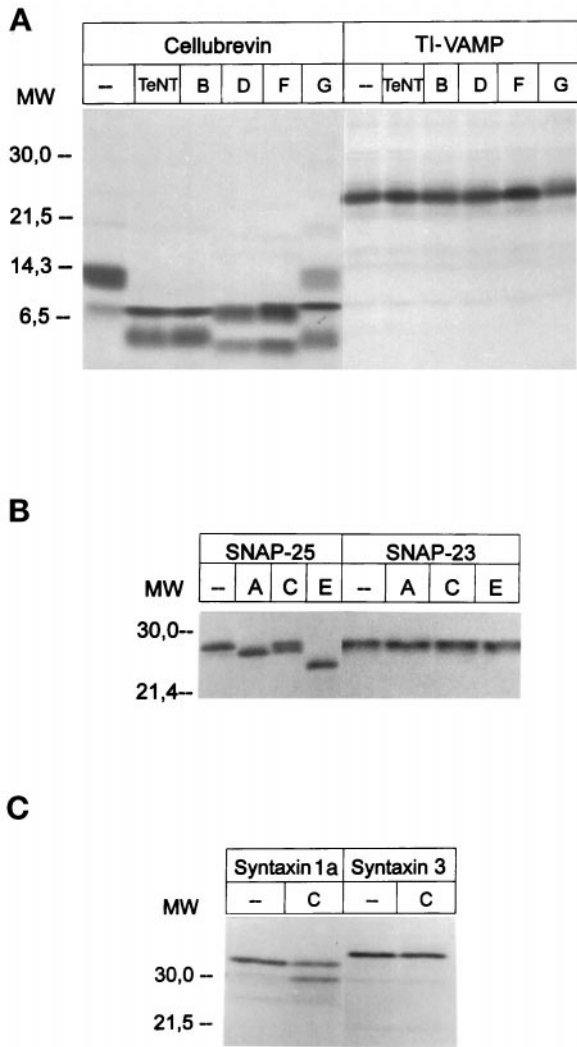


Figure 2. Effects of recombinant light chain of clostridial NTs on SNARE proteins. Cellubrevin, TI-VAMP (A), SNAP25, SNAP23 (B), syntaxin 1a, and syntaxin 3 (C) were translated in vitro in the presence of [³⁵S]methionine and then incubated with the various light chains of the NTs in 150 mM potassium glutamate, 10 mM HEPES-KOH, pH 7.2, for 12 h at 37°C. The cleavage was analyzed after the products were separated by SDS-PAGE and visualization of bands by autoradiography. The concentrations of the toxins used were as follows: TeNT, BoNT/B, D, F, and G, 2 μM; BoNT A, 0.8 μM; BoNT C, 0.9 μM; and BoNT E, 0.4 μM.

organelles (our unpublished observations) as seen by confocal microscopy. Because Tf receptor is known to recycle mainly between basolateral endosomes and plasma membrane (Hughson and Hopkins, 1990), we investigated whether TI-VAMP could be detected in the apical plasma membrane as expected if TI-VAMP-containing vesicles dock and fuse at this site. For this purpose, we have analyzed the localization of TI-VAMP by immunoelectron microscopy with gold-labeled antibodies in CaCo-2 cells, and we observed

labeling of the apical plasma membrane, suggesting the presence of a pool of this protein (Figure 5C).

We then studied the capacity of TI-VAMP and cellubrevin to interact with t-SNAREs in vitro. Using recombinant fragments of the cytoplasmic domains of these v-SNAREs fused to GST, we found that cellubrevin and TI-VAMP were both able to form SDS-resistant SNARE complexes (Hayashi *et al.*, 1994) with syntaxin 3 and SNAP23 translated in vitro in the presence of [³⁵S]methionine (Figure 6). SDS resistance of the brain syntaxin 1/SNAP25/synaptobrevin 2 SNARE complex is thought to be a key property of SNARE complexes that is probably attributable to the high stability of the coiled-coiled interactions (Hayashi *et al.*, 1994). We also found that both cellubrevin and TI-VAMP interacted poorly with syntaxin 3 alone (Figure 6) or SNAP23 alone (our unpublished results), in agreement with a previous report showing the lack of interaction between syntaxin 3 and the synaptobrevins (Calakos *et al.*, 1994). Interestingly, a shorter fragment of the cytoplasmic domain of cellubrevin, excluding the second coiled coil fused to GST (GST-hCB1), was only slightly less efficient in this assay (Figure 6), as was expected, because deletion of the second putative α helix in synaptobrevin 2 did not prevent association of SNAP25 (Regazzi *et al.*, 1996). These data clearly indicate that cellubrevin and TI-VAMP have the capacity to form SNARE complexes with the same t-SNAREs, including the NT-insensitive syntaxin 3 and SNAP23.

To show that exocytic organelles can dock at the apical plasma membrane, we set up an immunoprecipitation assay based on the observation that syntaxin 3 is found strictly at the apical plasma membrane (see above). NEM treatment was used to block NEM-dependent ATPases, including NEM-sensitive NSF (Beckers *et al.*, 1989), and therefore should elicit accumulation of assembled SNARE complexes as seen in PC12 cells (Banerjee *et al.*, 1996). NEM has also been shown to inhibit the binding of nSec1 to syntaxin 1 in vitro (Meffert *et al.*, 1996), an effect that is also expected to enhance SNARE complex formation, because nSec1 prevents syntaxin 1 from forming SNARE complexes (Pevsner *et al.*, 1994). To compare a control condition with a condition in which SNARE complexes are accumulated, we pretreated CaCo-2 cells grown on filters at 4°C with either 1) 1 mM NEM plus 2 mM DTT for 30 min (control conditions) or 2) 1 mM NEM (15 min) followed by 2 mM DTT (15 min) and allowed vesicles to accumulate at their target membrane by returning the cells to culture medium at 37°C as reported previously in the case of PC12 cells (Banerjee *et al.*, 1996). First, we observed by immunofluorescence that syntaxin 3 and SNAP23 are still localized at the apical plasma membrane after treatment with NEM. Second, using this experimental approach, we could immunoprecipitate virtually all of the syntaxin 3 present in the cell lysate using anti-syntaxin 3 immu-

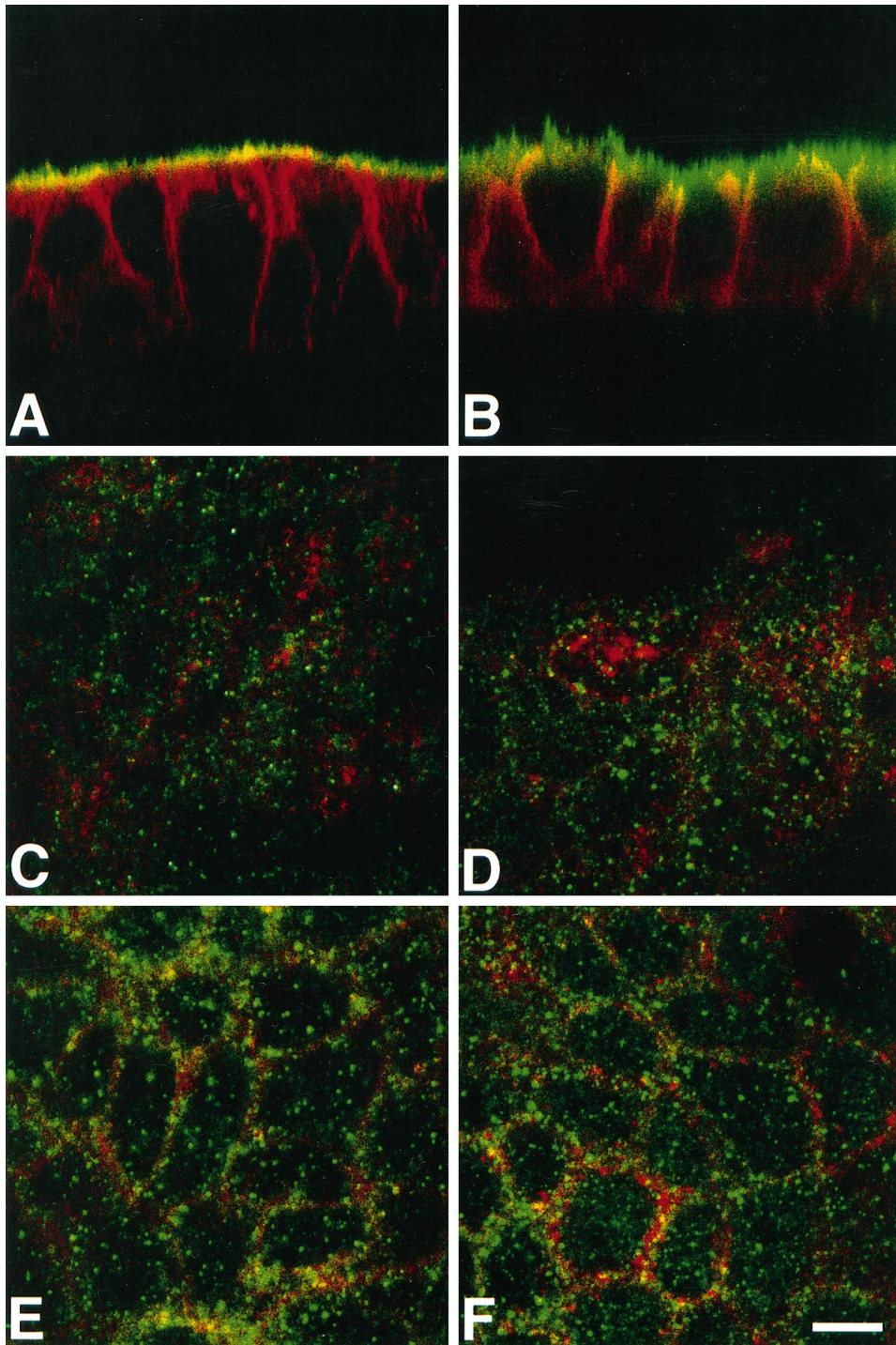


Figure 3. Localization of v- and t-SNAREs in vertical (A and B) and horizontal (C–F) confocal sections of CaCo-2 cells. Syntaxin 3 (green, A) and SNAP23 (green, B) are present at the apical plasmalemma. The staining for E-cadherin, a protein of the basolateral membrane, is in red (A and B). Note the occurrence of a low amount of SNAP23 in the top part of the lateral plasma membrane (yellow, B). Cellubrevin (green, C, E) and TI-VAMP (green, D, F) are present on intracellular organelles localized both in the lateral plane (C and D) and in the apical domain (E and F). Note the very low degree of colocalization (yellow) with transferrin receptor (monoclonal antibody H68.4, red, C and D) of both v-SNAREs. Bar, 10 μm .

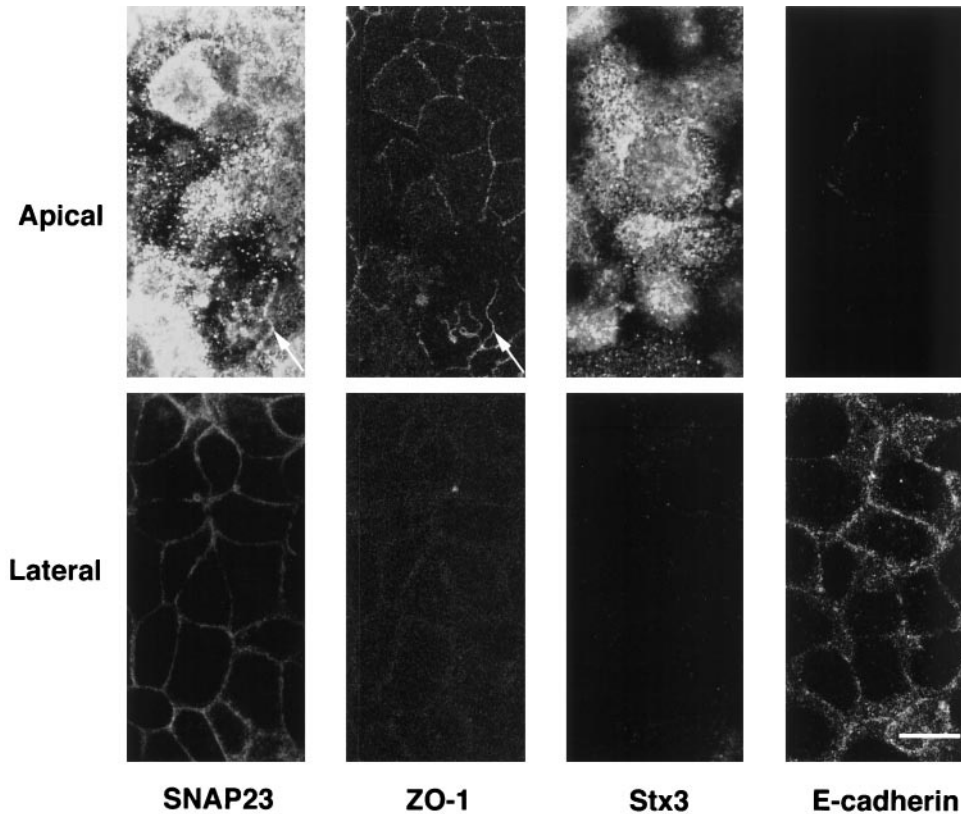


Figure 4. Localization of t-SNAREs in horizontal confocal sections of CaCo-2 cells. Double immunofluorescence micrographs of CaCo-2 cells stained for SNAP23 and ZO-1, a tight junction marker (left) or syntaxin 3(Stx3) and E-cadherin, a basolateral marker (right). Syntaxin 3 is restricted to the apical plasma membrane (note the lack of staining of the lateral plasma membrane that is positive for E-cadherin in bottom right micrographs). SNAP23 is highly concentrated at the apical plasmalemma and is present in tight junctions (top left micrographs; arrow indicates colocalization with ZO-1) and at low level in the lateral plasma membrane below tight junctions (bottom left). Bar, 10 μm .

nobeads (Figure 7). Western-blotting analysis of the fractions bound to the syntaxin 3 immunobeads showed a dramatic simultaneous increase of the amounts of SNAP23 and cellubrevin coimmunoprecipitated with syntaxin 3 when the cells were pretreated with NEM (Figure 7). Third, TI-VAMP was found to coimmunoprecipitate with syntaxin 3 only from NEM-pretreated cell extracts (Figure 7). None of the SNAREs were detected in pellet fractions recovered with anti-GST immunobeads, and Sec8, a protein that can be found in association with syntaxin 1 to a small extent (Hsu *et al.*, 1996), was also absent from all bead fractions (Figure 7), therefore showing the high specificity of the experimental procedure. This result clearly indicates that the t-SNAREs present at the apical plasma membrane, syntaxin 3 and SNAP23, allow NEM-dependent SNARE complex formation, which includes cellubrevin or TI-VAMP (Figure 7).

DISCUSSION

Our results suggest the involvement of the clostridial NT-resistant t-SNAREs syntaxin 3 and SNAP23 in

exocytosis at the apical plasma membrane of epithelial cells.

First, we have found that in contrast to their neuronal counterparts, SNAP23 and syntaxin 3 were not proteolyzed by clostridial NTs. Our finding that syntaxin 3 was insensitive to BoNT C1 is in contrast to a report showing the cleavage of a synaptosomal protein recognized by an anti-syntaxin 3 antibody by BoNT C1 (Schiavo *et al.*, 1995). It should be mentioned that the antibody used in the latter study also weakly recognizes syntaxin 1 (Gaisano *et al.*, 1996). In the present article, we report the lack of effect of the toxins on full-length *in vitro*-translated human SNARE proteins. This method avoids the kind of technical problems described above. The resistance of SNAP23 and syntaxin 3 to clostridial NTs is surprising given the high degree of similarity between SNAP25 and SNAP23 (72%) and between syntaxin 1 and syntaxin 3 (77%). The lack of cleavage by NTs of SNAP23 and syntaxin 3 might be the result of the inability of the toxins either to bind to the putative coiled coils of these SNAREs or to

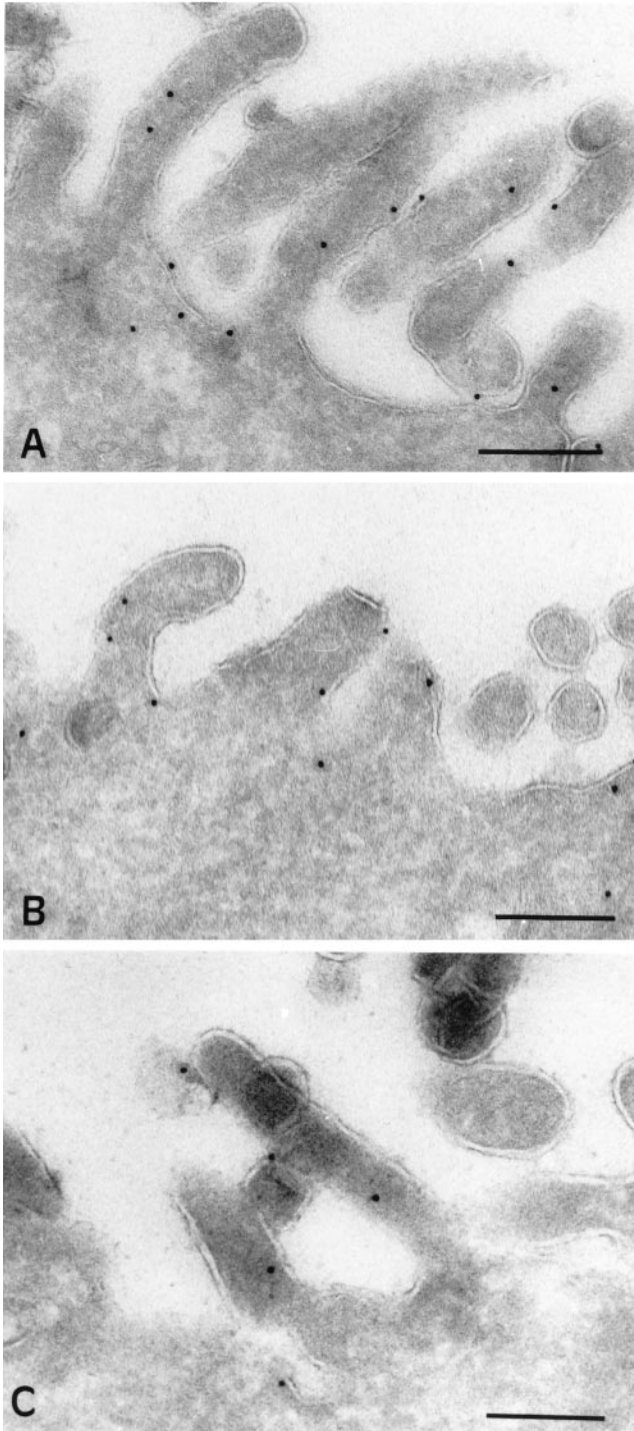


Figure 5. Immunogold localization of syntaxin 3, SNAP23, and TI-VAMP in CaCo-2 cells. Syntaxin 3 (A), SNAP23 (B), and TI-VAMP (C) visualized with their respective affinity-purified antibody and PAG 10 are localized at the cytoplasmic side of the microvillar membrane of polarized CaCo-2 cells. Bars, 200 nm.

recognize them as substrates because of key amino acid substitutions within the cleavage sites (Pellizzari *et al.*, 1996).

Second, by confocal and immunoelectron microscopy, we have found that endogenous syntaxin 3 and SNAP23 were both localized at the apical plasma membrane of CaCo-2 cells. In the case of syntaxin 3, we were unable to detect any intracellular or basolateral plasma membrane staining either by confocal microscopy or by immunoelectron microscopy on fully polarized and differentiated cells. This is in contrast to two studies on MDCK (Low *et al.*, 1996) and CaCo-2 cells (Delgrossi *et al.*, 1997) overexpressing syntaxin 3 in which significant intracellular labeling was detected, including lysosome labeling (Low *et al.*, 1996). In our case, we observed syntaxin 3-positive vesicular structures only in CaCo-2 cells that were not confluent and not yet fully polarized (our unpublished observations). We believe therefore that intracellular syntaxin 3 labeling might represent the newly synthesized pool or a pool resulting from overexpression of the protein or both. In the case of the partner t-SNARE SNAP23, we found that this protein is localized mainly at the apical plasma membrane on fully polarized CaCo-2 cells, with a minor labeling of the lateral plasma membrane as shown by longitudinal confocal sections. We found a major association of SNAP23 with syntaxin 3 by coimmunoprecipitation. This demonstrates that syntaxin 3 and SNAP23 act in concert as apical plasma membrane t-SNAREs. The lateral pool of SNAP23 is probably associated with another syntaxin isoform, because SNAP23 was already found to associate with syntaxin 4 (Ravichandran *et al.*, 1996), a basolateral t-SNARE in MDCK cells (Low *et al.*, 1996). Because we could not detect syntaxin 4, 2, or 1 by immunofluorescence microscopy in CaCo-2 cells (our unpublished observations), we have not investigated this point in the present study.

Third, we show that syntaxin 3 and SNAP23 form apical SNARE complexes and provide the first evidence for the involvement of cellubrevin and a new v-SNARE, TI-VAMP, in apical SNARE complex formation. We have taken advantage of the specific localization of syntaxin 3 at the apical plasma membrane of polarized CaCo-2 cells to immunoprecipitate apical SNARE complexes containing syntaxin 3. We found that NEM pretreatment of CaCo-2 cells increased the recovery of cellubrevin and SNAP23 associated with syntaxin 3. We have also found that in NEM-pretreated cell extracts, SNAP23 and syntaxin 3 form SNARE complexes with TI-VAMP, a new member of the VAMP/brevin family that is the first to be shown to be insensitive to all clostridial NTs that proteolyze the brevins. Because of the restricted localization of syntaxin 3 to the apical plasma membrane and cellubrevin and TI-VAMP on intracellular vesicles, we believe that the most likely explanation of our immu-

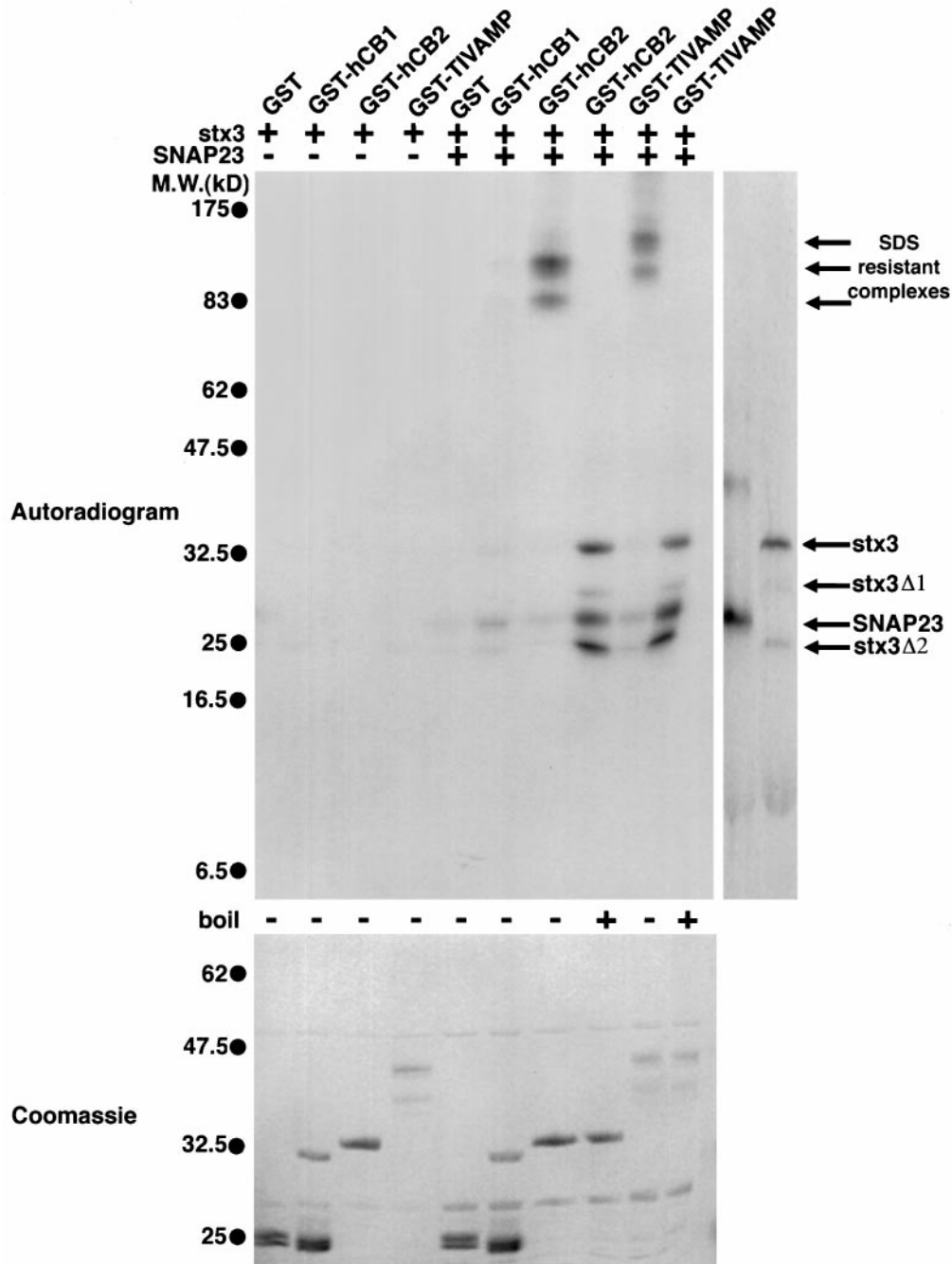


Figure 6. In vitro formation of SNARE complexes of cellubrevin and TI-VAMP with SNAP23 and syntaxin 3. Glutathione beads coated with GST, GST-hCB1 (first coiled coil only), GST-hCB2 (whole cytoplasmic domain), or GST-TI-VAMP (whole cytoplasmic domain) (1 μ M, 100 μ l) were incubated overnight with radiolabeled in vitro-translated human syntaxin 3 (stx3) alone or together with human SNAP23 (8 μ l of translated proteins). The fractions bound to glutathione beads were eluted, separated by SDS-PAGE, and visualized by autoradiography. The shorter forms of stx3 (stx3 Δ 1 and stx3 Δ 2) are likely to be generated by initiation of translation at internal methionine residues. Binding of syntaxin 3 alone to the v-SNAREs is at the limit of detection. The molecular weights of the SDS stable complexes are 85 and 120 kDa in the case of GST-hCB2 and 115 and 150 kDa in the case of GST-TI-VAMP. Note also that the GST-hCB1 SNARE complex is not SDS stable.

noprecipitation experiments is that cellubrevin- and TI-VAMP-containing vesicles dock at the apical plasma membrane through the NEM-dependent for-

mation of SNARE complexes, which include SNAP23 and syntaxin 3. Further evidence was obtained in the case of TI-VAMP by our observation of a pool of the

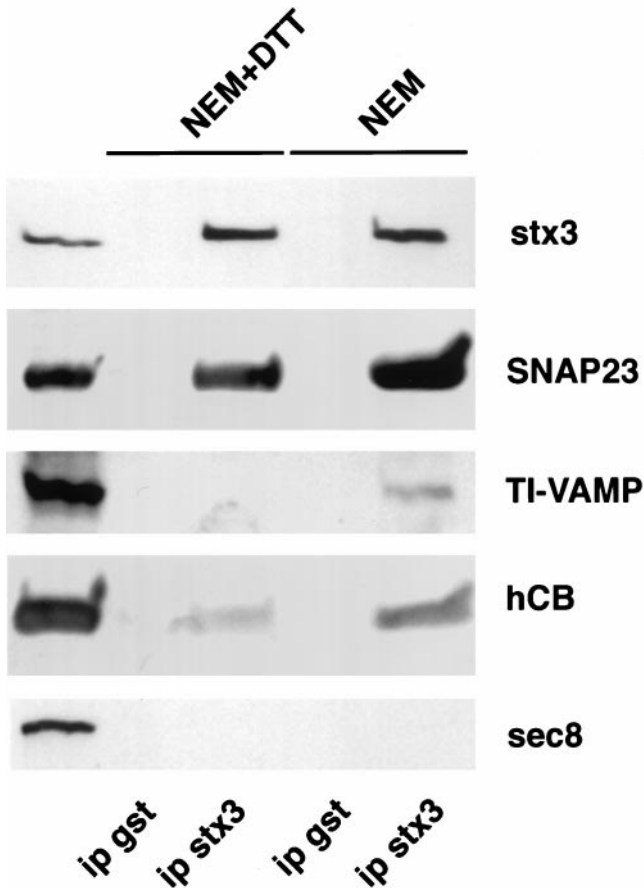


Figure 7. Effect of NEM on the formation of SNARE complexes in CaCo-2 cells. CaCo-2 cells grown to confluency were treated with 1 mM NEM for 15 min and then with 2 mM DTT for 15 min (NEM) or with 1 mM NEM plus 2 mM DTT for 30 min (NEM+DTT) on ice. The cells were then further incubated in culture medium for 30 min at 37°C and lysed in the presence of 1% Triton X-100 in conditions in which proteins are diluted enough so that the SNARE complex does not form spontaneously (Banerjee *et al.*, 1996). The cell extract was immunoprecipitated with anti-syntaxin 3 (ip stx3) or anti-GST (ip gst) (the pellet fractions represent a fivefold enrichment compared with the amount in the extract). The bound fractions were run on SDS-PAGE (Schagger and von Jagow, 1987) and analyzed by Western blotting with specific SNARE (syntaxin 3, stx3; hCB, human cellubrevin) antibodies. Note that syntaxin 3 is entirely immunoprecipitated and that NEM treatment greatly stimulates the recovery of SNAP23, cellubrevin, and TI-VAMP in the syntaxin 3 immunobead pellets.

protein at the apical plasma membrane revealed by immunogold labeling on ultrathin cryosections. In CaCo-2 cells, the syntaxin 3/SNAP23/cellubrevin and syntaxin 3/SNAP23/TI-VAMP SNARE complexes appear as the analogues of the syntaxin 1/SNAP25/synaptobrevin complex found in the nerve terminal.

The properties of TI-VAMP, a new member of the VAMP/brevin family, have not been examined previously. It is insensitive to NTs, binds to plasma membrane t-SNAREs, and localizes to apical organelles in

epithelial cells. This v-SNARE has a unique N-terminal domain that resembles the lipid/ Ca^{2+} binding domain of annexin XIII and therefore may be a v-SNARE with additional lipid/ Ca^{2+} binding properties. In any case, the N-terminal domain of TI-VAMP probably confers to this molecule specific features that would distinguish it from other members of the VAMP/brevin family. It is intriguing that the weak but nonetheless significant similarity we found in the N-terminal part of TI-VAMP establishes a relationship between this new v-SNARE and annexin XIII, an intestine-specific annexin (Wice and Gordon, 1992), which has been proposed to play a role in apical transport of HA in MDCK cells (Fiedler *et al.*, 1995). Future studies should explore the functional relevance of the similarity between TI-VAMP and annexin XIII. Another remarkable property of TI-VAMP is that it is resistant to TeNT and to BoNTs B, D, F, and G. As in the case of SNAP23 and syntaxin 3, this might result from lack of binding of the NTs or from the differences in amino acid sequence observed in the NT cleavage sites (see the legend of Figure 1A). In this study in epithelial cells, despite the biochemical specificities of TI-VAMP depicted above, we have not found major localization or biochemical differences regarding the t-SNARE partners between TI-VAMP and cellubrevin. This may be because these proteins have overlapping functions in the same apical exocytotic pathway or because our level of analysis could not distinguish different apical exocytotic pathways. Our observations 1) that cellubrevin and TI-VAMP could both form SNARE complexes with the same apical t-SNAREs but that these v-SNAREs were not complexed together and 2) that TI-VAMP was found associated with syntaxin 3 only in NEM-pretreated cells could be seen to favor the second hypothesis. In CaCo-2 cells, sucrase isomaltase was shown to be transported directly from the trans-Golgi network to the apical plasma membrane, whereas dipeptidyl-peptidase IV is first transported to the lateral membrane and then sorted to the apical plasma membrane (Matter *et al.*, 1990). Given the role of cellubrevin in transferrin receptor recycling in fibroblasts (Galli *et al.*, 1994) and the lack of effect of TeNT on transport of HA from the trans-Golgi network to the apical plasma membrane in MDCK cells (Ikonen *et al.*, 1995), an attractive but speculative hypothesis could be that cellubrevin is involved in recycling of apical proteins and TI-VAMP in the direct trans-Golgi network to the apical plasma membrane pathway. Obviously, it will be important to characterize by electron microscopy the vesicular pools of cellubrevin and TI-VAMP in epithelial cells, to test whether TI-VAMP distributes on HA-containing vesicles and whether it is involved in apical docking/fusion of these vesicles with the apical plasma membrane in MDCK cells.

In conclusion, our data are in favor of the existence of SNARE-dependent exocytotic events at the apical plasma membrane of epithelial cells. They also imply that NTs should be unable to impair multiple exocytotic events in different cell types. The NT-resistant SNAREs described here have a very broad tissue and cell distribution. Our work opens the way for studying NT-resistant secretory pathways in nonneuronal cells and in neurons (Osen-Sand *et al.*, 1996) and in particular the role played by TI-VAMP in these cell types.

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