INTRODUCTION

The soluble NSF attachment protein receptor (SNARE) hypothesis of vesicle transport postulates that specific membrane-anchored proteins on the transport vesicle and on the target membrane form a central part of the machinery responsible for specific docking/fusion of transport vesicles (Söllner et al., 1993a; Rothman, 1994). The vesicle SNAREs (v-SNAREs) are related to the synaptic vesicle synaptobrevin/VAMP proteins, and the target membrane SNAREs (t-SNAREs) to the synaptic plasma membrane syntaxins 1A, B and the synaptosomal-associated protein of 25 kDa (SNAP-25). The association of the t-SNAREs syntaxin and SNAP-25 creates a high affinity binding site for the v-SNARE synaptobrevin (Pevsner et al., 1994; Hayashi et al., 1994). The SNARE complexes function as binding sites for the general components of the cellular membrane fusion machinery, the N-ethylmaleimide sensitive fusion protein (NSF) and the soluble NSF attachment proteins (SNAPs). Proteins homologous to the synaptic SNAREs have been identified in numerous eukaryotic organisms from yeast to man, and their central role in intracellular membrane trafficking has been established (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Hay and Scheller, 1997). In mammalian cells the syntaxins constitute a large family of proteins, some of which are ubiquitous while some display more restricted expression patterns (Bennett et al., 1993; Bock and Scheller, 1997). SNAP-23 represents a counterpart of SNAP-25 in non-neuronal cells (Ravichandran et al., 1996), and cellubrevin is a ubiquitously expressed v-SNARE with suggested functions in exocytotic and recycling processes (Galli et al., 1994; Volchuk et al., 1995).

The Sec1-related proteins constitute a family of molecules that interact with syntaxins and are suggested to play a crucial role in the assembly of vesicle docking/fusion complexes (Aalto et al., 1992; Halachmi and Lev, 1996; Pevsner, 1996). Loss-of-function mutations in the Sec1-related genes of Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster have been demonstrated to lead to specific blocks in vesicle transport events (Novick et al., 1980; Ossig et al., 1991; Hosono et al., 1992; Piper et al., 1994; Cowles et al., 1994; Harrison et al., 1994; Wu et al., 1998), indicating a positive role of the encoded proteins in membrane trafficking processes (see also Graham et al., 1997). On the other hand, overexpression of Sec1 proteins is reported to inhibit vesicle docking/fusion (Schulze et al., 1994; Wu et al., 1998); the negative aspect of Sec1 action is further demonstrated by the finding that the neuronal Sec1 in vitro inhibits the association of syntaxin 1A with VAMP and SNAP-25 (Pevsner et al., 1994). Together, these observations suggest...
that the interaction of the Sec1 proteins with syntaxins leading to successful vesicle docking/fusion is transient in nature (Lupashin and Waters, 1997).

In mammals, the family of cloned Sec1 homologues constitutes 9 proteins which display characteristic patterns of syntaxin binding specificity (Halachmi and Lev, 1996; Pevsner, 1996; Matsuo et al., 1997). Even though the mammalian Sec1 proteins have been available for study for several years, little is known of their functions in specific membrane trafficking events. Previously, two endogenous Sec1/syntaxin complexes have been characterized: The neuronal n-Sec1 coimmunoprecipitates with syntaxin 1 (Pevsner et al., 1994; Garcia et al., 1995), and the Sec1 homologue r-Sly1 forms a functional complex with syntaxin 5, thereby regulating endoplasmic reticulum to Golgi trafficking (Dascher and Balch, 1996). In addition, Munc-18c (a ubiquitously expressed Sec1 protein) putatively interacts with syntaxin 4 to regulate GLUT-4 translocation in adipocytes (Tellam et al., 1997). We have previously identified Munc-18-2/Munc-18b (Katagiri et al., 1996; Tellam et al., 1995; Hata and Südhof, 1995) as a Sec1 homologue that is predominantly expressed in epithelial cells (Riento et al., 1996). In this study we present evidence for a novel functional complex consisting of Munc-18-2 and syntaxin 3, which is located on the apical plasma membrane of the epithelial cell line Caco-2. Furthermore, we show that the amount of the partner SNAREs SNAP-23 and cellubrevin bound to syntaxin 3 decreases upon Munc-18-2 overexpression, suggesting the involvement of Munc-18-2 in the regulation of vesicle transport at the apical plasma membrane of epithelial cells.

**MATERIALS AND METHODS**

**Cell culture**

The human colon carcinoma cell line Caco-2 was cultured in Eagle’s minimum essential medium (MEM) supplemented with 10% foetal bovine serum (Life Technologies), 2 mM L-glutamine, 1% non-essential amino acids, 100 i.u/ml penicillin and 100 μg/ml streptomycin. Culture of Caco-2 cells on Costar Transwell polycarbonate filters was carried out according to the method of Pinto et al. (1983).

**Antibodies**

Glutathione S-transferase (GST, Pharmacia) fusion protein containing the cytosolic portion of rat syntaxin 3 (syntaxin 3αC) was expressed in *Escherichia coli* JM109(DE3) (Promega) and purified by affinity chromatography on glutathione-Sepharose (Pharmacia). The protein was used for immunization of New Zealand White rabbits. For affinity purification of anti-syntaxin 3 antibodies, the antisemur was first incubated with GST and then with GST-syntaxin 3 covalently attached to cyanogen bromide activated Sepharose 4B (Pharmacia). The antibodies were eluted at acidic pH, followed by dialysis against phosphate buffered saline (PBS). The rabbit antisemur against Munc-18-2 is described in detail elsewhere (Riento et al., 1996). Syntaxin 2 antisemur was obtained from Dr M. K. Bennett, University of California, CA, US. The SNAP-23 and cellubrevin antisera are described by Galli et al. (1998).

**Immunofluorescence microscopy**

Filter-grown Caco-2 cells were fixed for 20 minutes with 4% paraformaldehyde, 250 mM Hepes, pH 7.4, and permeabilized for 20 minutes with 0.1% Triton X-100, 0.1% SDS in PBS. The primary antibodies diluted in 5% serum/PBS were incubated for 14 hours at 4°C and the bound antibodies were detected with fluorescein-isothiocyanate-conjugated goat anti-rabbit F(ab)2 (Immunotech). All incubations were carried out from both sides of the filter support. The specimens were mounted in Mowiol (Calbiochem); 50 mg/ml 1,4-diazabicicyclo-[2.2.2]octane (Sigma), and studied with a Leica TCS NT confocal microscope. Staining of 17-day embryonic mouse intestine cryosections (thickness 6-7 μm) was performed essentially as above, and the specimens were viewed with a Zeiss Axioshot fluorescence microscope.

**Western blotting**

Proteins were resolved by SDS-PAGE and transferred to Hybond-C extra nitrocellulose (Amersham). Unspecific binding of antibodies was blocked with, and all antibody incubations were carried out in, 5% fat-free milk, 0.05% Tween-20 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl. The bound antibodies were visualized with horseradish-peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) and the enhanced-chemiluminescence system (ECL, Amersham), or with 35S-labeled Protein A (Amersham). Quantitation of radioactive blots was performed with the Fujifilm BAS-1500 Imaging system.

**Overexpression of Munc-18-2**

The full length canine Munc-18-2 cDNA was inserted into the Smal site of pSFV1 (Liljestrom and Garoff, 1991), or cloned as a S’ myc-tagged version into the BamHI site of the viral vector. A construct carrying the human amyloid precursor protein cDNA was obtained from Dr P. Tienari (Dept of Neurology, University of Helsinki). Recombinant Semliki Forest viruses were prepared in BHK cells (Ollkonen et al., 1994). Caco-2 cells grown on 6 cm plastic dishes were treated with 5 mM EGTA in PBS for 10 minutes at 37°C, and transfected with the recombinant viruses for 4 hours as described by Ollkonen et al. (1994).

**Immunoprecipitations**

For myc-Munc-18-2 immunoprecipitation, transfected or untransfected Caco-2 cells were lysed in 1% Triton X-100, 10 mM Hepes, pH 7.4, 140 mM KCl, 1 mM MgCl₂ and 0.1 mM EGTA (buffer A) on ice. After removal of insoluble material in a microcentrifuge, the cell lysate was incubated with anti-myc antibody (9E10) covalently linked to agarose (Santa Cruz). For syntaxin 3 immunoprecipitation, affinity purified syntaxin 3 antibody and Protein A-Sepharose (Pharmacia) were incubated with Caco-2 cell lysate in buffer A, 0.01% sodium dodecyl sulfate (SDS). Munc-18-2 immunoprecipitation was carried out in buffer A, 0.1% SDS. The bound proteins were, after thorough washes of the matrix with buffer A, detached by boiling in reducing Laemmli sample buffer, resolved by SDS-PAGE, and analyzed by western blotting. For analysis of complexes between syntaxin 3 and SNAP-23/cellubrevin, the MgCl₂ and EGTA in buffer A were replaced by 10 mM EDTA.

**Sucrose density gradient analysis**

Caco-2 cells were incubated with [35S]methionine for 14 hours, after which they were lysed in 1% Triton X-100, 10 mM Hepes, pH 7.4, 140 mM KCl. After removal of insoluble material, the cell lysate was loaded onto 5-20% w/w sucrose gradients containing 10 mM Hepes, 140 mM KCl, 0.1% Triton X-100 and centrifuged in a SW40 rotor at 180 000 g for 18 hours. Fractions of 1 ml were collected, diluted threefold with buffer, and analyzed by immunoprecipitation with anti-syntaxin 3 or Munc-18-2 antibodies. For flotation sucrose gradients in the absence of detergent, Caco-2 cells were incubated with [35S]methionine for 14 hours, scraped in 250 mM sucrose, 10 mM Hepes, pH 7.4, 140 mM KCl, and broken by repeated passages through a 21 G needle. After removal of intact cells and nuclei, sucrose was added to 2 M (total volume 4.5 ml), which was overlayed with 5 ml of 1.7 M sucrose and 3.5 ml of 0.8 M sucrose, 10 mM Hepes, 140 mM KCl, and centrifuged in a SW40 rotor at 130 000 g.
for 20 hours, 10°C. Fractions of 1 ml were collected, diluted and analyzed by immunoprecipitation.

**Treatments of Caco-2 cells with pharmacological agents**

The medium of Caco-2 cells grown in flasks was changed to Na2HCO3-free MEM, 20 mM Hepes, pH 7.4, 0.2% BSA, and the cells were incubated at 37°C for 30 minutes in the presence of 10 μM nocodazole (before this the cells were kept at 4°C for 15 minutes), 5 μg/ml brefeldin A, 0.1 μM wortmannin, or 40 μM cytochalasin D. For N-ethylmaleimide (NEM) treatment, cells were incubated on ice with 1 mM NEM (Sigma), 2 mM dithiothreitol (DTT) for 30 minutes (control), or 15 minutes with 1 mM NEM and then 15 minutes with 2 mM DTT (Beckers et al., 1989). After change into fresh medium, the cells were incubated for 30 minutes at 37°C. Treatment with 1 μM phorbol myristate acetate was 15 minutes at 37°C. The cells were lysed in buffer A and protein complexes immunoprecipitated with anti-syntaxin 3 antibodies. The amounts of syntaxin 3 and Munc-18-2 in the precipitates were quantitated from immunoblots by the Fujifilm BAS-1500 system.

**In vitro binding of Munc-18-2 and syntaxin 3**

Canine Munc-18-2 was translated in vitro from pBluescript SK(-) in the presence of [35S]methionine using the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions; the reaction was terminated by addition of 1 mM cycloheximide. GST-syntaxin 3ΔC was covalently attached to CNBr-activated Sepharose 4B (Pharmacia). The proteins were separately incubated on ice with NEM or DTT + NEM as described above for Caco-2 cells, and thereafter incubated together for 60 minutes at room temperature. Alternatively, the proteins were allowed to associate before NEM or DTT + NEM treatment. After the incubations, the syntaxin 3 matrix was washed with PBS and the bound Munc-18-2 was detached by boiling with reducing Laemmli buffer, resolved by SDS-PAGE, and quantitated by the Fujifilm BAS-1500 Imaging system.

**RESULTS**

**Association of Munc-18-2 with syntaxin 3**

Our previous observation that Munc-18-2 is predominantly expressed in the epithelial cell layers of several tissues and localizes apically in the intestinal epithelium (Riento et al., 1996), prompted us to identify SNARE proteins associating with Munc-18-2 in epithelial cells. Of the t-SNAREs known to bind Munc-18-2 in vitro (Hata and Söd Hof, 1995; Riento et al., 1996), syntaxins 2 and 3 are expressed at significant levels in non-neuronal tissues. While the syntaxin 2 mRNA is expressed at similar levels in different mammalian tissues, the distribution of the syntaxin 3 message is more restricted, resembling that non-neuronal tissues. While the syntaxin 2 mRNA is expressed in the epithelial cell layers of several tissues and localizes apically in the intestinal epithelium (Riento et al., 1996), we verified the presence of a Munc-18-2 antisense immunoprecipitates the native protein poorly (Riento et al., 1996), we verified the presence of a Munc-18-2/syntaxin 3 complex by a transfection approach. myc-tagged Munc-18-2 was expressed in Caco-2 cells using the Semliki Forest virus (SFV) vector, and the complexes of myc-Munc-18-2 with endogenous proteins were isolated using the monoclonal anti-myc antibody 9E10 coupled to agarose. The bound proteins were resolved by SDS-PAGE and analyzed by western blotting. Syntaxin 3 was present in the immunoprecipitates of the transfected cells, whereas syntaxin 2 (using the antibody described by Low et al., 1996) was not being devoid of fluorescence signal. The apical immunostainings were completely inhibited by preincubation of the antibodies with the respective recombinant proteins (Fig. 2C,D). Also in polarized Caco-2 cells, Munc-18-2 and syntaxin 3 were detected on the apical plasma membrane (Fig. 2E,F).

To determine whether Munc-18-2 and syntaxin 3 form a physical complex, lysates of Caco-2 cells were immunoprecipitated using the affinity-purified syntaxin 3 antibody, and the presence of Munc-18-2 in the precipitates was assayed by western blotting. Munc-18-2 was specifically coprecipitated with syntaxin 3 (Fig. 3A). Under conditions in which all syntaxin 3 was recovered in the precipitate, 5% of the total Munc-18-2 present in the lysates coprecipitated (as quantitated by the BAS-1500 system). The result was the same for Caco-2 cells grown on plastic or on filter support. Further, the degree of cell confluence or disruption of the tight junctions of confluent cells by chelating Ca2+ with EGTA had no significant effect on the Munc-18-2/syntaxin 3 complex (data not shown), indicating that the presence of the complex is not dependent on cell polarity. In addition, the time of cell lysis incubation in the lysis buffer (containing 1% Triton X-100) before the precipitation did not affect the quantity of Munc-18-2 recovered in the complex (data not shown). Since our Munc-18-2 antisense immunoprecipitates the native protein poorly (Riento et al., 1996), we verified the presence of a Munc-18-2/syntaxin 3 complex by a transfection approach. myc-tagged Munc-18-2 was expressed in Caco-2 cells using the Semliki Forest virus (SFV) vector, and the complexes of myc-Munc-18-2 with endogenous proteins were isolated using the monoclonal anti-myc antibody 9E10 coupled to agarose. The bound proteins were resolved by SDS-PAGE and analyzed by western blotting. Syntaxin 3 was present in the immunoprecipitates of the transfected cells, whereas syntaxin 2 (using the antibody described by Low et al., 1996) was not being devoid of fluorescence signal. The apical immunostainings were completely inhibited by preincubation of the antibodies with the respective recombinant proteins (Fig. 2C,D). Also in polarized Caco-2 cells, Munc-18-2 and syntaxin 3 were detected on the apical plasma membrane (Fig. 2E,F).

**Fig. 1.** Characterization of the rabbit antibody against syntaxin 3. (A) Caco-2 cell total protein (10 μg/lane) was subjected to SDS-PAGE (12.5%) and western blotted with the affinity purified syntaxin 3 antibody (lane 1). The immunoreactivity was inhibited by preincubation of the antibody with purified GST-syntaxin 3ΔC (lane 2). (B) Syntaxins 1A, 2, 3, 4, and 5 (indicated above the top panel) were in vitro translated in the presence of [35S]methionine, resolved by SDS-PAGE, and either visualized by fluorography (top panel) or western blotted using the anti-syntaxin 3 antibody (bottom panel). The western blots were visualized by ECL detection.
detectable (Fig. 3B). The absence of syntaxin 2 in the complexes was probably due to a low expression level of the protein in Caco-2 cells (data not shown).

**Characterization of the Munc-18-2/syntaxin 3 complex**

To study the composition of the Munc-18-2/syntaxin 3 complex, the proteins of Triton X-100-solubilized, 35S-labeled Caco-2 cell lysates were sedimented in sucrose velocity gradients. Fig. 4 shows Munc-18-2 and syntaxin 3 immunoprecipitated from gradient fractions and analyzed by SDS-PAGE and fluorography. The majority of syntaxin 3 localized to fractions 2-6 (size range 20-100 kDa). Munc-18-2 migrated in fractions 4-6 (size range 60-100 kDa); the majority of the protein sedimented in the region where a soluble monomer should migrate. In order to visualize the fractions containing the Munc-18-2/syntaxin 3 complex, the syntaxin 3 immunoprecipitates were dissociated with glycine buffer, pH 2.5, neutralized, and immunoprecipitated with Munc-18-2 antibodies. The complex peaked in fraction 6, which corresponds to the molecular mass of a Munc-18-2/syntaxin 3 heterodimer (approximately 100 kDa). The large proportion of apparently monomeric Munc-18-2 prompted us to determine the distribution of the protein between soluble and membrane-bound forms. Post-nuclear supernatant of [35S]methionine labeled Caco-2 cells was fractionated by membrane flotation in sucrose gradients in the absence of detergent. The fractions were analyzed by immunoprecipitation, followed by SDS-PAGE and BAS-1500 quantitation. Munc-18-2 was almost exclusively found in the floating total membrane fraction; the fractions containing soluble proteins (2.0 M sucrose) were virtually devoid of the protein. The distribution of syntaxin 3, an integral membrane protein, was highly similar (Fig. 5).

To investigate whether the association of endogenous Munc-18-2 and syntaxin 3 is a dynamic process that can be affected by agents interfering with intracellular membrane trafficking, the immunoprecipitable complexes were analyzed after pretreatment of Caco-2 cells with nocodazole, cytochalasin D, wortmannin, phorbol myristate acetate, brefeldin A, or N-ethylmaleimide (NEM). The cells were lysed after the treatments and immunoprecipitated with the anti-syntaxin 3 antibodies.

**Fig. 2.** Localization of Munc-18-2 and syntaxin 3 in mouse intestine and Caco-2 cells by immunofluorescence microscopy. Mouse (embryonic day 17) intestine cryosections were stained with antibodies against Munc-18-2 (A,C) or syntaxin 3 (B,D). (C and D) The antibody dilutions were preincubated for 30 minutes at room temperature with the respective recombinant proteins (10 μg Munc-18-2 blotted on nitrocellulose/100 μl antibody dilution or 10 μg/ml of GST-syntaxin 3A/C). Caco-2 cells grown on polycarbonate filters were stained with the anti-Munc-18-2 (E) or syntaxin 3 antibodies (F). Panels E and F are XZ confocal images. The apical and basal domains of epithelial cells are indicated with a and b, respectively. Bars: 5 μm (A-D); 15 μm (E-F).

**Fig. 3.** Coimmunoprecipitation of Munc-18-2 and syntaxin 3. (A) The endogenous Munc-18-2/syntaxin 3 complex: immunoprecipitation of Caco-2 cell lysates with syntaxin 3 antibodies (anti-syn3), or a non-relevant rabbit IgG (IgG). The precipitates were western blotted using antibodies against either syntaxin 3 (anti-syn3) or Munc-18-2 (anti-Munc). (B) Complex of myc-Munc-18-2 and endogenous syntaxin 3: untransfected (-) Caco-2 cells or cells transfected for 14 hours with recombinant SFV expressing myc-tagged Munc-18-2 (T) were subjected to immunoprecipitation with monoclonal anti-c-myc. The precipitates were western blotted using either Munc-18-2 (anti-Munc), syntaxin 3 (anti-syn3) or syntaxin 2 (anti-syn2) antibodies. The blots were visualized by ECL detection. Syntaxin 3 is indicated with arrowheads, Munc-18-2 with arrows, and immunoglobulin heavy chains with H (The heavy chains are visible also in panel B due to a weak cross-reactivity of the secondary antibody conjugate with mouse IgG, together with a long exposure time used in the ECL detection).
Complex of Munc-18-2 and syntaxin 3

antibody. The precipitates were analyzed by western blotting with Munc-18-2 and syntaxin 3 antibodies (Fig. 6). Treatment of Caco-2 cells with phorbol myristate acetate (PMA), a stimulator of protein kinase C, increased the amount of Munc-18-2 in the complex by 60%. Disruption of the actin cytoskeleton with cytochalasin D or depolymerization of microtubules by nocodazole had no significant effect on the amount of Munc-18-2 bound to syntaxin 3. Likewise, treatments with the phosphoinositide-3-kinase inhibitor wortmannin or brefeldin A, a fungal toxin disturbing the cellular endomembrane organization, were without effect. The alkylating agent NEM, which inhibits a variety of membrane trafficking events, presumably by inactivating the NEM-sensitive fusion protein NSF (reviewed by Whiteheart and Kubalek, 1995), decreased the complex by 50% compared to the specific control (DTT + NEM). Inhibition of NSF activity leads to the stabilization of SNARE complexes (Söllner et al., 1993b). Therefore, the NEM effect detected could be due to sequestering of syntaxin 3 into complexes with other SNAREs (Galli et al., 1998), which would displace Munc-18-2. Alternatively, NEM could directly affect the ability of Munc-18-2 and syntaxin 3 to associate, or dissociate their complexes. The mode of NEM action was therefore studied in vitro using a purified GST-syntaxin 3ΔC fusion protein coupled to matrix and in vitro translated 35S-labeled Munc-18-2. The proteins were separately treated with NEM before the association reaction, or allowed to associate before the treatment. Pretreatment of Munc-18-2 with NEM before the association reaction, or allowed to associate before the treatment. Pretreatment of Munc-18-2 with NEM significantly interfered with the complex formation, resulting in a 45% reduction (Fig. 7). Treatment of syntaxin 3 caused only a minor inhibition, and preformed complexes were insensitive to the alkylating agent.

DISCUSSION

This study demonstrates that the Sec1-related protein Munc-18-2 and syntaxin 3 both localize on the apical plasma membranes of Caco-2 cells and the mouse intestinal

Fig. 4. Sucrose density gradient analysis of Munc-18-2, syntaxin 3, and their complex. Detergent lysates of Caco-2 cells labeled with [35S]methionine were subjected to centrifugation in 5-20% sucrose gradients in the presence of 0.1% Triton X-100. The fractions were immunoprecipitated using either syntaxin 3 (Syn3) or Munc-18-2 (Munc-18-2) antibodies, followed by SDS-PAGE and fluorography. To determine the mobility of the Munc-18-2/syntaxin 3 complex, the syntaxin 3 immunoprecipitates were dissociated at low pH, neutralized, and reprecipitated with anti-Munc-18-2 (Complex). Migration of molecular mass markers is indicated at the bottom.

Fig. 5. Flotation of Munc-18-2 and syntaxin 3 in sucrose gradients in the absence of detergent. The post-nuclear supernatant of Caco-2 cells labeled with [35S]methionine was loaded at the bottom of isopycnic sucrose step gradients. To analyze the distribution of the proteins in the heavy bottom fractions and the floating total membrane fraction, the gradient fractions were subjected to immunoprecipitation with Munc-18-2 (■) or syntaxin 3 (□) antibodies, and quantitation using the Fuji BAS-1500. The protein amounts in the fractions are given in arbitrary units. The sucrose concentrations in the gradient are indicated at the bottom.

The effect of Munc-18-2 overexpression on the association of apical SNAREs

The formation of SNARE complexes is suggested to be regulated by Sec1-related proteins (for reviews see Halachmi and Lev, 1996; Pevsner, 1996). The t-SNARE SNAP-23 and the v-SNARE cellubrevin are enriched in the apical domain of Caco-2 cells, and can be detected in a complex with syntaxin 3 (Galli et al., 1998). We therefore determined the effect of overexpressed Munc-18-2 on the association of syntaxin 3 with these partner SNAREs. Munc-18-2 or a non-relevant protein, the amyloid precursor protein, were expressed in Caco-2 cells using the Semliki Forest virus vector. After 4 hours of transfection using the viral vector, the quantity of Munc-18-2 in the cells was 6-fold compared to the endogenous protein level. Syntaxin 3 complexes were immunoprecipitated, followed by quantitation of Munc-18-2, SNAP-23, and cellubrevin in the precipitates. The transfection frequency was 90-100% as determined by immunofluorescence microscopy. The viral overexpression of Munc-18-2 increased the amount of the protein in the syntaxin 3 immunoprecipitates 4.5-fold (Fig. 8A). This increase coincided with a decrease of both SNAP-23 (62%) and cellubrevin (33%) bound to syntaxin 3, compared to cells infected with the control virus (Fig. 8B). The result demonstrates that moderate changes in the cellular amount of Munc-18-2 are able to modulate the association of syntaxin 3 with its cognate SNARE partners.
epithelium, and form a complex as demonstrated by coimmunoprecipitation. Munc-18-2 is, in addition to syntaxin 3, capable of binding syntaxins 1A and 2. However, in the intestinal Caco-2 cells studied here we were unable to detect a Munc-18-2/syntaxin 2 complex, probably due to a low expression level of syntaxin 2 in this cell type. Syntaxin 1A is primarily neuronal, and it is not expressed at detectable levels in Caco-2 cells (Némoz-Gaillard et al., 1998). Our previous demonstration that overexpressed syntaxins can recruit Munc-18-2 onto membranes indicated that a compatible syntaxin is sufficient for Munc-18-2 membrane association (Riento et al., 1996). It is thus possible that the apical localization of Munc-18-2 is due to an interaction with the apical syntaxin 3, and that on the basolateral plasma membrane there is no syntaxin capable of interacting with Munc-18-2 present at comparably high levels.

Upon immunoprecipitation in the presence of detergent, a minor portion (5%) of Munc-18-2 was recovered in a complex with syntaxin 3. Similarly, only a minor fraction of the Munc-18-2 was found in a complex with syntaxin 3 while the majority sedimented in the monomer position in velocity sucrose gradients containing detergent. However, cell fractionation by sucrose flotation in the absence of detergent demonstrated that Munc-18-2, like syntaxin 3, is almost quantitatively recovered in the total membrane fraction. Thus, the apparently monomeric Munc-18-2 detected following velocity centrifugation in the presence of detergent must have been released from membranes. Whether it originally was bound to syntaxin 3 cannot be deduced from these results. It is possible that a specific subpopulation of Munc-18-2/syntaxin 3 complexes dissociates during the immunoprecipitation protocol, or that part of the Munc-18-2 molecules interact with a membrane component different from syntaxin 3. Several proteins other than syntaxins have been reported to associate with Sec1-related proteins. These include the S. cerevisiae Mso1p (Aalto et al., 1997), the neuronal-specific Cdk5 (Shetty et al., 1995) and Mint proteins (Okamoto and Südhof, 1997).

**Fig. 6.** Effect of pharmacological agents on the quantity of Munc-18-2 bound to syntaxin 3. Untreated Caco-2 cells (control) or cells treated with nocodazole (10 μM), cytochalasin D (40 μM), phorbol myristate acetate (PMA; 1 μM), brefeldin A (BFA; 5 μg/ml), wortmannin (0.1 μM), or N-ethylmaleimide (NEM; 1 mM) in the presence or absence of dithiothreitol (DTT; 2 mM), as described in detail in Materials and Methods, were lysed with detergent and immunoprecipitated with anti-syntaxin 3 antibody. Syntaxin 3 and Munc-18-2 in the precipitates were quantitated by western blotting, [35S]Protein A and the Fuji BAS-1500. The relative ratio of Munc-18-2 signal to that of syntaxin 3 (± s.e.m.) determined from four independent experiments is shown. The values obtained with PMA and NEM differ significantly (t-test; P<0.05) from the appropriate controls.

**Fig. 7.** The effect of NEM on the in vitro association of Munc-18-2 and syntaxin 3. GST-Syntaxin 3AC coupled to Sepharose 4B and rabbit reticulocyte lysate containing in vitro translated [35S]methionine labeled Munc-18-2 were incubated together at room temperature for 60 minutes, after which the matrix was separated by centrifugation. The bound Munc-18-2 was analyzed by SDS-PAGE and Fuji BAS-1500 quantitation. The columns represent: Munc-18-2 and syntaxin 3 treated separately before the binding reaction with 1 mM NEM and 2 mM DTT (1); only syntaxin 3 treated with NEM before the binding (2); only Munc-18-2 treated with NEM before the binding (3); a preformed complex treated with NEM in the presence (4) or absence (5) of DTT. The values (± s.e.m.) are given relative to that of the preformed complex, which was set at 100 (n=3).

**Fig. 8.** The effect of Munc-18-2 overexpression on the complex of syntaxin 3 with SNAP-23 or cellubrevin. Caco-2 cells were transfected for 4 hours using either a control SFV expressing a nonrelevant protein (control) or the Munc-18-2 recombinant SFV (Munc-18-2). The cells were lysed and immunoprecipitated with antibodies against syntaxin 3. Thereafter, the amounts of Munc-18-2 and SNAP-23 (B) or cellubrevin (C) in the precipitates relative to that of syntaxin 3 were quantitated by western blotting and the Fuji BAS-1500 quantitation. The columns represent: control SFV expressing a nonrelevant protein (control) or the Munc-18-2 recombinant SFV (Munc-18-2). The differences in all cases are statistically significant (t-test; A,B, P<0.01; C, P<0.05).
and the mammalian DOC2 proteins (Verhage et al., 1997). The DOC2 proteins associate with membranes in a calcium dependent manner through their C2 domains and are thus potentially capable of anchoring Sec1 proteins to membranes. However, we have previously shown that chelation of divalent cations does not detach Munc-18-2 from membranes (Riento et al., 1996), suggesting that binding through DOC2 is unlikely.

Treatment of Caco-2 cells with the protein kinase C (PKC) activator phorbol myristate acetate (PMA) significantly increased the quantity of the Munc-18-2/syntaxin 3 complex, suggesting that PKCs are at least indirectly involved in regulation of its assembly/stability. PKCs are known to be involved in numerous Ca\(^{2+}\)-dependent exocytotic events (Takai et al., 1996), but they are also reported to regulate constitutive membrane trafficking events, such as transcytosis and recycling in epithelial cells (Cardone et al., 1994). PKC mediated phosphorylation of n-Sec1 in vitro was shown to inhibit its association with syntaxin 1A (Fujita et al., 1996). On the other hand, PMA treatment of PC12 cells was reported to enhance the PKC mediated phosphorylation of SNAP-25 and thereby inhibit its association with syntaxin 1A (Shimazaki et al., 1996). This could lead to increased binding of Sec1 to syntaxin. The alkylating agent N-ethylmaleimide (NEM) had a marked inhibitory effect on the Munc-18-2/syntaxin 3 association. The treatment has been shown to stabilize the complex(es) of syntaxin 3 with its cognate SNARE partners (Galli et al., 1998). Therefore, sequestration of syntaxin 3 into such complexes, leading to displacement of Munc-18-2, was regarded a plausible explanation for the NEM effect. However, treatment of Munc-18-2 with NEM was also found to inhibit the interaction of the two proteins in vitro. This observation, together with the previous findings that the neuronal Sec1 (Meffert et al., 1996) and the Golgi stacking protein GRASP65 (Barr et al., 1997) are inactivated by NEM, demonstrates that NEM is not the only target of NEM action relevant for cellular membrane dynamics.

The identity of the machineries responsible for the apical trafficking in epithelia is under debate. Ikonen et al. (1995) presented data suggesting that exocytosis at the apical membrane of the MDCK kidney epithelial cell line is independent of NSF, α-SNAP, Rab GTPases, and insensitive to neurotoxins which inactivate v-SNAREs. However, the delivery of transcytosed material to the apical cell surface in the same cell line was suggested to be dependent on the SNARE-based vesicle docking/fusion machinery (Apodaca et al., 1996). Further, t-SNARE proteins have been demonstrated to localize to the apical plasma membrane domains of several polarized cell types (Gaisano et al., 1996; Mandon et al., 1996; Low et al., 1996; Delgrossi et al., 1997; Galli et al., 1998). Therefore, the SNARE machinery is most likely functional at least in some apically directed vesicle transport events. The specific SNARE protein combinations used seem to depend on the cell type under study and the characteristics of its transport pathway organisation. The apical localization of Munc-18-2 and syntaxin 3 and the presence of a complex between these proteins suggest that the Munc-18-2/syntaxin 3 assembly has a specialized function in apical membrane trafficking. Further, the ability of Munc-18-2 to modulate the accessibility of syntaxin 3 for its cognate SNARE partners illustrates the regulatory role of the Sec1 homologue. The present work thus provides tools for detailed study of the molecular interactions essential for the docking/fusion events at the apical plasma membrane of epithelial cells.

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