Munc18-2, a Functional Partner of Syntaxin 3, Controls Apical Membrane Trafficking in Epithelial Cells*

Kirsti Riento‡‡§§, Maria Kauppi§§, Sirkka Keränen†, and Vesa M. Olkkonen‡**

From the ‡Department of Biochemistry, National Public Health Institute (KTL), Mannerheimintie 166, FIN-00300 Helsinki, Finland and §VTT Biotechnology, P. O. Box 1500, FIN-02044 VTT, Finland

The Sec1-related proteins bind to syntaxin family t-SNAREs with high affinity, thus controlling the interaction of syntaxins with their cognate SNARE partners. Munc18-2 is a Sec1 homologue enriched in epithelial cells and forms a complex with syntaxin 3, a t-SNARE localized to the apical plasma membrane. We generated here a set of Munc18-2 point mutants with substitutions in conserved amino acid residues. The mutants displayed a spectrum of different syntaxin binding efficiencies. The in vitro and in vivo binding patterns were highly similar, and the association of the Munc18-2 variants with syntaxin 3 correlated well with their ability to displace SNAP-23 from syntaxin 3 complexes when overexpressed in Caco-2 cells. Even the Munc18-2 mutants that do not detectably bind syntaxin 3 were membrane associated in Caco-2 cells, suggesting that the syntaxin interaction is not the sole determinant of Sec1 protein membrane attachment. Overexpression of the wild-type Munc18-2 was shown to inhibit the apical delivery of influenza virus hemagglutinin (HA). Interestingly, mutants unable to bind syntaxin 3 behaved differently in the HA transport assay. While one of the mutants tested had no effect, one inhibited and one enhanced the apical transport of HA. This implies that Munc18-2 function in apical membrane trafficking involves aspects independent of the syntaxin 3 interaction.

It is generally accepted that intracellular membrane trafficking requires compartment-specific membrane-associated proteins denoted collectively as soluble NSF attachment protein receptors (SNAREs), as well as the general factors N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (SNAPs), or their homologues (1, 2). The SNARE proteins present on the transport vesicles (v-SNAREs; related to the neuronal synaptobrevin/VAMP proteins) and the target membranes (t-SNAREs; homologues of the neuronal syntaxin and SNAP-25 proteins) assemble into stable core complexes through formation of coiled coil helix bundles, a process which is closely connected to the fusion of membrane bilayers (3–5).

Proteins of the Sec1 family (reviewed in Ref. 6) are suggested to play a crucial role in the control of SNARE complex assembly. These proteins bind with high affinity to specific syntaxins, thus modulating the capability of these t-SNAREs to interact with their cognate SNARE partners. In vitro binding assays or in vivo overexpression of Sec1 homologues have provided evidence for an inhibitory role of Sec1 proteins in SNARE complex formation and membrane trafficking (7–12). On the other hand, a wealth of evidence shows that Sec1 action is required for normal physiological function of the intracellular trafficking pathways: loss-of-function mutations in the Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster Sec1-related proteins have been demonstrated to lead to specific blocks in vesicle transport, the phenotypic effects often implying disturbance in consumption of transport vesicles (13–20).

Three mammalian Sec1 homologues are suggested to control specific vesicle transport events at the plasma membrane of different cell types: Munc18n-1Sec1rbSec1 is a predominantly neuronal protein, which binds the neuronal syntaxins 1A and 1B as well as syntaxins 2 and 3, and is essential for neurotransmission (21–25). Munc18-2/Munc18b (26–28) is expressed predominantly in epithelial cells, where it localizes at the apical plasma membrane and interacts with the apical t-SNARE syntaxin 3 (9, 29, 30). The ubiquitously expressed Munc18c binds to syntaxins 2 and 4, and has been shown to regulate glucose transporter trafficking in adipocytes through modulation of the function of syntaxin 4-based SNARE complexes (11, 12, 28, 31). Furthermore, a protein (denoted as PSP) probably representing human Munc18c was suggested to control platelet granule exocytosis (32).

The exact mode of action of the Sec1 proteins is even today unclear, but recent work has provided us with valuable clues to the principles of Sec1 function: Dulubova et al. (33) demonstrated by NMR spectroscopy that syntaxin 1A can adopt two different conformations, one of which (the “closed” conformation) binds Munc18 and appears to be crucial for the exocytic event. The results suggest that syntaxin undergoes a conformational switch from a Munc18-bound closed form to an “open” conformation present in the SNARE core complex.

How is Sec1 protein function linked to upstream regulatory signals? There is increasing evidence that one mechanism controlling the Sec1-syntaxin binding is phosphorylation of the Sec1 proteins, which appears to dissociate them from the t-SNARE, thus permitting positive interactions of syntaxin with other components of the vesicle docking/fusion apparatus (32,
Beta counter. To obtain the specific binding, the counts/min values of the GST-coated wells were subtracted from those of the GST-synaxin 3AC coated ones. For verification of specificity of the assay, N-terminal His6-tagged Munc18-2 was produced in SF9 cells using the Bac to Bac system (Life Technologies Inc.) and purified on Ni-NTA-agarose (Qia-nex) according to the manufacturer’s instructions. The purified 6×His-Munc18-2, or bovine serum albumin, were added in increasing amounts to the in vitro translated 35S-labeled wild-type Munc18-2 before applying the samples in the coated wells. After assessing the linearity of signal over the counts/min range studied for all the Munc18-2 variants, the actual measurements were carried out using 150,000 cpm of Munc-182 radioactivity per well.

**Generation of Recombinant SFVs and the Viral Infections—** For expression studies in Caco-2 cells the myc-Munc18-2 mutant cDNAs were transferred as BamHI fragments from pBluescript to pSFV1 (48). Constructs carrying the human amyloid precursor protein cDNA or the influenza A WSN/33 HA cDNA were gifts from Dr. P. Tienari (Dept. of Neurology, University of Helsinki, Finland) and Dr. P. Gleeson (Dept. of Pathology and Immunology, Monash University Medical School, Australia), respectively. A cDNA encoding the human CLN3 protein was kindly provided by Dr. Irma Järvelä (Dept. of Human Molecular Genetics, National Public Health Institute, Finland). For constructs carrying two inserts under independent 26 S promoters (49), the Munc18-2 inserts together with promoter and partial nsP4 open reading frame sequences were removed from pSFV1 using EcoR1, the cohesive ends filled-in, and XhoI. These fragments were cloned into pSFV1. HA digested with BglIII, followed by fill-in, and XhoI. Recombinant Semliki Forest viruses were prepared in baby hamster kidney cells, and infections were carried out as described (50). Prior to infection of Caco-2 cells grown on plastic the cells were treated for 10 min with 5 mM EGTA in PBS at 37 °C (9). Filter grown Caco-2 cells were infected directly from the basal side by placing the filter units on a droplet of virus dilution on Parafilm. After 1 h incubation at 37 °C, the filter units were moved to fresh complete medium. To assess the level of Munc18-2 overexpression, samples of the cells were directly lysed in SDS-PAGE loading buffer, and the quantity of Munc18-2 relative to that in cells infected with the control (amyloid precursor protein) SFV was determined by Western blotting. 35S-protein A, and Fujifilm BAS-1500 quantitation.

**Assay of Munc18-2 Stability—** For analysis of Munc18-2 stability, Caco-2 cells grown on plastic were infected with recombinant SFVs for 4 h. Thereafter the cells were pulse labeled with [35S]Met and Cys (Amersham Pharmacia Biotech AGQ0080; 30 μCi/cm2 dish) for 30 min using a medium containing 5% dialyzed fetal bovine serum, and either lysed after PBS washes in the EDTA-containing immunoprecipitation buffer described in In vitro Binding Assay for 2 h or chased for 2 h with an overlay containing Met and Cys. The myc-Munc18-2 proteins were immunoprecipitated using the 9E10 anti-myc mAb, and analyzed by SDS-PAGE, autoradiography, and Fujifilm BAS-1500 quantitation.

**Flotation of Membranes in Sucrose Gradients—** Caco-2 cells grown on 6-cm plastic dishes were infected with recombinant Semliki Forest viruses for 4 h. After PBS washes the cells were scraped in 250 mM sucrose, 5 mM KCl, 2 mM NaCl, pH 7.4, 1 mM ethylenediamine tetraacetic acid, passages through a 21-gauge needle. After removal of intact cells and nuclei, sucrose was added to 2 M (total volume of 2.5 ml), and overlaid with 2 ml of 1.7 M sucrose and 0.5 ml of 0.8 M sucrose, 140 mM KCl, 10 mM Hepes. The gradients were centrifuged at 130,000 × g in a SW50.1 rotor for 17 h, 10 °C. Fractions of 1 ml were collected, proteins were precipitated with trichloroacetic acid, and the specimens were analyzed by Western blotting using anti-Munc18-2, anti-syntaxin 3, or anti-gelsolin.

**Assay of HA Trafficking by Surface Immunoprecipitation—** Filter-grown Caco-2 cells were infected with recombinant SFVs expressing both HA and a Munc18-2 variant (or in the control virus the CLN3 protein) for 5 h, labeled with [35S]Met and Cys (70 μCi/12-mm filter) for 2 h in a medium containing 5% dialyzed fetal bovine serum, followed by a 2-h chase. For surface immunoprecipitation of HA the cells were washed once with ice-cold PBS with 0.5 mM MgCl2, 1 mM CaCl2 (PBS−) and placed on ice. The cells were incubated with anti-HIN1 antibody diluted in serum-free culture medium on either the apical or the basolateral side of the filter at 4 °C for 1 h. Unbound antibody was removed by washing with cold 0.1% bovine serum albumin in PBS−, and PBS−. The cells were lysed in 1% Triton X-100, 0.1% SDS, 20 mM Tris, pH 8.0, 0.1% 2-mercaptoethanol, 0.1% protease inhibitor cocktail, on ice. After removal of insoluble material in a microcentrifuge, Protein A-Sepharose (Amersham Pharmacia Biotech) was added in the lysates (incubation overnight at +4 °C). For total HA immunoprecipitation, anti-HIN1 antibody and Protein A-Sepharose were directly added in lysates of pulsed and chased cells. The bound proteins were, after thorough washes of the matrix with the lysis buffer, detached by boiling.
in reducing Laemmli sample buffer, resolved by SDS-PAGE, and quantified by the Fujifilm BAS-1500 system.

RESULTS

Design of Site-specific Mutants—In order to gain further insight to the function of Munc18-2, a set of mutants with substitutions in parts conserved in the Sec1 protein family was generated (Fig. 1, Table I). Most of the mutations correspond to ones characterized in functional terms in the literature for other Sec1-related proteins, while others were designed in conserved residues based on sequence alignment and secondary structure prediction without pre-existing functional information. A multiple sequence alignment generated using the GCG Pileup program was used in the mutant design, and the PHD software (Columbia University) was used for protein secondary structure considerations.

The D34N/M38V double mutant has been described in Munc18 (D34N/M38V) as one abolishing syntaxin 1A binding (51). The G416E corresponds to the temperature-sensitive (ts) S. cerevisiae sec1–1 mutation G443E causing post-Golgi secretion arrest at the restrictive temperature (16, 52),2 and G416W was designed to test the effect of a bulky hydrophobic residue in the same amino acid position. R405P corresponds to the S. cerevisiae sec1–1 mutation R432P resembling sec1–1 in phenotype (52).2 E467K was designed as a counterpart of the S.c. SLY1–20 mutation E532K described by Dascher et al. (53) as a dominant single copy suppressor of deletion of YPT1. This identification is, however, not absolutely positive, since the sequences of Sly1p and the other Sec1 family members are markedly divergent in this region (Fig. 1). P242S and H293Y correspond to mutations described in the Dro sophila Rop protein (P254S and H302Y). They display different phenotypes in the fly, causing an increase in evoked neurotransmission and a dramatic reduction in both evoked and spontaneous neurotransmitter release, respectively (20). In addition, three entirely novel mutants were generated. The K314L/R315L double mutation destroys a pair of conserved basic residues predicted to localize in a solvent accessible loop, G541E substitutes a highly conserved glycine predicted to be buried in the fold of Munc18-2, and P187A substitutes a highly conserved proline which can be envisioned to create a kink that affects the positioning of neighboring secondary structure units.

Binding of the Munc18-2 Variants to Syntaxin 3—The previous observation that Munc18-2 and syntaxin 3 form a complex in epithelial cells (9) prompted us to analyze the association of the Munc18-2 variants with syntaxin 3. An in vitro assay was set up to measure the amount of in vitro translated [35S]Met-labeled Munc18-2 bound to syntaxin 3 coated on 96-well plates (Fig. 2A). The specificity of the assay was confirmed by competition of the signal by unlabeled Munc18-2 produced in insect cells, and the assay was highly linear in the entire radioactivity range studied. Analysis of syntaxin binding using this assay revealed that the Munc18-2 mutant proteins differ greatly in their ability to bind syntaxin 3 (Fig. 2B; Table I). As expected, D34N/M38V showed only negligible association with syntaxin 3. Similar results were obtained for R405P (counterpart of sec1–11), H293Y (inhibitory mutation in Rop), and the novel double mutant K314L/R315L. The S.c. sec1–1 mutation homologue, G416E, behaved differently, binding to syntaxin at roughly 50% efficiency compared with the wild-type protein. Incubation of the binding assays at 24 °C instead of 37 °C did not change the binding of G416E and R405P (the corresponding yeast mutants are ts) relative to the other mutants or the wt protein (data not shown). Substituting Gly416 with tryptophan (W) caused a more dramatic (90%) inhibition of syntaxin binding, suggesting that insertion of a bulky hydrophobic residue in this position causes more extensive structural distortion than a smaller charged residue. The E467K (tentative counterpart of SLY1–20) mutant protein displayed somewhat enhanced binding to syntaxin 3.

To study the interaction of the Munc18-2 variants with syntaxin 3 in vivo, Caco-2 cells were infected with recombinant Semliki Forest viruses expressing the myc-tagged Munc18-2 protein variants. Before carrying out these experiments, the stability of the mutant proteins was assessed by experiments employing a 30-min pulse and a chase of 2 h, followed by immunoprecipitation with anti-myc mAb. The mutants fell into three stability categories (Fig. 3, Table I): E467K and K314L/R315L did not differ significantly from the wild-type protein; G416E, G416W, R405P, P242S, and H293Y showed a mild decrease in stability relative to the wild-type protein (50–80% remaining after chase); D34N/M38V, P187A, and G541E were remarkably unstable (20–25% remaining after chase), indicating that these three mutations significantly interfered with the correct folding of the protein. This result thus confirmed the important structural role of the conserved Pro187 and Gly541 residues selected as targets of novel mutations. However, the instability of these mutants did not hamper the subsequent overexpression studies, since the expression time point used (4 h after SFV infection) represents a stage at which the recombinant protein has started to appear within the past hour and

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2 M. Brummer and S. Keränen, unpublished data.
its amount increases rapidly. Therefore, upon quantitation of the total amount of the expressed Munc18-2 variants in the cell lysates, the proteins of reduced stability were present at the total amount of the expressed Munc18-2 variants in the cell, its amount increases rapidly. Therefore, upon quantitation of that it interacts with syntaxin in a metastable manner susceptibility measured using the infected cells (Fig. 4) coincided well with the binding efficiency in vitro assay, the main difference being the apparently greater sensitivity of the in vitro assay. Several mutants with weak but detectable binding in vitro displayed no measurable signal in vivo. It is noteworthy that the mild increase in the syntaxin binding of E467K seen in vitro was also detected in vivo, strengthening the notion that the difference in binding efficiency is real. The mutants with low stability, D34N/M38V, P187A, and G541E, also failed to bind syntaxin 3, which is a plausible consequence of the apparent structural distortion of these proteins. Importantly, stability equal to that of the wt protein and absence of syntaxin interaction are combined in the new double mutant K314L/R315L.

The Effect of Overexpressed Munc18-2 Variants on the SNAP-23 Syntaxin 3 Complex—Overexpression of Munc18-2 disrupts apical SNARE complexes containing syntaxin 3, SNAP-23, and cellubrevin (9, 47). Therefore, we used the Semliki Forest virus expression system to study to what extent the mutant Munc18-2 proteins were able to interfere with the association of SNAP-23 and syntaxin 3 in Caco-2 cells (Fig. 5; Table I). Overexpression of the wt Munc18-2 decreased the amount of SNAP-23 bound to syntaxin 3 by 50% compared with the control, a recombinant SFV expressing a non-relevant product, the amyloid precursor protein. The ability of the mutant proteins to displace SNAP-23 correlated well with their ability to bind syntaxin 3. The strongest binder, E467K, displaced SNAP-23 with similar efficiency as the wt protein, whereas most of the variants incapable of syntaxin interaction also failed to displace the partner t-SNARE. The only exception was H293Y, which showed no syntaxin binding in vivo but was, nevertheless, capable of significantly reducing the amount of SNAP-23 in the immunoprecipitates. However, this protein did bind detectably to syntaxin in the in vitro assay, which may indicate that it interacts with syntaxin in a metastable manner susceptible to the immunoprecipitation conditions.

Intriguingly, Carr et al. (45) recently reported that the S. Sec1p preferentially binds to the exocytic SNARE core complex consisting of Sso1p, Sec9p, and Snc2p, rather than to the syntaxin homologue Sso1p alone. Therefore, we investigated whether any SNAP-23 could be detected in the Munc18-2 immunocomplexes pulled down with the myc-antibody. In Caco-2 cells cultured under standard or ATP depletion conditions (45), no SNAP-23 communoprecipitated with myc-Munc18-2 (data not shown).

Association of the Munc18-2 Variants with Cellular Membranes—It has previously been shown that overexpressed Sec1 proteins require simultaneous overexpression of an appropriate syntaxin to localize to the correct cellular membranes (29, 31). Furthermore, upon co-expression of syntaxin 3 and Munc18-2 in polarized Caco-2 cells the Sec1 homologue is recruited onto the apical plasma membrane. However, it has been unclear if Munc18-2 expressed in the absence of syntaxin overexpression is cytosolic or associates with intracellular membranes. To clarify this issue, post-nuclear supernatants of Caco-2 cells expressing the Munc18-2 variants were subjected to flotation in sucrose gradients. Fig. 6 shows the results for the wt Munc18-2 and three mutants displaying no or weak syntaxin binding, D34N/M38V, K314L/R315L, and P242S. A major portion of the endogenous Munc18-2 present in the control infected cells was found in the top fraction containing the floated membranes, while some remained in the interface of 2 and 1.7 M sucrose and a small amount in the soluble fractions. The overexpressed wt protein showed a similar distribution, demonstrating that a majority of the expressed protein associated with cellular membranes. Interestingly, the mutant proteins defective in syntaxin binding displayed similar results. A major portion of all these proteins was recovered in the floated membranes. Under these conditions, the integral membrane protein syntaxin 3 was quantitatively found in the membranes and the cytosolic protein gelsolin in the soluble bottom fractions.

The Effect of Munc18-2 Overexpression on the Apical Transport of Influenza Virus HA—The role of Munc18-2 in the apical transport processes has not been characterized. To monitor the possible effects of the protein on apical exocytic events, we

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**Table I**

| Mutant         | Corresponds to | Stability | Binding to Syn3 in vitro | Binding to Syn3 in vivo | SNAP23 displacement |
|----------------|----------------|-----------|--------------------------|-------------------------|---------------------|
| wt Munc 18–2   |                |           |                          |                         |                     |
| G416E          | G443E, sec1–1f | ++        | ++                       | ++                      | ++                 |
| E467K          | E532K, SLY1–20 | ++        | ++                       | ++                      | ++                 |
| G416W          |                | ++        | +/−                      | ND                      | ND                  |
| D34N/M38V      | D34N/M38V (Munc 18) | +/−     | ND                      | ND                      | ND                  |
| G541E          |                | +         | +/−                      | ND                      | ND                  |
| K314L/R315L    |                | ++        | +/−                      | ND                      | ND                  |
| R187A          |                | +/−       | +/−                      | ND                      | ND                  |
| R242S          |                | +/−       | +/−                      | ND                      | ND                  |
| R293Y          |                | +         | +/−                      | ND                      | ND                  |
| R405P          | R432P, sec1–1l | ++       | ND                      | ND                      | ND                  |

* ++ + >80%.
* + + >50% ≤ 80%.
* − − ≤ 20%.
* +/− ≥ 20% ≤ 50%.
* − − ≥ 50%.
* ND, not detectable.
* Wu et al. (20).
* Aalto et al. (52).

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3 K. Riento and V. Olkkonen, unpublished data.
devised an assay measuring the transport of influenza hemagglutinin (HA) expressed from recombinant SFV. This viral vector causes shut-off of host protein synthesis (48), and is thus incompatible with pulse-chase analysis of endogenous protein transport. We therefore constructed recombinant SFVs carrying both influenza virus HA cDNA and Munc18-2 variant cDNAs under independent 26 S promotors. This approach guaranteed the expression of both proteins exclusively in the same cells, and allowed us to reliably monitor the impact of the co-expressed Munc18-2 on the transport of HA to the apical or basolateral plasma membrane domains of Caco-2 cells (Fig. 7).

A majority of the HA precipitated from cell surfaces had an approximated size of 80 kDa, corresponding to fully glycosylated protein, whereas in the total cellular immunoprecipitates a smaller, 70-kDa band obviously representing high mannose forms of the protein in the endoplasmic reticulum, was predominant (Fig. 7A). In cells infected with the control SFV expressing a non-relevant protein together with HA, slightly more HA was found on the basolateral than on the apical surface (Fig. 7B). This distribution was highly similar to that observed when a recombinant SFV expressing HA alone was used.

FIG. 2. In vitro binding of the myc-Munc18-2 variants to syntaxin 3. A, validation of the binding assay. [35S]Met-labeled Munc18-2 protein variants were incubated for 30 min at 37 °C on 96-well plates coated with either GST or GST-syntaxin 3A, and the radioactivity bound specifically to the syntaxin was determined. The inset shows linearity of the response over the radioactivity range used (a representative experiment performed in triplicate), and the main panel competition of the signal by addition of increasing amounts of unlabeled recombinant Munc18-2 (f, protein mass added per well is indicated on the x axis); A, bovine serum albumin added as a control. B, binding assay of the in vitro translated Munc18-2 mutants. The data presented represent values obtained using 150,000 cpm of Munc18-2 radioactivity per well (±S.E., n = 3). The values are shown relative to that of the wild type myc-Munc18-2 (WT), which was set at 100.

deviation of the wt Munc18-2 reduced the amount of HA transported to the apical surface by an average of 30%, whereas no effect on the basolateral delivery of HA was detected. To elucidate if the observed inhibition was simply due to sequestration of syn-
In the present study we have addressed the function and syntaxin 3 interaction of the epithelia en- enriched Sec1-related protein, Munc18-2, by site-directed mutagenesis. To analyze the mutated proteins, a solid phase ligand binding assay meas- uring Munc18-2/syntaxin 3 interaction was set up. On the other hand, association of the protein variants with syntaxin 3 was assessed in vitro by expressing them in Caco-2 cells using recombinant SFVs. The results obtained with the in vitro and in vitro binding assays were highly similar, demonstrating the usability of the novel in vitro assay as a simple and rapid screen for interesting mutations in Sec1-related proteins. It is noteworthy that all the mutants, except for E467K, displayed defective binding to syntaxin. The fact that the mutations designed based on data from different Sec1 proteins in different organisms have similar molecular consequences supports the idea that interaction with syntaxins is a major cellular function of the Sec1 proteins.

Among the mutants generated some proteins showed marked- ly reduced stability. One of these was the D34N/M38V mutant used previously to assess the role of syntaxin binding by Munc18-2 (51), indicating that this specific mutant may be severely misfolded and is thus not an optimal control protein for analysis of the Sec1-syntaxin interaction. Instead, the novel K314L/R315L mutant was perfectly stable and yet showed no significant syntaxin binding, suggesting that these conserved residues predicted to be exposed on the surface of the protein are specifically involved in the syntaxin interaction. We therefore surmise that this novel mutant is an excellent tool for future studies not only on Munc18-2 but also other mammalian Sec1 homologues as well as the Drosophila Rop protein.

The two mutants corresponding to the S.c. ts mutations sec1-1 and sec1-11, G416E and R405P, respectively, showed quite different syntaxin binding properties: G416E bound at 50–70% efficiency compared with the wt protein, while R405P displayed hardly detectable binding. This may indicate that the similar phenotypes of sec1-1 and sec1-11 (16, 52) may involve slightly different mechanisms. However, one should not draw detailed conclusions from this data since the amino acid identity between yeast Sec1p and Munc18-2 is only 29%. The E467K mutant tentatively corresponding to yeast SLY1-20 (53) displayed mildly increased binding to syntaxin 3. The implications of this finding are, however, unclear since Grabowski and Gallwitz (54) observed no change in the in vitro binding affinity of the yeast mutant protein to the syntaxin homologue Ssd5p.

Syntaxin 3 localizes apically in epithelial cells and forms complexes with another t-SNARE, SNAP-23, and the v-SNAREs cellubrevin and tetanus toxin-insensitive VAMP.
cell precipitates, one-fifth of the amount present in the representative data set is shown.

...and 70 kDa (closed arrow). A...}

The present data demonstrate that the syntaxin binding capacity of the Munc18-2 variants correlates well with their ability to displace SNAP-23 from syntaxin 3 complexes and/or to inhibit the association of the two SNAREs. The only exception was H293Y, which showed hardly any syntaxin binding but still displaced SNAP-23 to a significant extent. This may be explained by a change in the syntaxin interaction of the protein making the complex susceptible to the conditions used in the binding assays. Another possibility is that Sec1 proteins may also affect SNARE complexes through indirect mechanisms independent of direct syntaxin interaction. One potential pathway by which such effects could be mediated is regulation of the activity of specific protein kinases (56). Furthermore, disturbance of interactions of Sec1 proteins with other polypeptides involved in the control of membrane trafficking (39, 43) can be envisioned to cause indirect effects on the SNARE machinery.

Analysis of the membrane association of the Munc18-2 variants revealed that they were capable of association with the cellular membranes, independently of their capacity to bind syntaxin. This brings up the idea that the Sec1 proteins display two modes of membrane interaction, syntaxin dependent and independent ones, the latter obviously being of lower affinity. In this case regulated exchange of interaction partners could form an important part of the functional cycle of the Sec1-related proteins. An observation consistent with this idea has previously been made with Munc18c. Dissociation of Munc18c from syntaxin 4 induced by insulin treatment of adipocytes did not release the Sec1 homologue from the cellular membranes (31).

The functional effects of wt and mutant Munc18-2 proteins were assayed by measuring the transport of recombinant SFV-expressed influenza HA protein to the apical and basolateral surfaces of polarized Caco-2 cells. Previous work employing influenza virus infection has shown that a large majority of HA is distributed to the apical plasma membrane in Caco-2 cells (57, 58). We detected a similar distribution of HA in preliminary experiments using infection with A/Beijing/353/89 influenza virus (data not shown). However, in our transport assay based on SFV expression of influenza WSN33 HA cDNA the viral glycoprotein was detected in similar amounts on both plasma membrane domains. This variation may be due to differences between the viral strains and the cell line subclones used. The less polarized HA distribution provided a means of simultaneously monitoring transport to the basolateral surface. Our results demonstrated that wt Munc18-2 overexpression distinctly inhibited the apical transport of HA, without affecting the basolateral delivery. Intriguingly, mutants incapable of binding syntaxin 3 behaved differently in the assay. While one had no effect, one inhibited apical trafficking, and one enhanced it. This indicates that the main parameter used in characterization of Sec1 protein function, the syntaxin interaction, only provides a very narrow view to a complex network of molecular interactions. It is impossible to predict how the mutations abolishing syntaxin binding affect the association of Sec1 proteins with other interaction partners, which are not necessarily directly involved in SNARE function. This is well illustrated by the finding that the D34N/M38V mutant, even though it does not bind syntaxin detectably, caused inhibition of apical transport similar to the wt protein. The unexpected behavior of the other non-syntaxin binding mutant K314L/R315L, which enhanced apical HA delivery, indicates that this protein variant, when present in excess amounts, may either bind and sequester a factor imposing a negative regulatory effect on apical transport, or activate a positive regulator more efficiently than the wt protein.

Caco-2 cells are known to deliver material to the apical plasma membrane both by direct exocytosis and the transcytotic route (59, 60). With the methodology used in the present study we cannot exclusively conclude that the observed effect on HA distribution is solely due to inhibition of the direct exocytic route. However, overexpression of syntaxin 3, the major interaction partner of Munc18-2, caused disturbances of the apical recycling and exocytosis, not transcytosis, in Madin-Darby canine kidney cells (61). Furthermore, we detected no accumulation of HA on the basolateral surface of Caco-2 cells in

FIG. 7. The effect of Munc18-2 overexpression on influenza virus HA transport to the different plasma membrane domains. Polarized Caco-2 cells were infected with recombinant SFVs expressing both HA and the wild type Munc18-2, or one of its variants, for 5 h. Proteins were labeled for 2 h with [35S]Met and Cys, chased for 2 h, and HA was surface immunoprecipitated from the apical or basolateral plasma membrane domains, or from total cell lysate, followed by SDS-PAGE and quantification using Fuji BAS-1500. A, SDS-PAGE analysis of the immunoprecipitated HA; A, apical surface; B, basolateral surface; T, total immunoprecipitable HA. The Munc18-2 variants are identified on the top; C, control SFV infection; WT, wt-type protein. Of the total cell precipitates, one-fifth of the amount present in the A and B lanes was loaded. The two HA forms detected display approximated molecular masses of 80 kDa (closed arrow) and 70 kDa (open arrow). A representative data set is shown. B, quantitation of HA transported to the distinct plasma membrane domains. The Munc18-2 variants are indicated on the bottom. The amount of apical or basolateral surface immunoprecipitated HA was divided by the total immunoprecipitated HA. The values obtained (± S.E., n = 4) are presented relative to that of apically delivered HA in the control infected (C) cells, which was set at 1.

(TI-VAMP) (47, 55). We have previously shown that overexpression of Munc18-2 modifies the quantity of the other SNAREs complexed with syntaxin 3 (9). The present data demonstrate that the syntaxin binding capacity of the Munc18-2 variants correlates well with their ability to displace SNAP-23 from syntaxin 3 complexes and/or to inhibit the association of the two SNAREs. The only exception was H293Y, which showed hardly any syntaxin binding but still displaced SNAP-23 to a significant extent. This may be explained by a change in the syntaxin interaction of the protein making the complex susceptible to the conditions used in the binding assays. Another possibility is that Sec1 proteins may also affect...
the presence of overexpressed Munc18-2, which could have been expected in case of a block in transcytosis. Therefore, we favor the hypothesis that Munc18-2 directly affects the exocytic delivery of HA from the TGN to cell surface.

The present results provide the first direct functional evidence for a role of Munc18-2 in apical membrane trafficking. The study also provides novel insight into the functional relationship of the Munc18-2-syntaxin 3 protein pair. Furthermore, the results indicate that Munc18-2, and perhaps Sec1 proteins in general, may have effector pathways independent of syntaxin interaction.

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