ABSTRACT We have investigated the relationships between the apical sorting mechanism using lipid rafts and the soluble N-ethyl maleimide-sensitive factor attachment protein receptor (SNARE) machinery, which is involved in membrane docking and fusion. We first confirmed that anti-alpha-SNAP antibodies inhibit the apical pathway in Madin–Darby canine kidney (MDCK) cells; in addition, we report that a recombinant SNAP protein stimulates the apical transport whereas a SNAP mutant inhibits this transport step. Based on t-SNARE overexpression experiments and the effect of botulinum neurotoxin E, syntaxin 3 and SNAP-23 have been implicated in apical membrane trafficking. Here, we show in permeabilized MDCK cells that antisyntaxin 3 and anti-SNAP-23 antibodies lower surface delivery of an apical reporter protein. Moreover, using a similar approach, we show that tetanus toxin-insensitive, vesicle-associated membrane protein (TI-VAMP; also called VAMP7), a recently described apical v-SNARE, is involved. Furthermore, we show the presence of syntaxin 3 and TI-VAMP in isolated apical carriers. Polarized apical sorting has been postulated to be mediated by the clustering of apical proteins into dynamic sphingolipid-cholesterol rafts. We provide evidence that syntaxin 3 and TI-VAMP are raft-associated. These data support a raft-based mechanism for the sorting of not only apically destined cargo but also of SNAREs having functions in apical membrane-docking and fusion events.

The main feature of polarized epithelial cells is the compartmentalization of their plasma membrane into apical and basolateral domains. These membrane domains differ both in lipid and protein composition (1–3). To generate and maintain this polarized membrane composition, specific vectorial flow of membrane carriers must be ensured. This implies that along the biosynthetic pathway, apically and basolaterally destined lipids and proteins must be segregated and sorted.

In Madin–Darby canine kidney (MDCK) cells, the first sorting event occurs in the trans-Golgi network (TGN) (1). Several signals have been identified that participate in the specific targeting of proteins to their proper location (3). A hierarchy of these signals has been proposed such that the basolateral signals are dominant over the apical ones. Moreover, it has been postulated that apically routed proteins are recruited into glycosphingolipid- and cholesterol-enriched microdomains, called rafts (4). Because of the high melting temperature \( T_m \) of their sphingolipids, rafts are resistant to Triton X-100 solubilization at low temperatures (5). The mechanism for rafts to coalesce into large domains upon detergent treatment has been investigated in detail. The study of the miscibility between liquid-ordered and crystalline phases demonstrated that rafts exist before detergent addition (6). Recently, using crosslinking and fluorescence resonance energy transfer (FRET) techniques, the size of a single raft was estimated to less than 70 nm (7, 8). A strict correlation between raft association and apical sorting cannot be established because some basolaterally routed proteins can partition into (basolateral) rafts, e.g., caveolin-2 (9). Rafts are clearly present basolaterally. In the basolateral plasma membrane, stable assembly of rafts occurs in caveolae (9, 10). The apical plasma membrane, on the other hand, has been proposed to constitute a percolating raft domain (11). Most importantly, caveolae are not observed at the apical surface of MDCK cells (9). In the TGN, rafts, together with apical cargo, would be clustered and packaged into apical transport carriers (12). Hence, rafts would constitute platforms for apical sorting of proteins devoid of basolateral sorting signals (2–4). Those rafts that are not clustered by the apical sorting machinery could be envisaged to be included into basolateral transport carriers either passively or as caveolae precursors. Therefore, raft lipids are delivered from the TGN to both plasma membrane domains although their organization might differ in these domains.

Because inclusion into rafts is a prerequisite for some apical proteins to be sorted properly, we asked whether apical SNAREs could be raft-associated. The SNARE proteins are known to play a general role in vesicular docking and fusion in the biosynthetic pathway and in regulated exocytosis (13). Cytosolic proteins, i.e., soluble N-ethyl maleimide-sensitive factor (NSF) and soluble NSF attachment protein (SNAP), prime SNAP receptor (SNARE) proteins located on the donor (or vesicular) and the acceptor (or target membrane) compartment for the process that leads to membrane docking and fusion (14, 15). Previous results have shown that in MDCK cells the basolateral pathway uses the NSF/SNAP/SNARE mechanism, whereas the apical route has been demonstrated to be insensitive to the \( N \)-ethyl maleimide (16, 17). New insights have come from the localization studies of SNAREs. For instance, the t-SNARE syntaxin 3 and the v-SNARE TI-VAMP [or VAMP7 (18)] were reported to be apically distributed whereas syntaxin 4 was found basolaterally and syntaxin 2 and SNAP-23 were observed in both compartments (19–22). Moreover, the apical route was shown to be inhibited after overexpression of syntaxin 3 in MDCK cells (17).

The aim of this study was to analyze whether or not apical SNAREs are raft-associated and to analyze their involvement in apical delivery.

Abbreviations: HA, hemagglutinin; MβCD, methyl-β-cyclodextrin; MDCK, Madin–Darby canine kidney; NSF, soluble N-ethyl maleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNAP23 and SNAP25, synaptosomal-associated protein of 23 and 25 kDa, respectively; SNARE, SNAP receptor; TI-VAMP, tetanus toxin-insensitive, vesicle-associated membrane protein; v- and t- SNARE, vesicular and tarbale membrane SNARE, respectively; TGN, trans-Golgi network; SLO, streptolysin-O. 

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Antibodies and Recombinant Proteins. The production and characteristics of the anti-alpha-SNAP antibodies (3E2 and 2F10) will be described elsewhere. Affinity-purified antibodies raised in rabbits against syntaxin 3 (TG0), SNAP-23 (TG7), and TI-VAMP (also called VAMP7; ref. 18) (TG11) were described previously (17). In Western blotting experiments performed on apical TGN-derived vesicles, TG16, an anti-TI-VAMP antibody prepared following the same protocol as TG11, was used. The TG15 antibody against endobrevin (23), also called VAMP8 (26), was obtained from rabbits injected with the N-terminal peptide MEEASGSAGNDRVRNC of human endobrevin coupled to keyhole limpet hemocyanin and affinity-purified on the peptide. Affinity-purified polyclonal antisyntaxin 4, 6, and 11 antibodies were generous gifts of M. K. Bennett (University of California, Berkeley), R. C. Piper (University of Iowa), and P. A. Roche (National Cancer Institute, Bethesda, MD), respectively. Polyclonal and monoclonal anti-HA antibodies used for Western blotting and immunoelectron microscopy, respectively, were obtained as described (24, 25). Polyclonal anticaeveolin 1 (N20) antibody was purchased from Santa Cruz Biotechnology. His6-tagged human endobrevin coupled to keyhole limpet hemocyanin and affinity-purified on the peptide. Affinity-purified polyclonal antisyntaxin 4, 6, and 11 antibodies were generous gifts of M. K. Bennett (University of California, Berkeley), R. C. Piper (University of Iowa), and P. A. Roche (National Cancer Institute, Bethesda, MD), respectively. Polyclonal and monoclonal anti-HA antibodies used for Western blotting and immunoelectron microscopy, respectively, were obtained as described (24, 25). Polyclonal anticaeveolin 1 (N20) antibody was purchased from Santa Cruz Biotechnology. His6-tagged human endobrevin coupled to keyhole limpet hemocyanin and affinity-purified on the peptide.

Immunolabeling Studies. Immunofluorescence labeling of filter-grown MDCK cells and immunoelectron labeling of isolated apical carriers were done according to ref. 26 and refs. 9 and 27, respectively.

Transport Assays. The transport assays were performed according to a published protocol (28), with the streptolysin-O (SLO) generously supplied by S. Bhakdi (University of Mainz, Germany). Experiments were performed by using duplicate filters, and SEM of representative independent experiments are reported; the cytosol-dependent transport is 100% (transport in the presence of cytosol minus transport in the absence of added cytosol).

Detergent Treatments and Gradient Density Floatation. The experiment was done according to Lafont et al. (27) with the following modifications. Fowl plague virus-infected, filter-grown MDCK cells were incubated at 20°C for 2 hr before being scraped on ice and homogenized in 25 mM Tris-HCl, pH 7.4/150 mM NaCl/5 mM EDTA (TNE) supplemented with 5 mM DTT and a mixture of protease inhibitors (CLAP: chymostatin, leupeptin, antipain, pepstatin A; final concentration, 10 μg/ml of each). The postnuclear supernatant was either treated with 10 mM methyl-β-cyclodextrin (MβCD) at 37°C for 30 min or not before a 1% Triton X-100 extraction performed on ice for 30 min. Samples were adjusted to 35 or 40% OptiPrep before being overlaid with solutions containing different percentages of OptiPrep (as indicated Fig. 4) and prepared in TNE containing 1% Triton X-100. After 4 hr of spinning at 40,000 rpm in SW 60 tubes (Beckman) at 4°C, fractions were collected from the top and proteins were methanol-chloroform-precipitated (29) and analyzed by SDS/PAGE and Western blotting. For detergent extraction performed on isolated TGN-derived apical carriers, carriers isolated as described in Lafont et al. (27) were either treated or not with MβCD as above. Treated and untreated samples then were extracted on ice for 30 min with 0.1% Triton X-100, and samples were adjusted to 30% OptiPrep. Samples were overlaid with 20, 10, and 5% OptiPrep prepared in TNE containing 0.1% Triton X-100 and submitted to gradient density flotation for 2 hr at 55,000 rpm and 4°C in a TLS 55 Beckman rotor. Fractions were collected from the top of the gradient, and proteins were methanol-chloroform-precipitated before SDS/PAGE and Western blot analysis.

RESULTS AND DISCUSSION

SNAP Involvement in the Apical Pathway. Previously, anti-NSF antibodies were used in an assay to follow the pathways from the TGN to either the apical or the basolateral surface (16). In this assay, the TGN to the plasma membrane transport of either apical or basolateral viral marker proteins [the hemagglutinin (HA) of the influenza fowl plague virus or the glycoprotein of the vesicular stomatitis virus (VSV), respectively] is monitored in SLO-permeabilized MDCK cells. This transport is cytosol-, temperature-, and energy-dependent (30). In contrast to the basolateral route, the apical route was found NSF-independent, as confirmed recently (17). To examine the role of the other cytosolic component of the SNARE machinery, i.e., the alpha-SNAP, we employed two newly developed mAbs, 3E2 and 2F10, which specifically recognize alpha-SNAP from HeLa and MDCK cells (C.W. and J. E. Rothman, unpublished results). When these antibodies were tested in the transport assay, we found a dose-dependent decrease of surface delivery in both pathways (Fig. 1A). Interestingly, in agreement with data published earlier, Ikonen et al. (16), we observed a different requirement for alpha-SNAP between the pathways insofar as the apical route was found less susceptible to the addition of the 3E2 antibody than the basolateral one. Our results are in agreement with data obtained by Low et al. (17) showing that the basolateral pathway is more affected by the anti-alpha-SNAP antibodies (cf. figure 5 in ref. 17 and Fig. 1A in our study).

Furthermore, we examined the role of alpha-SNAP taking advantage of the recently developed recombinant wild-type and mutated (L294A) alpha-SNAP (31). The L294A alpha-SNAP is unable to stimulate Ca2+-dependent exocytosis in chromaffin cells (31) and behaves as a dominant inhibitory mutant in endosome fusion (32). As shown in Fig. 1B, the

FIG. 1. Alpha-SNAP involvement in apical membrane trafficking. (A) Dose-dependent decrease of the TGN to the apical and to the basolateral plasma membrane transport by antibodies against alpha-SNAP. The transport was monitored in SLO-permeabilized, filter-grown MDCK cells that were infected with either influenza virus or VSV. HA and VSV G were used as apical and basolateral markers, respectively. Note that the basolateral transport is more affected than the apical transport by the addition of the 3E2 antibody. (B) Influence of the His-tagged wild-type and dominant inhibitory mutant (L294A) recombinant alpha-SNAP proteins on apical delivery.
apical transport was stimulated slightly by the addition of the wild-type alpha-SNAP (132 ± 5% of control transport) whereas the dominant inhibitory mutant was partially inhibiting this pathway (66.5 ± 2% of control transport). Although, in both cases, the quantity of recombinant protein used was 3 μM, the limited effects observed might be caused by technical reasons related to the amount of recombinant proteins that gained access to permeabilized cells. The buffer in which the eluted recombinant proteins were collected did not perturb the efficiency of the transport (90 ± 9% of control transport).

**Effect of SNAP-23, Syntaxin 3, and TI-VAMP on the Apical Pathway.** Next, we examined the role of t-SNAREs in the apical route, focusing first on the possible involvement of SNAP-23, the ubiquitously expressed homologue of the neuronal SNAP-25. SNAP-23 is localized to both apical and basolateral compartments in MDCK cells (22), although it is mainly apical in CaCo-2 cells (21). When added to permeabilized cells, the anti-SNAP-23 antibody decreased the efficiency of the surface delivery of HA (59 ± 8% of control transport; Fig. 2A). This result is in agreement with data obtained by Low et al. (17), who used a different approach based on the specific cleavage of the canine SNAP-23 by the botulinum neurotoxin E (BoNT-E) (22). These authors reported an inhibition of apical delivery after cleavage inactivation of SNAP-23 (17). It is worth mentioning that in this study both the basolateral and the apical pathways were susceptible to BoNT-E, but the apical transport of a reporter transmembrane protein was less susceptible to the effect of the toxin.

We also investigated whether syntaxins could be implicated in the apical pathway. Syntaxin 3 has been shown to be localized specifically to the apical compartment of MDCK cells (19, 20), and it is not susceptible to BoNT-C1 (21). Low et al. (17) reported that when syntaxin 3 was overexpressed ~10-fold, apical transport was inhibited by about 20–30% depending on the cellular clone. Here, we show that the addition of antisyntaxin 3 antibody in permeabilized cells decreased the apical transport (59 ± 12% of control transport; Fig. 2A). As controls, we used antibodies against the post-Golgi-localized syntaxin 11 and 4 (18, 19). Both antisyntaxin 11 and antisyntaxin 4 antibodies did not affect apical transport (100 ± 5% and 112.5 ± 3.5% of control transport, respectively). Interestingly, antisyntaxin 11 antibody did not affect the basolateral pathway whereas the antisyntaxin 4 antibody did (data not shown).

Then, we investigated whether pairing partners of SNAP-23 and syntaxin 3 also could be implicated in the apical route. Both cellubrevin and the recently described v-SNARE TI-VAMP have been suggested by immunoprecipitation experiments to form apical complexes with SNAP-23 and syntaxin 3 (21). Cellubrevin is unlikely to be implicated in the direct TGN to the apical surface pathway because, first, this pathway has been shown to be insensitive to the tetanus neurotoxin that cleaves cellubrevin (16, 33) and, second, cellubrevin has been suggested to act in transferrin receptor recycling (34). We took advantage of the development of antibodies against TI-VAMP. This protein is apically targeted in CaCo-2 cells (21). We first verified that TI-VAMP was localized in the apical compartment of MDCK cells grown on polycarbonate filters (Fig. 2B). Then, we tested the effect of the anti-TI-VAMP antibody on apical transport. When 1.3 μM of antibody was added to permeabilized cells, surface delivery of HA was lowered (65 ± 11.5% of control transport; Fig. 2C). The antibody was found to be ineffective on the basolateral route (data not shown).

Interestingly, all the v- and t-SNAREs identified to act in apical membrane trafficking are not cleaved by the clathrin neurotoxins used in the study of Ikonen et al. (16). Here, we mainly used bivalent antibodies and, hence, cannot exclude that the apical carriers were prevented to reach the surface because of a steric hindrance by the bound antibodies. It is possible that SNAREs would be transported as cargo to function, for instance, in recycling events. The apical recycling pathway has been suggested to involve cellubrevin and syntaxin 3 (17, 34), and TI-VAMP was implicated in membrane-trafficking events involving lysosomes (18). Also, basolateral-to-apical transcytosis, which includes a transport step through an endosomal station, is both SNAP-23- and NSF-dependent (17). A scenario in which the apical carriers include SNAREs as cargo molecules fits with data showing that the transport from the TGN to the apical plasma membrane is NSF-insensitive (16, 17). This interpretation also conforms with the weak impairment of the apical transport observed after syntaxin 3 overexpression (17). An alternative explanation is that TI-VAMP, syntaxin 3, and SNAP-23 constitute the SNARE fusion machinery involved in the apical delivery of TGN-derived exocytic carriers in MDCK cells, but our methods are not yet sufficiently sensitive to block transport completely. According to this interpretation, a chaperone different from NSF would be used to activate the SNAREs for function.

**Localization of Syntaxin 3 and TI-VAMP in Apical Carriers.** Given the previous results, both TI-VAMP and syntaxin 3 are expected to be present in apical carriers. Therefore, we used immunoelectron microscopy to visualize these SNAREs directly in apical TGN-derived vesicles obtained from influenza virus-infected and perforated cells. Apical carriers were isolated after gradient density floatation (9, 27). Both the v-SNARE TI-VAMP and the t-SNARE syntaxin 3 could be observed in apical HA-positive vesicles (Fig. 3). This vesicle preparation previously has been reported positive for the apically targeted VIP21/caveolin-1 (9) and annexin XIIIb (27). The same preparation also labeled weakly for SNAP-23 but was negative for post-Golgi-localized syntaxins 4, 6, and 11 (data not shown). Syntaxin 4 was shown to be basolaterally targeted in MDCK cells (19) whereas syntaxins 6 and 11 were suggested to distribute along the TGN-to-endosomes pathway (18, 35).
Raft Association of Apical v- and t-SNAREs. We then investigated whether syntaxin 3 and TI-VAMP could be included in sphingolipid–cholesterol rafts. A postnuclear supernatant, obtained from scraped filter-grown MDCK cells, was extracted at low temperature with Triton X-100 before gradient density floatation, using a simple two-step gradient. For both TI-VAMP and syntaxin 3, a fraction was found floating with detergent-resistant membranes together with caveolin-1, a well characterized raft protein (Fig. 4A). These results suggested strongly that TI-VAMP also was affected by the MβCD treatment, floatation of HA was reduced as reported previously (27, 37). Similarly, after MβCD treatment, floatation of syntaxin 3 was decreased (Fig. 4B). We next tested whether the apically destined TI-VAMP was raft-associated. The floatation of TI-VAMP also was affected by the MβCD treatment before the Triton extraction, arguing for its association with raft lipid microdomains (Fig. 4B).

To determine whether TI-VAMP and syntaxin 3 were recruited into rafts at the TGN level, we performed a similar detergent-extraction experiment with or without cyclodextrin treatment using isolated apical carriers as a starting material. We observed a cholesterol-dependent detergent resistance of these SNAREs when they were included in budded apical carriers (Fig. 4C). These results suggested strongly that TI-VAMP and syntaxin 3 are associated with rafts already in TGN-derived apical carriers. These results provide evidence that incorporation into rafts is a means to sort both apical cargo and the membrane-docking/fusion machinery playing a role in membrane-trafficking events occurring in the apical compartment.

Several important points raised by these results await further investigation. It remains to be defined precisely at which step(s) the SNAREs we studied act in apical membrane trafficking. Also, we cannot exclude that other SNAREs yet to be identified could be involved. Interestingly, endobrevin (or VAMP8) was shown as abundantly expressed in kidney vs. others tissues (18). However, endobrevin is distributed throughout MDCK cells, and the addition of an antiendobrevin antibody in the transport assay carried out in SLO-permeabilized cells did not interfere with apical delivery (F.L. and K.S., unpublished data). Although the antibody used might not have blocking activity, this result would fit with the involvement of endobrevin in other pathways such as endosome trafficking (18, 23). Proteins proposed as regulators of SNAREs’ function might also play an important role in apical delivery. For instance, Munc-18-2/secl has been shown to interact with syntaxin-3 and SNAP-23 (39). On the other hand, syntaxin 4 might possess a dominant basolateral signal in its cytoplasmic domain to be sorted basolaterally. Notwithstanding these points yet to be resolved, we conclude that we have identified post-Golgi v- and t-SNAREs associated with rafts. These SNAREs are present in apical carriers and are likely to be involved in apical membrane trafficking.

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