THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 6, Issue of February 6, pp. 3422–3430, 1998
Printed in U.S.A.

Targeting of SNAP-23 and SNAP-25 in Polarized Epithelial Cells*

(Received for publication, July 3, 1997, and in revised form, November 12, 1997)

Seng Hui Low‡§, Paul A. Roche¶, Howard A. Anderson¶, Sven C. D. van Ijzendoorn‡‖, Min Zhang‡, Keith E. Mostov‡, and Thomas Weimbs‡§‡‡**

From the ‡Department of Anatomy, Department of Biochemistry and Biophysics, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0452, ¶Experimental Immunology Branch, NCI, National Institutes of Health, Bethesda, Maryland 20892, ‖Department of Physiological Chemistry, Groningen University, 9713 AV Groningen, The Netherlands

SNAP-23 is the ubiquitously expressed homologue of the neuronal SNAP-25, which functions in synaptic vesicle fusion. We have investigated the subcellular localization of SNAP-23 in polarized epithelial cells. In hepatocyte-derived HepG2 cells and in Madin-Darby canine kidney (MDCK) cells, the majority of SNAP-23 was present at both the basolateral and apical plasma membrane domains with little intracellular localization. This suggests that SNAP-23 does not function in intracellular fusion events but rather as a general plasma membrane t-SNARE. Canine SNAP-23 is efficiently cleaved by the botulinum neurotoxin E, suggesting that it is the toxin-sensitive factor previously found to be involved in plasma membrane fusion in MDCK cells. The localization of SNAP-25 in transfected MDCK cells was studied for comparison and was found to be identical to SNAP-23 with the exception that SNAP-25 was transported to the primary cilia protruding from the apical plasma membrane, which suggests that subtle differences in the targeting signals of both proteins exist. In contrast to its behavior in neurons, the distribution of SNAP-25 in MDCK cells remained unaltered by treatment with dibutyryl cAMP or forskolin, which, however, caused an increased growth of the primary cilia. Finally, we found that SNAP-23/25 and syntaxin 1A, when co-expressed in MDCK cells, do not stably interact with each other but are independently targeted to the plasma membrane and lysosomes, respectively.

A conserved machinery, the "SNARE machinery," appears to be involved in the docking and fusion steps of a multitude of membrane traffic pathways in all eukaryotic cells (reviewed in Refs. 1–5). The main components of this machinery have been identified, and their biochemical interactions have been investigated, in most detail for the machinery mediating the fusion of synaptic vesicles. In the process of vesicle docking and/or fusion, a core complex is formed between the t-SNAREs syntaxin and SNAP-25,[1] localized at the target (presynaptic) membrane, and the vesicular v-SNAREs synaptobrevin/vesicle-associated membrane protein and probably also synaptotagmin (6). This complex can recruit the soluble factors NSF and SNAP (soluble NSF attachment protein; unrelated to the synaptosome-associated protein of 25 kDa, SNAP-25) to form the mature fusion complex, which can disassemble after NSF-mediated ATP hydrolysis, leading to membrane fusion. Most of the protein-protein interactions in the core fusion complex involve protein domains with a predicted high probability of forming coiled-coil structures. SNAP-25, which possesses two coiled-coil domains, must play a central role in this complex, since it is capable of directly binding to all other constituents of the complex, *i.e.* to syntaxin to form a t-SNARE heterodimer, to synaptobrevin/vesicle-associated membrane protein, and to synaptotagmin (6–11). By sequence analysis, we have previously shown that both the N- and C-terminal coiled-coil domains of members of the SNAP-25 protein family as well as the membrane-proximal coiled-coil domain of the members of the syntaxin family are homologous to each other, uniting both families into a new superfamily (12).

An attractive feature of the SNARE hypothesis is its proposal that vesicle docking and/or fusion can occur only when cognate v- and t-SNAREs bind to each other (13). This could provide a proofreading mechanism to prevent a transport vesicle from fusing with an incorrect target membrane (14). A prerequisite for this specificity aspect of the SNARE hypothesis is that different isoforms of t- and v-SNAREs exist in different membrane compartments and classes of transport vesicles, respectively. Indeed, different isoforms of v- and t-SNAREs have been identified, some of which are expressed only in neurons, whereas others are more ubiquitously expressed in other mammalian tissues.

The specificity of the v-SNAREs could be demonstrated in yeast where certain v-SNAREs (*e.g.* Bos1p, Bet1p, Sec22p) are involved in early steps of the secretory pathway, whereas others (Snc1/2) are specific for the latest step, *i.e.* the fusion with the plasma membrane (15). A similar specificity exists for the t-SNAREs of the syntaxin family. Several non-neuronal syntaxin isoforms have been identified in mammals two of which, syntaxin 5 and 6, are localized to the membranes of the Golgi apparatus (16, 17), whereas the isoforms 2, 3, and 4 are found at the plasma membrane (16, 18). It was recently discovered that the localization of these plasma membrane-specific syntaxins is further segregated in epithelial cells. Polarized epithelial cells possess two plasma membrane domains, the apical and basolateral domains, with different protein and lipid compositions and a variety of different membrane traffic pathways leading to either surface (14, 19, 20). We have demonstrated

* This work was supported by National Institutes of Health Grants R01 AI25144, AI39161, and AI36953 (to K. E. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by the Irvington Institute for Immunology.

** Supported by a travel grant from the Groningen Institute for Drug Studies.

‡‡ Supported by a Feodor-Lynen-Fellowship of the Alexander von Humboldt-Foundation. To whom correspondence should be addressed: Dept. of Anatomy, University of California San Francisco, 513 Parnassus Ave., San Francisco, CA 94143-0452. E-mail: weimbs@itsa.ucsf.edu.

[1] The abbreviations used are: SNAP-25, synaptosomal-associated protein of 25 kDa; NSF, *N*-ethylmaleimide-sensitive fusion protein; BC, bile canaliculi; Bt_2cAMP, dibutyryl cAMP; MDCK, Madin-Darby canine kidney; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis.

recently that syntaxins 3 and 4 are restricted to the apical and basolateral plasma membrane domains, respectively, whereas syntaxin 2 was found on both surfaces in MDCK cells (18). This suggested that syntaxins 3 and 4 would serve only polarized pathways to their respective surfaces. It has been hypothesized earlier that apical fusion might be generally SNARE-independent and would utilize a different mechanism (21, 22) but our finding of two syntaxin isoforms, 2 and 3, being present at the apical plasma membrane in MDCK cells suggests that vesicle fusion at this surface is also mediated by the SNARE machinery (18).

Information on the specificity that might be provided by the second t-SNARE subunit, the SNAP-25 proteins, has lagged behind mostly because until recently only one member of this family has been identified in yeast (Sec9p) and one in mammals (SNAP-25). Sec9p is involved in plasma membrane fusion in yeast (23). The mammalian SNAP-25 is differentially expressed in neuronal subpopulations and is found at the axonal plasma membrane including the presynaptic terminals (24, 25). It appears to be utilized only for the fusion of synaptic vesicles in the nervous system and for similar regulated secretion events in neuroendocrine cells (26). Recently, however, a non-neuronal homologue of SNAP-25, termed SNAP-23, has been identified that is 59% identical to SNAP-25 at the amino acid level (27). SNAP-23 is expressed ubiquitously in all tissues examined and appears to bind the syntaxin isoforms 1, 2, 3, and 4 with equal efficiency *in vitro*. To date, it is not known whether SNAP-23 is present on all membrane compartments in the cell, where it could act as a general constituent of all SNARE complexes, or if it is specifically localized to only one particular compartment.

We have investigated the latter question by studying the subcellular localization of SNAP-23 in polarized epithelial cells. The majority of endogenous SNAP-23 in HepG2 cells as well as transfected SNAP-23 in MDCK cells was present at the plasma membrane. SNAP-23 was found on both the basolateral and apical domains, suggesting that it may play a role in determining the possible proofreading specificity with respect to plasma membrane/non-plasma membrane vesicle fusion but not with respect to apical *versus* basolateral polarity. Also, the neuron-specific SNAP-25 was efficiently transported to both plasma membrane domains when expressed in MDCK cells. In addition, only SNAP-25 could be detected at the plasma membrane surrounding the primary cilia that protrude from the apical surface of MDCK cells. This points to differences in the targeting of these two homologues.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media were from Cell Gro, Mediatech (Washington, DC), and the UCSF Cell Culture Facility. Fetal bovine serum (FBS) was from Hyclone (Logan, UT). G418 was obtained from Life Technologies, Inc. Transwell polycarbonate cell culture filters were purchased from Corning Costar Corporation (Massachusetts, MA). Rat monoclonal antibody ascites against ZO-1 was obtained from Chemicon International (Temecula, CA). The monoclonal antibody against SNAP-25 was from Sternberger Monoclonals (Baltimore, MD). Rabbit antiserum against detyrosinated tubulin (28) was a kind gift from Gregg Gundersen (Columbia University). Fluorescein isothiocyanate- or Texas red-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Horseradish peroxidase-conjugated, affinity-purified goat anti-rabbit IgG was from Southern Biotechnology Associates (Birmingham, AL). Propidium iodide was purchased from Molecular Probes (Eugene, OR). The monoclonal antibody against syntaxin 1A (HPC-1) and other chemicals and reagents were from Sigma. cDNA for the bacterial expression of His-tagged botulinum toxin E light chain was a kind gift of Drs. Heiner Niemann and Thomas Binz (Medizinische Hochschule, Hannover, Germany). The toxin was expressed in *Escherichia coli* and purified as described (29).

Antibodies against Human SNAP-23—Rabbit antisera (R.SNAP-23N and R.SNAP-23C) against human SNAP-23 were generated by immunization with synthetic peptides corresponding to the amino-terminal 17 (MDNLSSEEIQQRAHQIT) or the C-terminal 15 (RIDIANARAKKLIDS) amino acids of human SNAP-23 plus an additional C- or N-terminal cysteine residue, respectively. The peptides were covalently coupled to maleimide-activated keyhole limpet hemocyanin (Pierce) prior to immunization.

Both R.SNAP-23N and R.SNAP-23C recognized human SNAP-23 in immunoblot analysis. R.SNAP-23C but not R.SNAP-23N also cross-reacts with canine SNAP-23. R.SNAP-23N could be used for immunofluorescence analysis, while R.SNAP-23C gave only very weak signals and was not used for this technique. In all cases, the signals could be completely blocked by competition with the respective peptide.

R.SNAP-23N was affinity-purified by passing the diluted serum over a column of SulfoLink agarose (Pierce) chemically coupled to the SNAP-23 amino-terminal peptide. The column was then washed successively with 10 mM Tris, pH 7.5, and with 10 mM Tris, pH 7.5, containing 500 mM NaCl, and the bound antibody was eluted in 0.1 M glycine, pH 2.8. Eluted antibody was immediately neutralized by the addition of ⅒ volume of 1 M Tris, pH 8.0, and the samples were dialyzed against PBS.

SDS-PAGE and Immunoblotting—Adherent cells were recovered by trypsinization, washed, and resuspended in PBS containing 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM 1-chloro-3-tosylamido-7-amino-2-heptanone, 5 mM iodoacetamide, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cells were sonicated for 10 s on ice and boiled for 5 min in SDS-PAGE sample buffer containing 1% β-mercaptoethanol (25×10^6 cells/ml). Chromosomal DNA was sheared by repeated passage through a 22-gauge needle, and approximately 20 μg of total cell protein per lane was analyzed by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The blot was probed with R.SNAP-23N (1:3000), and the bound antibody was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (NEN Life Science Products).

Expression Vectors—Human SNAP-23 cDNA (27) was subcloned into pcDNA3 by adding *Eco*RI and *Xho*I sites at the 5′- and 3′-ends of the cDNA using the polymerase chain reaction. The sequence of pcDNA3-SNAP-23 was confirmed by automated sequence analysis. The cDNA for murine SNAP-25 was kindly provided by Dr. Michael Wilson (University of New Mexico, Albuquerque). It was subcloned from pSNAP 8.52 (24) into the *Eco*RI site of the expression vector pcDNA3.

Culture of HepG2, HT-29, and Caco-2 Cells—HepG2 and Caco-2 cells were obtained from the UCSF Cell Culture Facility. HT-29.74 cells were a kind gift of Dr. Charlotte S. Kaetzel (University of Kentucky, Lexington). HT-29.74 cells were grown in RPMI 1640 with 25 mM Hepes, 2 mM glutamine, 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (4.5 g/liter glucose), supplemented with 20% heat-inactivated FBS, 1% nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were grown in Dulbecco's modified Eagle's medium-H21 (4.5 g/liter glucose) containing 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (30).

Culture and Transfection of MDCK Cells—MDCK strain II cells were maintained in minimal essential medium, supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2, 95% air. Newly transfected cells or pooled stable clones were cultured on 12-mm, 0.4-μm pore size Transwell polycarbonate filters so that they reached confluency after 1 day and were grown for a further 3 or 4 days with regular medium changes.

MDCK cells or MDCK cells that stably express syntaxin 1A (described by Low *et al.* (18)) were transfected with the expression vectors for SNAP-23 or SNAP-25 by the calcium phosphate method as described previously (31). 3 days after transfection, cells were either directly plated onto filters for immunocytochemistry, or stable clones were selected in medium containing 0.35 mg/ml G418.

Immunocytochemistry, Confocal Microscopy, and Image Analysis—Samples were fixed with 4% paraformaldehyde, permeabilized with 0.025% (w/v) saponin in phosphate-buffered saline, and blocked with 10% FBS followed by sequential incubations with primary antibodies and fluorescein isothiocyanate- and/or Texas red-conjugated secondary antibodies. In some cases, nuclei were stained with 5 μg/ml propidium iodide after treatment with 100 μg/ml RNase A. The samples were analyzed using a Bio-Rad MRC-1000 confocal microscope.

For measurement of the length of the primary cilia, cells were labeled with an antibody against detyrosinated tubulin (28). Serial *x-y* confocal sections were recorded in 1-μm increments covering the entire height of the cell including the region above the apical membranes and projected into a single image. The apparent length of individual cilia in randomly

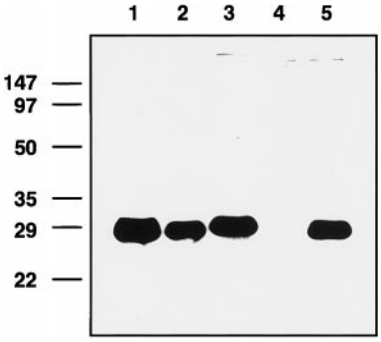

FIG. 1. **SNAP-23 is expressed in epithelial cell lines.** Equal amounts of total protein extracts from the cultured human epithelial cell lines HepG2 (*lane 1*), HT-29 (*lane 2*), Caco-2 (*lane 2*), and the canine kidney cell line MDCK (*lane 3*) were investigated by Western blot analysis with the antibody against human SNAP-23 (R.SNAP-23N). Total proteins from a stable clone of MDCK cells transfected to express human SNAP-23 were analyzed for comparison (*lane 5*).

selected fields was measured using NIH Image version 1.61 (National Institutes of Health). Since the length of three-dimensional cilia was measured after projection into a two-dimensional image, the obtained values are slightly shorter than the real length. We therefore refer to them as the "apparent" lengths.

Botulinum Toxin E Cleavage—Radiolabeled human SNAP-23 and murine SNAP-25 were produced by *in vitro* transcription/translation from the expression vectors described above using the TNT coupled *in vitro* translation/transcription kit (Promega, Madison, WI) using T7 RNA polymerase, reticulocyte extract, and [^{35}S]methionine. 1 μl of the products were incubated with 0–1 μg of purified His-tagged botulinum toxin E light chain (see "Experimental Procedures") for 1 h at 37 °C in 10 μl of 115 mM potassium acetate, 25 mM Hepes-KOH, pH 7.4, 2.5 mM magnesium acetate. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min. The proteins were separated by standard SDS-PAGE (15% acrylamide) and visualized by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

For the analysis of endogenous canine SNAP-23, cultured MDCK cells or MDCK cells stably expressing SNAP-25 or syntaxin 3 (18) were washed with ice-cold phosphate-buffered saline, scraped off, and homogenized by passing through a 23-gauge needle in 1 ml of 20 mM Tris-HCl, pH 7.5, including protease inhibitors. Membranes were pelleted and solubilized in 115 mM potassium acetate, 25 mM Hepes-KOH, pH 7.4, 2.5 mM magnesium acetate, 0.75% Triton X-100, and protease inhibitors for 25 min at 4 °C. Insoluble material was removed by centrifugation, and the supernatant was used for cleavage experiments. To 15 μl of this lysate, 0–1 μg of purified His-tagged botulinum toxin E light chain was added and incubated for 2 h at 37 °C. The samples were processed for Western blot analysis using antibodies against SNAP-23 (R.SNAP-23C), SNAP-25, and syntaxin 3.

RESULTS

SNAP-23 Is Expressed in Epithelial Cell Lines—SNAP-23 has been previously shown to be expressed in many different human tissues including tissues that are rich in epithelial cells like lung, liver, kidney, and pancreas (27). To study the localization of SNAP-23 in epithelial cells, a polyclonal antibody was raised against a peptide of the N-terminal 17 amino acids of the human SNAP-23 sequence. Total cellular protein extracts of different cultured human cell lines were prepared and examined by Western blot analysis (Fig. 1). A single band with an apparent molecular mass of 28 kDa could be detected in the three human epithelial cell lines examined: HepG2 (hepatocyte-derived) and Caco-2 and HT-29 (both colon carcinoma-derived). No band was detectable in the dog kidney cell line MDCK due to the inability of the antibody to cross-react with the canine protein as determined by Western blot analysis of different dog tissues (data not shown). However, using an antibody directed against a C-terminal peptide of human SNAP-23, which cross-reacts with the canine isoform (see "Experimental Procedures"), we could confirm that MDCK cells also express SNAP-23 endogenously (data not shown). The observed apparent molecular weight of SNAP-23 is higher than the calculated molecular weight, which may be due to the post-translational palmitoylation of several cysteine residues in analogy to SNAP-25 (32, 33). The results are in agreement with the previously observed wide tissue distribution of SNAP-23 and show that the protein is expressed in several pure epithelial cell lines.

Localization of Endogenous SNAP-23 in HepG2 Cells—The human hepatoma-derived HepG2 cells can be grown in culture so that they become polarized. After the initiation of cell-cell contact, neighboring cells form a structure that resembles the bile canaliculi (BC) of liver tissue (30). The plasma membrane surrounding the BC contains numerous microvilli and is equivalent to the apical plasma membrane domain of other epithelial cells. It is separated by tight junctional complexes from the basolateral domain. Polarized HepG2 cells were analyzed by confocal immunofluorescence microscopy after co-labeling with the affinity-purified SNAP-23 antibody and an antibody against villin, a microvillar protein (Fig. 2). Both the basolateral and the apical (BC) plasma membrane domains were clearly stained with the SNAP-23 antibody. The staining was specific, since the addition of the N-terminal SNAP-23 peptide completely eliminated all staining (not shown). The concentration of SNAP-23 appears higher in the apical than in the basolateral plasma membrane domain. However, if the high degree of folding in the apical surface due to the densely packed microvilli is taken into account, the SNAP-23 concentrations are probably similar in both domains. Some SNAP-23 staining was often observed intracellularly in HepG2 cells, but no distinct pattern was apparent for this staining. Some of the "intracellular" staining is attributable to basolateral plasma membranes reaching into the optical confocal section from the top or the bottom of the cells, but a fraction might also result from newly synthesized or endocytosed SNAP-23. The apical plasma membrane domains were in most cases positive for both SNAP-23 and villin. Occasionally, however, BC-like structures were observed that were stained for SNAP-23 but not for villin (*open arrowheads* in Fig. 2, *A* and *B*), suggesting that these are newly formed apical domains that have not yet developed fully mature microvilli.

Transfected SNAP-23 Is Targeted to the Basolateral and Apical Plasma Membrane Domains in MDCK Cells—MDCK cells are the one epithelial cell line in which membrane trafficking pathways have been most extensively characterized (14, 19). In contrast to hepatocytes, they are capable of direct biosynthetic membrane protein transport to the apical surface (34, 35). Since we had previously found that different syntaxin isoforms are differentially localized in MDCK cells (18), it was of great interest to also determine the localization of SNAP-23, the member of the second family of t-SNAREs, in this cell line. Because our SNAP-23-specific antibody did not cross-react with the canine protein (see "Experimental Procedures"), human SNAP-23 was expressed in MDCK cells by transfection. The transfected cells were grown as a polarized monolayer on a permeable filter support and investigated by confocal immunofluorescence microscopy. Figs. 3 and 4*A* show that the majority of SNAP-23 is transported to both the basolateral and apical domains of the plasma membrane. Both surfaces are stained to a similar extent, indicating that SNAP-23 is not preferentially targeted to either domain. Only transfected cells are labeled for SNAP-23, whereas neighboring nontransfected cells are unlabeled, demonstrating the specificity of the antibody. Again, some minor, undefined intracellular staining was observed. The average level of exogenous SNAP-23 expression in MDCK cells was comparable with the endogenous level in HepG2 cells as judged by the relative intensities of the immunofluorescence

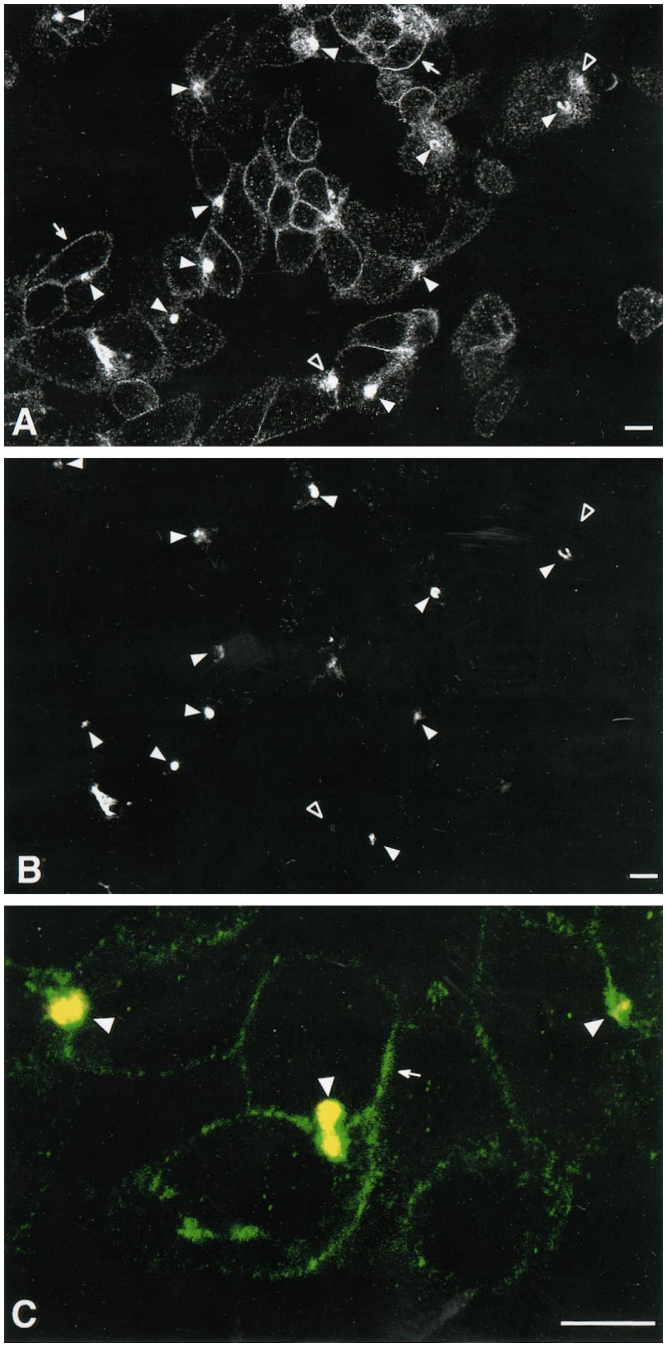

FIG. 2. **Endogenous SNAP-23 is expressed at the apical and basolateral plasma membrane domains of HepG2 cells.** HepG2 cells were double-labeled with antibodies directed against SNAP-23 and villin, a microvillar protein, and investigated by confocal fluorescence microscopy. *Panel A* shows the localization of SNAP-23. Bile canaliculi-like structures, which correspond to the apical plasma membrane, are highlighted by *arrowheads*. Some basolateral plasma membranes are marked by *arrows*. *Panel B* shows the staining for villin of the same field as in *panel A*. Villin is present almost exclusively at the apical membranes. Occasionally, SNAP-23-positive bile canaliculi-like structures can be seen that do not contain villin (*open arrowheads*). *Panel C* shows a higher magnification of a different field. The SNAP-23 channel (*green*) and the villin channel (*red*) are merged, resulting in *yellow* where they colocalize. *Bars*, 5 μm.

signals and by Western blot analysis (Fig. 1). Furthermore, the localization pattern did not change over a wide range of expression levels.

Together, these results suggest that SNAP-23 does not function in fusion events at intracellular organelles but only at the basolateral and apical plasma membrane domains in epithelial cells. This is in agreement with the observed ability of SNAP-23 to bind to all plasma membrane syntaxin isoforms *in vitro* (27).

Canine but Not Human SNAP-23 Is a Substrate for Botulinum Neurotoxin E—The neuronal SNAP-25 is a target of the botulinum toxins type A and E, zinc-dependent proteases that cleave SNAP-25 at two different sites close to its C terminus (29, 36). This action of the toxins inhibits neurotransmitter secretion efficiently *in vivo* and *in vitro* (37). Botulinum E-toxin has also been shown recently to specifically inhibit the fusion of transcytotic vesicles with the apical plasma membrane as well as recycling vesicles with the basolateral plasma membrane in streptolysin O-permeabilized MDCK cells (38). In that study, the substrate of the toxin remained unknown, but it was suggested to be a non-neuronal isoform of SNAP-25 that was not known at the time. The fact that the E-toxin cleavage site in SNAP-25 (Arg^{180}-Ile^{181}) is conserved in human SNAP-23 (27) and our finding that SNAP-23 is localized at both plasma membrane domains of MDCK cells suggest that it might be the botulinum E-toxin-sensitive SNARE involved in transcytosis and recycling. To investigate this possibility, we first treated *in vitro* translated human SNAP-23 (and SNAP-25 as a control) with recombinant, bacterially expressed botulinum E-toxin light chain. As shown in Fig. 5*A*, SNAP-25 was efficiently cleaved by the toxin, whereas no cleavage of SNAP-23 could be detected even with a large excess of the toxin, 100-fold more than needed to cleave >90% of the SNAP-25. Under similar conditions, human SNAP-23 was also not cleaved by botulinum toxin A (data not shown).

This result suggests that either SNAP-23 is not the botulinum E-toxin-sensitive factor involved in transcytosis and that therefore another SNAP-25 homologue might be expressed in MDCK cells or that the endogenous canine SNAP-23 of MDCK cells might differ in the toxin-binding and/or -cleavage sites from the human protein that might render it sensitive to the toxin. To distinguish between these possibilities, a second antibody was raised against a C-terminal peptide of human SNAP-23. This antibody cross-reacts well with the canine homologue in immunoblot analysis. It specifically recognizes a band of 28 kDa, and this signal completely disappears by competition with the C-terminal peptide (Fig. 5*B* and data not shown). Total membrane extracts of MDCK cells and MDCK cells stably expressing SNAP-25 or syntaxin 3 were treated with botulinum E-toxin. As shown in Fig. 5*B*, both the endogenous canine SNAP-23 and the positive control SNAP-25 are efficiently cleaved by the toxin, whereas syntaxin 3, as a negative control, is not affected. In contrast to SNAP-25, the cleavage of canine SNAP-23 is observed as a disappearance of the protein band, since the C-terminal domain recognized by the antibody is removed by the toxin.

This result shows that canine SNAP-23 is susceptible to botulinum toxin E and strongly suggests that SNAP-23 is the E-toxin-sensitive protein shown to be involved in vesicle fusion at the apical and basolateral plasma membrane domains in MDCK cells (38).

Transfected SNAP-25 Is Targeted to both the Basolateral and Apical Plasma Membrane Domains as Well as to the Cilium in MDCK Cells—For comparison with SNAP-23, the neuron-specific isoform SNAP-25 was also expressed by transfection in MDCK cells. Its steady state localization was very similar to that of SNAP-23. SNAP-25 was found mostly at the basolateral and apical plasma membrane domains with little intracellular staining (Figs. 4*B* and 6). One apparent difference, however, was that SNAP-25 could be consistently detected at the primary cilia that protrude from the apical surface of MDCK cells (Figs. 4*B* and 6*A*). Neither SNAP-23 (see Fig. 3 and 4*A*) nor the

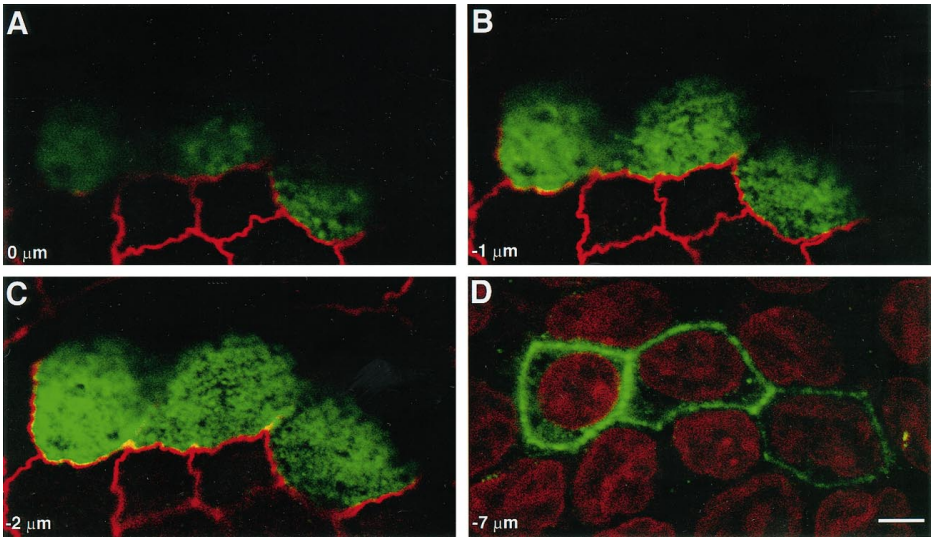

FIG. 3. **SNAP-23 is localized at the apical and basolateral plasma membrane domains in transfected MDCK cells.** MDCK cells transfected for human SNAP-23 were grown on a permeable filter support. Cells were labeled for SNAP-23 (*green*), for ZO-1 (*red*) (a tight junction protein), and for DNA with propidium iodide (*red*). Consecutive optical sections through the cell monolayer were recorded by confocal fluorescence microscopy at 1-μm intervals, and sections at the levels of the apical surface (*A–C*) and of the nuclei (*D*) are shown. Both the apical and the basolateral surfaces are clearly labeled for SNAP-23 in transfected cells, whereas neighboring nontransfected cells are unlabeled. *Bar*, 5 μm.

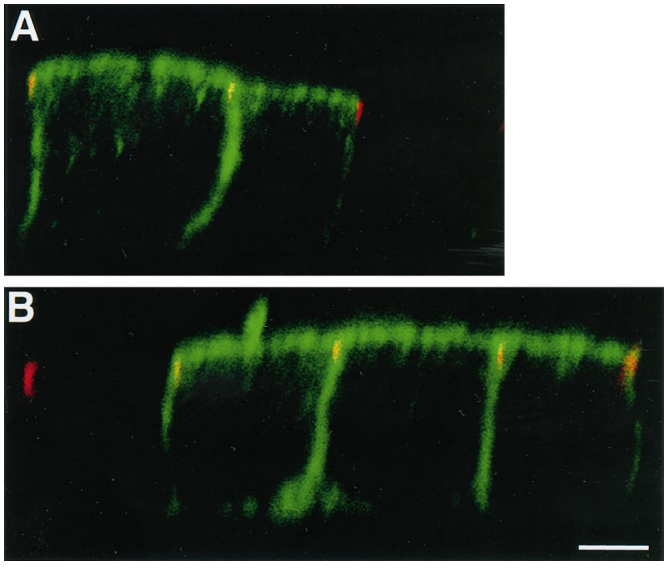

FIG. 4. **Comparison of SNAP-23 and SNAP-25 localization in transfected MDCK cells.** Filter-grown MDCK cells expressing either SNAP-23 (*A*) or SNAP-25 (*B*) were double-labeled for SNAP-23/25 (*green*) and the tight junction protein ZO-1 (*red*) and investigated by confocal fluorescence microscopy. Vertical optical sections are shown with the apical surface on top. A primary cilium that emerges from the apical surface and stains for SNAP-25 can be seen in *panel B*. *Bar*, 5 μm.

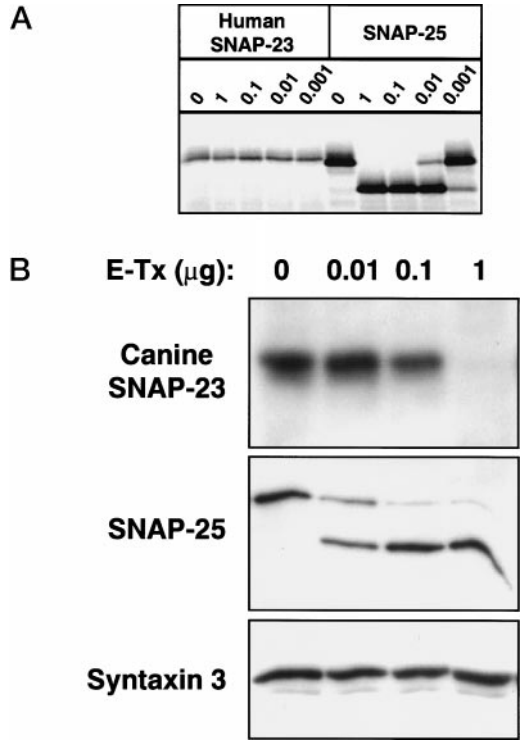

FIG. 5. **Canine but not human SNAP-23 is a substrate of botulinum neurotoxin E.** *A*, *in vitro* translated, [^{35}S]methionine-labeled human SNAP-23 or murine SNAP-25 were incubated with 0, 0.001, 0.01, 0.1, or 1 μg of purified, recombinant botulinum E-toxin light chain as indicated. The samples were separated on a 15% SDS-polyacrylamide gel and detected with a PhosphorImager screen. More than 90% of SNAP-25 is cleaved by 0.01 μg of toxin, but SNAP-23 is resistant to even a 100-fold higher amount under the same conditions. *B*, detergent extracts of total membranes from MDCK cells (*top panel*) or MDCK cells stably expressing SNAP-25 (*middle panel*) or syntaxin 3 (*bottom panel*) were incubated with 0, 0.01, 0.1, or 1 μg of botulinum E-toxin light chain as indicated. Samples were analyzed by immuno blot using antibodies against SNAP-23 (R.SNAP-23C), SNAP-25, and syntaxin 3, respectively. Both SNAP-23 and -25 are cleaved by the toxin, while the negative control syntaxin 3 is unaffected. Note that the cleavage product of SNAP-23 cannot be detected because the C terminus, which is recognized by the antibody, is removed by the toxin.

apical syntaxin isoforms 2 and 3 (18) were detected on the cilia. Again, the observed localization was independent of the expression level. The possibility that SNAP-25 expression altered the occurrence of cilia in MDCK cells was excluded by co-labeling studies with an antibody directed against detyrosinated tubulin, which labeled primary cilia of indistinguishable morphology in SNAP-23- and SNAP-25-expressing as well as nontransfected cells (see below and data not shown). These results demonstrate that the neuron-specific SNAP-25 is efficiently targeted to the plasma membrane of MDCK cells in contrast to the neuron-specific syntaxin 1A, which we previously found to be localized to lysosomes when expressed in MDCK cells (18). The finding that SNAP-25 but not SNAP-23 is targeted to the primary cilia suggests that subtle differences in their targeting signals exist.

cAMP Causes Growth of Primary Cilia but No Preferential Translocation of SNAP-25—The subcellular localization of SNAP-25 in rat brain has been reported to shift during development from cell bodies and axons to presynaptic terminals (39). Moreover, it has been shown that SNAP-25 is preferentially transported to the endings of neurites of differentiated PC12 cells only when the cells were treated with dibutyryl-cAMP (40). Together, these results suggest that the intracellular targeting of SNAP-25 is altered during neuron maturation

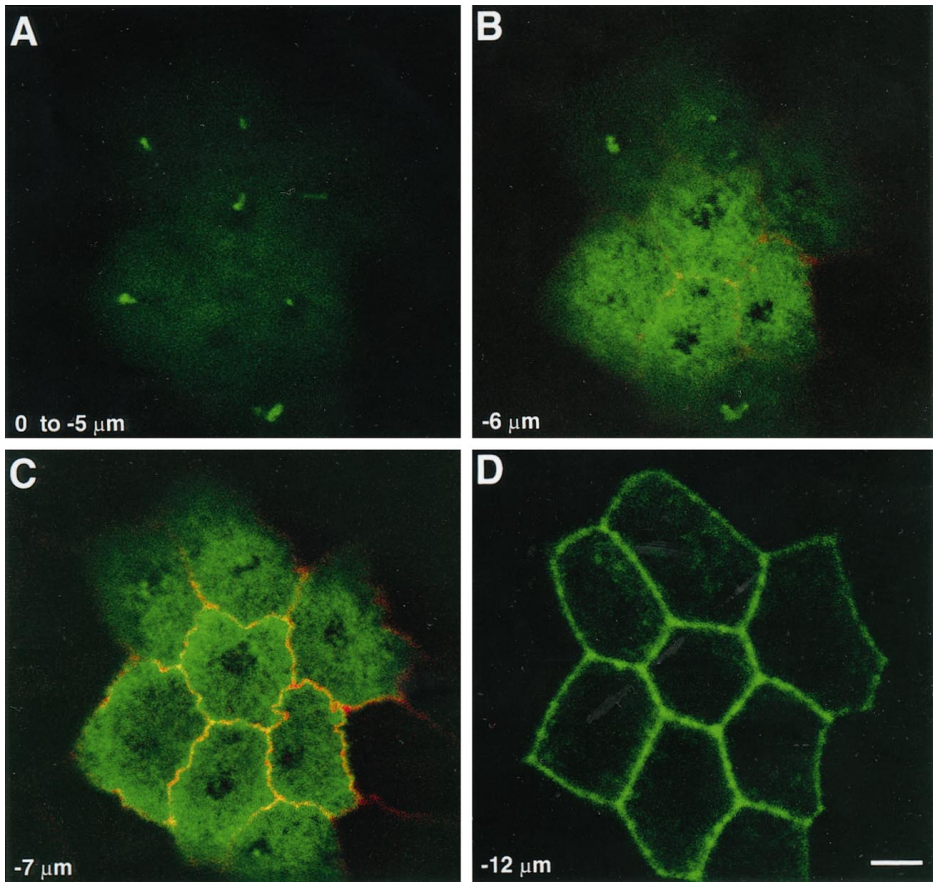

FIG. 6. **SNAP-25 is localized at the apical and basolateral plasma membrane domains and at the primary cilia in transfected MDCK cells.** Filter-grown MDCK cells transfected for SNAP-25 were double-labeled for SNAP-25 (*green*) and the tight junction protein ZO-1 (*red*) and investigated by confocal fluorescence microscopy. Consecutive optical sections through the cell monolayer were recorded at 1-μm intervals. The six uppermost sections above the apical surface were projected into one image and are shown in *panel A*. SNAP-25-positive primary cilia can clearly be seen. *Panels B* and *C* show sections at the level of the apical membrane, and *panel D* shows a section at the level of the nuclei. *Bar*, 5 μm.

by a process that is perhaps regulated by a cAMP-dependent signaling pathway. We investigated whether raising the intracellular cAMP concentration would alter the steady-state localization of SNAP-25 in transfected MDCK cells. Cells expressing SNAP-25 were treated for 16 h or 3 days with the membrane-permeable analog dibutyryl-cAMP or with the adenylyl cyclase activator forskolin and investigated by immunofluorescence confocal microscopy (Fig. 7). No changes in the plasma membrane localization of SNAP-25 were observed, and the relative labeling between the basolateral and the apical domains was unaltered. However, strikingly, the length of the primary cilia was increased significantly, and the average length had approximately doubled as compared with the control (Table I). The cilia were co-labeled using an antibody directed against detyrosinated tubulin. SNAP-25 was distributed evenly along the entire length of the cilia. No concentration at the endings was observed. Note that for these experiments mixed clones of stably transfected cells were used consisting of cells with a wide range of expression levels, which allowed us to control for a correlation between SNAP-25-expression and cilia length. No such correlation was found. The increase in cilia length was also identical for transfected and nontransfected MDCK cells (not shown).

SNAP-25 and Syntaxin 1A Are Targeted Independently of Each Other—SNAP-25 and syntaxin 1A bind tightly to each other to form the heterodimeric t-SNARE at the presynaptic nerve terminal. It is not known at which stage in their biosynthetic trafficking these two t-SNARE subunits bind to each other for the first time. We investigated whether SNAP-25 and syntaxin 1A, when co-expressed in the same MDCK cell, would mutually alter their targeting. If they could form a stable complex, we would expect to find them co-localized most likely either at the plasma membrane or in lysosomes. MDCK cells stably expressing syntaxin 1A (18) were transfected with SNAP-25, and the localization of both proteins was investigated by double labeling immunofluorescence microscopy. As shown in Fig. 8, the overall localization of syntaxin 1A and SNAP-25 was unchanged in cells expressing both proteins. The majority of SNAP-25 was still found at the plasma membrane, and syntaxin 1A remained localized to lysosomes. Only occasionally, intracellular regions were observed in which a fraction of the SNAP-25 was located in proximity but not perfectly overlapping with syntaxin 1A. No syntaxin 1A could be detected in the primary cilia despite the presence of SNAP-25 (not shown). The same result was obtained when syntaxin 1A and SNAP-23 were expressed together in MDCK cells, indicating that SNAP-23 also does not interact stably with syntaxin 1A under these conditions (data not shown).

This result suggests that syntaxin 1A and SNAP-25 are not stably associated when exogenously expressed in MDCK cells and do not form a complex en route to their final destination.

DISCUSSION

SNAP-23 Functions at the Plasma Membrane—Our finding that the majority of SNAP-23 localizes to the plasma membrane of different cell lines suggests that it functions as a t-SNARE in the docking and/or fusion of transport vesicles with this membrane compartment. Since very little SNAP-23 could be detected intracellularly, it is unlikely that it has a specific function in any compartment other than the plasma membrane. Therefore, the characterized t-SNAREs of the SNAP-25 family (SNAP-25, SNAP-23, and the yeast Sec9p) appear to function exclusively at the plasma membrane. However, since most of the other membrane fusion events in eukaryotic cells also seem to involve the SNARE machinery, the question arises as to whether these intracellular fusion events may either not require a SNAP-25-like t-SNARE or whether they may involve as yet unknown SNAP-25 homologues. We have recently iden-

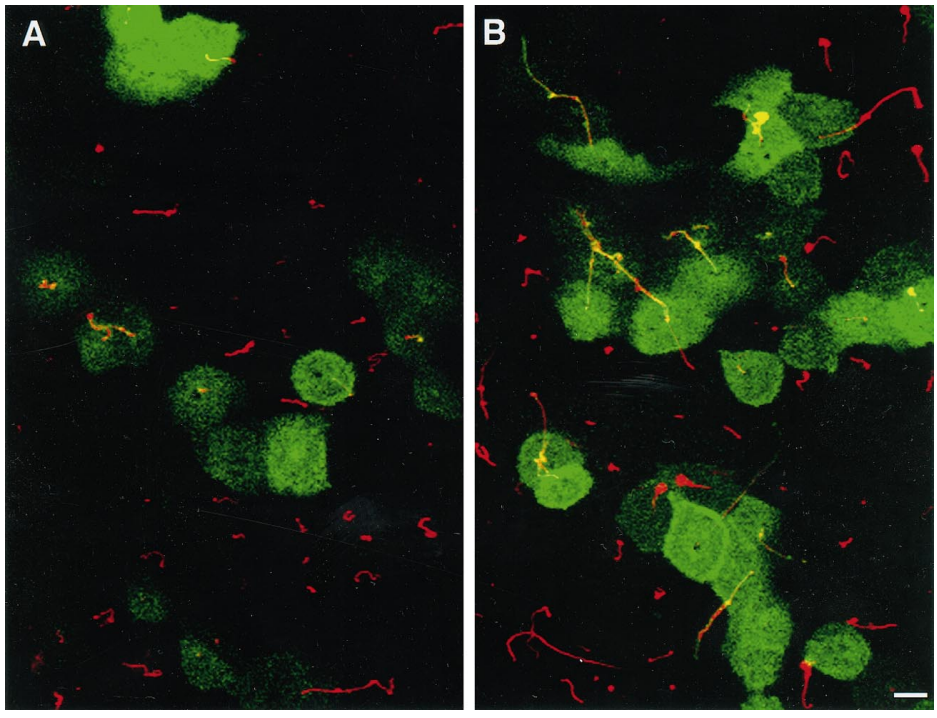

FIG. 7. **Elevated cAMP concentration causes an increase in cilia length but no redistribution of SNAP-25.** Pooled clones of MDCK cells stably expressing SNAP-25 were treated for 16 h without (*A*) or with (*B*) 1 mM Bt_2cAMP. Cells were then double-labeled for SNAP-25 (*green*) and detyrosinated tubulin (*red*) and investigated by confocal fluorescence microscopy. Representative fields are shown. Consecutive optical sections were recorded at 1-μm intervals. Ten images, above and partially including the level of the apical membranes, were projected into one image. The effect on cilia morphology of treatment with forskolin or Bt_2cAMP was nearly indistinguishable (see Table I). *Bar*, 5 μm.

TABLE I
cAMP stimulation results in increased length of primary cilia

Confluent MDCK monolayers on polycarbonate filters were treated for 16 or 72 h with either 1 mM Bt_2cAMP, 50 μM forskolin (FSK) or without drug (control). Cells were fixed and permeabilized, and the primary cilia were labeled with an antibody against detyrosinated tubulin. The apparent lengths of the primary cilia, which differ slightly from the real lengths, were measured as described under "Experimental Procedures" from randomly selected micrograph fields.

Treatment	Average apparent cilia length		S.E.	Sample size (number of cilia measured)
	Median	Mean		
	μm			
Control	2.3	2.8	0.1	380
Bt_2cAMP, 16 h	4.2	5.7	0.4	162
FSK, 16 h	3.6	5.4	0.2	471
FSK, 72 h	5.3	7.0	0.2	503

tified two new distantly related members of the SNAP-25 family by profile-based data base searches (12) that are possible candidates for t-SNAREs involved in intracellular membrane fusion events.

Our finding that canine SNAP-23 can be efficiently cleaved by botulinum neurotoxin E links the previously reported functional effects of this toxin on membrane fusion events in MDCK cells to SNAP-23. Treatment of streptolysin-O-permeabilized MDCK cells with the E-toxin inhibits the fusion of transcytotic vesicles with the apical plasma membrane and of recycling vesicles with the basolateral membrane in an *in vitro* reconstituted transport assay (38). Our findings that canine SNAP-23 is a substrate of this toxin and that SNAP-23 is localized on both the basolateral and apical surfaces strongly suggest that it is involved at least in these two membrane traffic pathways.

The finding that human SNAP-23 can not be cleaved by the E-toxin suggests that it differs from the canine homologue in a critical amino acid(s) in the toxin binding or cleavage sites.

SNAP-23 and the Specificity of Vesicular Traffic—The SNARE hypothesis predicts that different membrane compartments should contain different t-SNAREs, which would provide a mechanism to allow only the fusion of correctly targeted

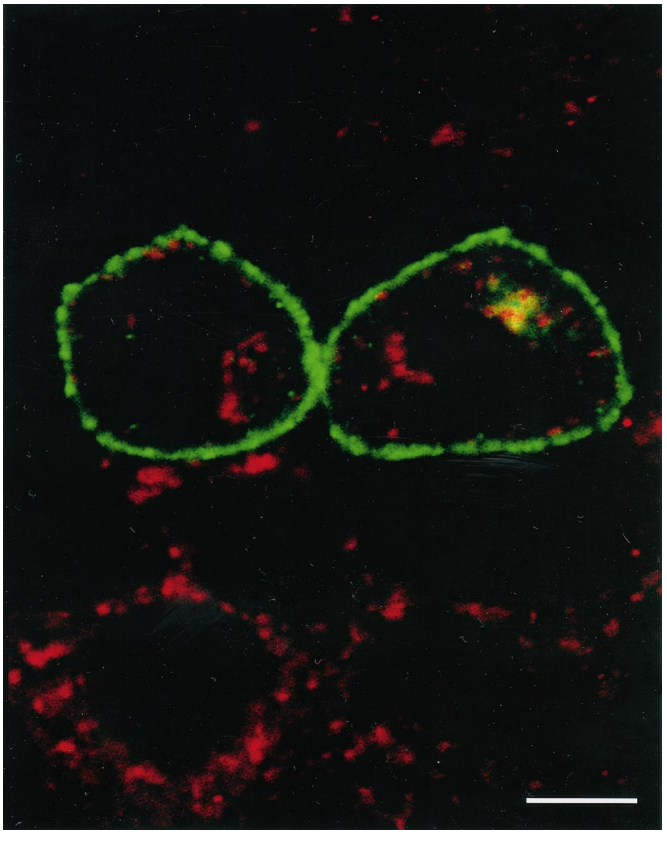

FIG. 8. **SNAP-25 and syntaxin 1A do not co-localize significantly when co-expressed in MDCK cells.** MDCK cells stably expressing syntaxin 1A (18) were transfected for SNAP-25 and grown on permeable filters. The cells were double-labeled for SNAP-25 (*green*) and syntaxin 1A (*red*) and investigated by confocal fluorescence microscopy. The *figure* shows a representative horizontal optical section at the level of the nuclei. Two SNAP-25-expressing cells are shown that are surrounded by cells only expressing syntaxin 1A. Neither the localization of syntaxin 1A nor of SNAP-25 (compare with Fig. 6) was affected by the co-expression of the other, and no significant co-localization was observed. *Bar*, 5 μm.

transport vesicles possessing matching v-SNAREs. Since SNAP-23 localizes to both the basolateral and the apical plasma membrane domains of polarized epithelial cells, it cannot contribute directly to the specificity of the fusion of vesicles to either domain. Although it is possible that additional SNAP-23-homologues exist that might be localized specifically to either plasma membrane domain, we find it more likely that the specificity in the v-SNARE/t-SNARE interaction is based on the second t-SNARE subunit, the syntaxins. This is supported by our previous findings that the syntaxin isoforms 2, 3, and 4 are indeed differentially localized at the plasma membrane domains of MDCK cells (18) and that SNAP-23 binds equally well to these syntaxin isoforms *in vitro* (27). It is therefore likely that SNAP-23 is a general binding partner for all plasma membrane syntaxins in non-neuronal cells.

The presence of SNAP-23, in addition to the syntaxins 2 and 3 (18), on the apical plasma membrane of polarized epithelial cells, including MDCK cells, further demonstrates that this membrane possesses components of the SNARE machinery and makes it unlikely that vesicular fusion with the apical domain is generally independent of this machinery as previously proposed (21, 22).

Our finding that SNAP-23 is abundantly present at the apical plasma membrane of HepG2 cells is the first demonstration that a t-SNARE is present at this membrane domain of hepatocytes. Hepatocytes lack a direct biosynthetic pathway to the apical surface, and most proteins reach this surface by transcytosis. It is therefore likely that SNAP-23 is involved in the apical fusion of transcytotic vesicles in hepatocytes.

SNAP-25 in Primary Cilia—When expressed in MDCK cells, SNAP-25 but not SNAP-23 was targeted to the primary cilia that protrude from the apical plasma membrane. MDCK cells possess a single primary cilium as do most cells in mammalian tissues (41–45). These cilia are of the 9 + 0 nonmotile type, and their function is still unknown, although a sensory role as mechano- or chemoreceptors has been suggested. In our experience with various endogenously or exogenously expressed apical membrane proteins in MDCK cells, we have never observed a protein that was associated with the primary cilia; nor are we aware of any report by others demonstrating that an apical plasma membrane protein was targeted to the cilia membrane in MDCK cells. It therefore appears as if the membrane surrounding the cilia excludes most membrane proteins. This membrane could therefore be regarded as a distinct subdomain of the apical plasma membrane with a different protein and perhaps lipid composition.

Three possible mechanisms could account for the observed localization of SNAP-25 in the cilia membrane: (i) SNAP-25 cannot be efficiently excluded from this domain, (ii) it possesses an intrinsic targeting signal, or (iii) it binds to another protein with such a signal, which in turn would target it to this domain. Cilia are in some ways similar to neuronal axons; both are long, narrow membrane protrusions with a core of microtubules whose (+)-ends are directed toward the distal end. Since SNAP-25 is efficiently targeted to the axonal membrane of neurons and enriched in the nerve terminals, it is conceivable that, when expressed in MDCK cells, it is mistakenly transported to the apical cilia, perhaps by vesicular transport. Small vesicles of approximately 50 nm have been observed within primary cilia (46). Interestingly, the outer segment of photoreceptor cells, which contains the membranous disks, is regarded as a highly modified cilium. SNAP-25 is expressed in photoreceptor cells (47, 48), and an attractive hypothesis is that SNAP-25 is involved in membrane traffic into the outer segment and must therefore itself be targeted into this segment or to the connecting cilia, possibly by a mechanism that is similar between the modified cilium of the photoreceptor cell and the primary cilium of MDCK cells.

In contrast to its behavior in undifferentiated and neural growth factor-differentiated PC12 cells, in which SNAP-25 translocates to the endings of neurites after treatment with Bt_2cAMP (40), we observed no shift in localization toward the basolateral or apical plasma membrane nor to the endings of the primary cilia of transfected MDCK cells after treatment with Bt_2cAMP or forskolin. The applied concentrations of Bt_2cAMP and forskolin were in the same range as previously used to stimulate apical membrane transport pathways (49, 50). Our results indicate that the proposed developmental changes in the targeting of SNAP-25 (39, 40) cannot be mimicked in this epithelial cell system.

Surprisingly, the average cilia length increased approximately 2-fold after stimulation with either Bt_2cAMP or forskolin. To our knowledge, such an effect of cAMP on primary cilia has not been reported before. Further studies characterizing this effect might prove valuable in elucidating the as yet elusive function of primary cilia that are abundantly present in most tissues. Interestingly, treatment of neurons in primary culture or PC12 cells with drugs that cause an increase in the intracellular cAMP concentration results in neurite outgrowth (51–53).

How are SNAP-23 and -25 Targeted to the Plasma Membrane?—SNAP-23 and -25 are hydrophilic proteins that do not possess a hydrophobic proteinacious membrane anchor. SNAP-25 (and by analogy presumably SNAP-23) is membrane-anchored after several clustered cysteine residues become post-translationally modified by palmitic acid groups (32, 33). It is not known when this modification occurs during the biosynthesis of SNAP-23 and -25. Consequently, newly synthesized SNAP-23 and -25 do not necessarily have to be integrated into the membrane of the endoplasmic reticulum and transported through the entire secretory pathway. Thus, their intracellular targeting could be independent of membrane transport. However, SNAP-25 appears to be membrane-bound during its rapid axonal transport, and indirect evidence suggests that it is palmitoylated before axonal transport (32). Also, syntaxins are not classical integral membrane proteins with a signal sequence but instead belong to a class of "tail-anchored" proteins that may be generally integrated into the endoplasmic reticulum membrane posttranslationally and then transported along the secretory pathway (54).

It is believed that the sorting mechanisms in neurons and epithelial cells share common features and that the apical and basolateral plasma membrane domains of epithelial cells are equivalent to the axonal and somatodendritic membranes, respectively, of neurons (55, 56). It is therefore surprising that we found SNAP-25, which localizes to the axonal membrane of mature neurons, being targeted to both domains of MDCK cells. However, recently other examples of axonal membrane proteins have been found that do not localize to the apical domain when expressed in MDCK cells (57). Moreover, we have found previously that the neuron-specific syntaxin 1A is targeted to late endosomes and lysosomes when expressed in MDCK cells (18). This was interpreted as a mistargeting due to the lack of a necessary neuron-specific factor(s). It would have been conceivable that syntaxin 1A could not be efficiently retained at the plasma membrane in the absence of its t-SNARE binding partner SNAP-25, leading to its destruction in lysosomes. In that case, SNAP-25 would be the missing factor. Large fractions of syntaxin 1 and SNAP-25 have been found to be stably bound to each other in a heterodimeric complex in adrenal chromaffin cells (58). Our present study shows, however, that this is not the case in MDCK cells. Since we observed

no significant co-localization of SNAP-25 and syntaxin 1A when both were co-expressed in MDCK cells, both proteins must be targeted independently of each other without stably interacting. It is likely that this is also the case in neurons and that both t-SNARE subunits interact only after they have reached their final destination. t-SNAREs have to be "turned off" efficiently during their biosynthetic transport to prevent premature vesicle fusion. It is possible that both syntaxin 1 and SNAP-25 are kept from binding to each other by binding to n-Sec1 (25, 59) or the recently discovered Hrs-2 (60), respectively, or non-neuronal homologues (61–63) of these proteins. An additional, even more effective, security mechanism to prevent premature binding between syntaxin and SNAP-25 might be that both proteins are transported along different nonintersecting pathways to the axonal plasma membrane.

In conclusion, our findings presented here establish SNAP-23 as a general plasma membrane t-SNARE. If other intracellular membrane fusion reactions involve members of the SNAP-25 family, these remain to be discovered. We are currently investigating the possible roles of new candidates of intracellular SNAP-25-like t-SNAREs.

Acknowledgments—We thank Steven Chapin for valuable discussions. We also thank Heiner Niemann and Thomas Binz for providing the cDNA for botulinum E-toxin light chain; Michael Wilson for the SNAP-25 cDNA; Charlotte Kaetzel for HT-29 cells; and Gregg Gundersen for the antibody against detyrosinated tubulin.

REFERENCES

1. Bennett, M. K. (1995) *Curr. Opin. Cell Biol.* **7,** 581–586
2. Rothman, J. E. (1994) *Nature* **372,** 55–63
3. Südhof, T. C. (1995) *Nature* **375,** 645–653
4. Calakos, N., and Scheller, R. H. (1996) *Physiol. Rev.* **76,** 1–29
5. Ferro-Novick, S., and Jahn, R. (1994) *Nature* **370,** 191–193
6. Schiavo, G., Stenbeck, G., Rothman, J. E., and Söllner, T. H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 997–1001
7. Chapman, E. R., An, S., Barton, N., and Jahn, R. (1994) *J. Biol. Chem.* **269,** 27427–27432
8. Fasshauer, D., Bruns, D., Shen, B., Jahn, R., and Brünger, A. T. (1997) *J. Biol. Chem.* **272,** 4582–4590
9. Kee, Y., Lin, R. C., Hsu, S. C., and Scheller, R. H. (1995) *Neuron* **14,** 991–998
10. McMahon, H. T., and Südhof, T. C. (1995) *J. Biol. Chem.* **270,** 2213–2217
11. Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T. C., and Niemann, H. (1994) *EMBO J.* **13,** 5051–5061
12. Weimbs, T., Low, S. H., Chapin, S. J., Mostov, K. E., Bucher, P., and Hofmann, K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 3046–3051
13. Rothman, J. E., and Warren, G. (1994) *Curr. Biol.* **4,** 220–233
14. Weimbs, T., Low, S. H., Chapin, S. J., and Mostov, K. E. (1997) *Trends Cell Biol.* **7,** 393–399
15. Pelham, H. R. B., Banfield, D. K., and Lewis, M. J. (1995) *Cold Spring Harbor Symp. Quant. Biol.* **60,** 105–111
16. Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hazuka, C. D., and Scheller, R. H. (1993) *Cell* **74,** 863–873
17. Bock, J. B., Lin, R. C., and Scheller, R. H. (1996) *J. Biol. Chem.* **271,** 17961–17965
18. Low, S. H., Chapin, S. J., Weimbs, T., Kömüves, L. G., Bennett, M. K., and Mostov, K. E. (1996) *Mol. Biol. Cell* **7,** 2007–2018
19. Mostov, K. E., Altschuler, Y., Chapin, S. J., Enrich, C., Low, S. H., Luton, F., Richman-Eisenstat, J., Singer, K. L., Tang, K., and Weimbs, T. (1995) *Cold Spring Harbor Symp. Quant. Biol.* **60,** 775–781
20. Simons, K., and Wandinger-Ness, A. (1990) *Cell* **62,** 207–210
21. Ikonen, E., Tagaya, M., Ullrich, O., Montecucco, C., and Simons, K. (1995) *Cell* **81,** 571–580
22. Fiedler, K., Lafont, F., Parton, R. G., and Simons, K. (1995) *J. Cell Biol.* **128,** 1043–1053
23. Brennwald, P., Kearns, B., Champion, K., Keranen, S., Bankaitis, V., and Novick, P. (1994) *Cell* **79,** 245–258
24. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989) *J. Cell Biol.* **109,** 3039–3052
25. Garcia, E. P., McPherson, P. S., Chilcote, T. J., Takei, K., and De Camilli, P. (1995) *J. Cell Biol.* **129,** 105–120
26. Sadoul, K., Lang, J., Montecucco, C., Weller, U., Regazzi, R., Catsicas, S., Wollheim, C. B., and Halban, P. A. (1995) *J. Cell Biol.* **128,** 1019–1028
27. Ravichandran, V., Chawla, A., and Roche, P. A. (1996) *J. Biol. Chem.* **271,** 13300–13303
28. Gundersen, G. G., Kalnoski, M. H., and Bulinski, J. C. (1984) *Cell* **38,** 779–789
29. Binz, T., Blasi, J., Yamasaki, S., Baumeister, A., Link, E., Südhof, T. C., Jahn, R., and Niemann, H. (1994) *J. Biol. Chem.* **269,** 1617–1620
30. Zaal, K. J., Kok, J. W., Sormunen, R., Eskelinen, S., and Hoekstra, D. (1994) *Eur. J. Cell Biol.* **63,** 10–19
31. Breitfeld, P., Casanova, J. E., Harris, J. M., Simister, N. E., and Mostov, K. E. (1989) *Methods Cell Biol.* **32,** 329–337
32. Hess, D. T., Slater, T. M., Wilson, M. C., and Skene, J. H. (1992) *J. Neurosci.* **12,** 4634–4641
33. Veit, M., Söllner, T. H., and Rothman, J. E. (1996) *FEBS Lett.* **385,** 119–123
34. Casanova, J. E., Mishumi, Y., Ikehara, Y., Hubbard, A. L., and Mostov, K. E. (1991) *J. Biol. Chem.* **266,** 24428–24432
35. Low, S. H., Wong, S. H., Tang, B. L., Subramaniam, V. N., and Hong, W. J. (1991) *J. Biol. Chem.* **266,** 13391–13396
36. Schiavo, G., Rossetto, O., Catsicas, S., Polverino de Laureto, P., DasGupta, B. R., Benfenati, F., and Montecucco, C. (1993) *J. Biol. Chem.* **268,** 23784–23787
37. Niemann, H., Blasi, J., and Jahn, R. (1994) *Trends Cell Biol.* **4,** 179–185
38. Apodaca, G., Cardone, M. H., Whiteheart, S. W., DasGupta, B. R., and Mostov, K. E. (1996) *EMBO J.* **15,** 1471–1481
39. Oyler, G. A., Polli, J. W., Wilson, M. C., and Billingsley, M. L. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5247–5251
40. Sanna, P. P., Bloom, F. E., and Wilson, M. C. (1991) *Brain Res. Dev. Brain Res.* **59,** 104–108
41. Wheatley, D. N. (1995) *Pathobiology* **63,** 222–238
42. Wheatley, D. N., Wang, A. M., and Strugnell, G. E. (1996) *Cell Biol. Int.* **20,** 73–81
43. Roth, K. E., Rieder, C. L., and Bowser, S. S. (1988) *J. Cell Sci.* **89,** 457–66
44. Valentich, J. D. (1981) *Ann. N. Y. Acad. Sci.* **372,** 384–405
45. Bacallao, R., Antony, C., Dotti, C., Karsenti, E., Stelzer, E. H., and Simons, K. (1989) *J. Cell Biol.* **109,** 2817–2832
46. Poole, C. A., Flint, M. H., and Beaumont, B. W. (1985) *Cell Motil.* **5,** 175–93
47. Brandstatter, J. H., Wassle, H., Betz, H., and Morgans, C. W. (1996) *Eur. J. Neurosci.* **8,** 823–828
48. Ullrich, B., and Südhof, T. C. (1994) *J. Physiol. Paris* **88,** 249–257
49. Pimplikar, S. W., and Simons, K. (1994) *J. Biol. Chem.* **269,** 19054–19059
50. Eker, P., Holm, P. K., van Deurs, B., and Sandvig, K. (1994) *J. Biol. Chem.* **269,** 18607–18615
51. Gunning, P. W., Landreth, G. E., Bothwell, M. A., and Shooter, E. M. (1981) *J. Cell Biol.* **89,** 240–245
52. Richter-Landsberg, C., and Jastorff, B. (1986) *J. Cell Biol.* **102,** 821–829
53. Rydel, R. E., and Greene, L. A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1257–1261
54. Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B., and Rapoport, T. A. (1995) *EMBO J.* **14,** 217–223
55. de Hoop, M. J., and Dotti, C. G. (1993) *J. Cell Sci. (Suppl.)* **17,** 85–92
56. Ahn, J., Mundigl, O., Muth, T. R., Rudnick, G., and Caplan, M. J. (1996) *J. Biol. Chem.* **271,** 6917–6924
57. Gu, H. H., Ahn, J., Caplan, M. J., Blakely, R. D., Levey, A. I., and Rudnick, G. (1996) *J. Biol. Chem.* **271,** 18100–18106
58. Höhne-Zell, B., and Gratzl, M. (1996) *FEBS Lett.* **394,** 109–116
59. Pevsner, J., Hsu, S. C., and Scheller, R. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1445–1449
60. Bean, A. J., Seifert, R., Chen, Y. A., Sacks, R., and Scheller, R. H. (1997) *Nature* **385,** 826–829
61. Hata, Y., and Südhof, T. C. (1995) *J. Biol. Chem.* **270,** 13022–13028
62. Tellam, J. T., McIntosh, S., and James, D. E. (1995) *J. Biol. Chem.* **270,** 5857–5863
63. Katagiri, H., Terasaki, J., Murata, T., Ishihara, H., Ogihara, T., Inukai, K., Fukushima, Y., Anai, M., Kikuchi, M., Miyazaki, J., Yazaki, Y, and Oka, Y. (1995) *J. Biol. Chem.* **270,** 4963–4966