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Differential Localization of Syntaxin Isoforms in Polarized Madin-Darby Canine Kidney Cells

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Syntaxins, integral membrane proteins that are part of the ubiquitous membrane fusion machinery, are thought to act as target membrane receptors during the process of vesicle docking and fusion. Several isoforms of the syntaxin family have been previously identified in mammalian cells, some of which are localized to the plasma membrane. We investigated the subcellular localization of these putative plasma membrane syntaxins in polarized epithelial cells, which are characterized by the presence of distinct apical and basolateral plasma membrane domains. Syntaxins 2, 3, and 4 were found to be endogenously present in Madin-Darby canine kidney cells. The localization of syntaxins 1A, 1B, 2, 3, and 4 in stably transfected Madin-Darby canine kidney cell lines was studied with confocal immunofluorescence microscopy. Each syntaxin isoform was found to have a unique pattern of localization. Syntaxins 1A and 1B were present only in intracellular structures, with little or no apparent plasma membrane staining. In contrast, syntaxin 2 was found on both the apical and basolateral surface, whereas the plasma membrane localization of syntaxins 3 and 4 were restricted to the apical or basolateral domains, respectively. Syntaxins are therefore the first known components of the plasma membrane fusion machinery that are differentially localized in polarized cells, suggesting that they may play a central role in targeting specificity.

INTRODUCTION

Eukaryotic cells possess various membrane domains and membrane-bound compartments that differ in protein and lipid composition and are involved in distinct cellular functions. This membrane system is connected by trafficking of vesicular intermediates that bud from a donor compartment, conveying soluble and membrane cargo to an acceptor compartment by fusing with it. A ubiquitous fusion machinery, the soluble *N*-ethylmaleimide-sensitive factor (NSF[1]) attachment protein (SNAP) receptor (SNARE) machinery, has been identified in recent years and appears to mediate membrane fusion in most vesicular trafficking pathways (Rothman, 1994; Bennett, 1995). This fusion machinery consists of integral membrane proteins on the vesicle (v-SNAREs) and on the target membrane (t-SNAREs) that are thought to interact with each other in the process of vesicle docking. In mammalian cells, t-SNAREs include members of the syntaxin and SNAP-25 families while v-SNAREs are members of

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[1] Abbreviations used: MDCK, Madin-Darby canine kidney; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, SNAP receptor; TGN, trans-Golgi network.

the VAMP/synaptobrevin family. The formation of a t-SNARE/v-SNARE complex results in the recruitment of the soluble proteins αSNAP (unrelated to SNAP-25) and NSF, forming the so-called "fusion complex." Membrane fusion is finally achieved after ATP hydrolysis mediated by the ATPase NSF, which leads to the disassembly of the fusion complex (Südhof *et al.*, 1993; Bennett, 1995; Südhof, 1995; Calakos and Scheller, 1996; Rothman and Wieland, 1996).

It has been hypothesized that different classes of transport vesicles and different acceptor membranes possess distinct isoforms of v- and t-SNAREs, respectively, and that only the pairing of a matching combination would lead to successful vesicle fusion (Rothman and Warren, 1994). This so-called "SNARE hypothesis" therefore postulates a proofreading mechanism in which the SNAREs would contribute to the specificity of vesicular fusion. The still speculative notion that SNAREs may be responsible for the specificity of vesicle targeting originated from the finding that different SNAREs are involved in different steps in the biosynthetic pathway in yeast, from the ER to the Golgi, Golgi to plasma membrane, and Golgi to vacuole (Söllner *et al.*, 1993). This hypothesis therefore predicts that different t-SNAREs are localized to and define different membrane compartments in the cell. Indeed, several isoforms of t-SNARES have been identified in mammalian cells (Bennett *et al.*, 1993; Ravichandran *et al.*, 1996). The syntaxin isoforms 1, 2, and 4 have been shown previously to be located, at least partially, at the plasma membrane of neurons or nonpolarized cells (Bennett *et al.*, 1993), and the closely related syntaxin 3 is another candidate for a plasma membrane t-SNARE. However, the majority of work in this area has been on the mechanism of fusion in systems where there is only one major type of vesicle and one type of target, e.g., fusion of synaptic vesicles with the axonal plasma membrane (Bennett, 1995). Similarly, this work has generally examined the role of a single v-SNARE/t-SNARE combination. To our knowledge, only two reports have examined the role of multiple syntaxin isoforms in a single, nonpolarized cell type, i.e., in adipocytes (Volchuk *et al.*, 1996) and macrophages (Hackam *et al.*, 1996).

Polarized epithelial cells offer a useful but so far unexamined system to analyze the localization and roles of the various t-SNAREs and their possible involvement in the mechanisms underlying the specificity of vesicular targeting. These cells have apical and basolateral plasma membrane domains. Each domain receives vesicles from several pathways, including direct delivery from the trans-Golgi network (TGN), ipsilateral recycling from endosomes, and transcytosis from the contralateral surface. Furthermore, some of these transport steps may actually involve two parallel pathways. For instance, TGN to basolateral plasma membrane delivery in both Madin-Darby canine kidney (MDCK) cells and rat hepatocytes seems to use separate vesicles for transporting membrane proteins or secretory proteins (Boll *et al.*, 1991; Saucan and Palade, 1994). Recent studies by Ikonen *et al.*, 1995 have demonstrated that the fusion of biosynthetic vesicles (carrying the vesicular stomatitis virus G protein as a marker protein) with the basolateral surface required NSF, αSNAP, and VAMP/synaptobrevin (Ikonen *et al.*, 1995; Wilson, 1995). In contrast, the fusion of influenza hemagglutinin-containing biosynthetic vesicles with the apical surface was shown to be independent of these components of the SNARE machinery (Ikonen *et al.*, 1995). This led to the hypothesis that only the basolateral plasma membrane domain utilizes the SNARE machinery, whereas fusion with the apical domain requires a different, so far unidentified machinery that might involve annexin 13b (Fiedler *et al.*, 1995). Recently, this view has been modified by the finding that the transport of basolaterally internalized IgA to both the basolateral and the apical surface was dependent on NSF and the t-SNARE, SNAP-25, or a SNAP-25-like protein in MDCK cells (Apodaca *et al.*, 1996). This suggests that the SNARE machinery is indeed also used at the apical surface, although possibly only for a subset of incoming vesicles. It is, however, unknown whether t-SNAREs are present at the apical surface in polarized epithelial cells and, if so, whether distinct apical and basolateral isoforms exist, as would be predicted by the SNARE hypothesis.

In the present study, we have investigated the expression and localization of syntaxin isoforms in MDCK cells, a polarized epithelial cell line. Each syntaxin isoform localized to distinct membrane compartments in agreement with the SNARE hypothesis. Of the three syntaxins found to be endogenously expressed by MDCK cells (syntaxins 2, 3, and 4), two isoforms (syntaxins 2 and 3) were found at the apical plasma membrane domain, demonstrating that this domain does possess at least this element of the SNARE machinery. Syntaxins 3 and 4 exhibited a mutually exclusive localization at the apical and basolateral surface, respectively, making them good candidates for t-SNAREs involved in the specific fusion of apically or basolaterally targeted transport vesicles.

MATERIALS AND METHODS

Materials

Cell culture media was obtained from Mediatech (Washington, DC), and the University of California, San Francisco, Cell Culture Facility. Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Hygromycin was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Transwell polycarbonate membrane filters and Transwell-COL collagen-coated membrane filters were purchased from Corning Costar (Cambridge, MA). Rat monoclonal antibody ascites against ZO-1 was obtained from Chemicon

(Temecula, CA). AC17, a mouse monoclonal antibody against a lysosomal membrane glycoprotein (Nabi *et al.*, 1991), was a kind gift from E. Rodriguez-Boulan (Cornell University Medical College, New York, NY). The anti-gp 135 antibody against an apical plasma membrane protein (Herzlinger and Ojakian, 1984) was a generous gift from G.K. Ojakian (State University of New York Health Science Center, Brooklyn, NY). The 12CA5 monoclonal antibody against the hemagglutinin (HA) epitope was kindly provided by I. Wilson (Scripps Institute, La Jolla, CA). The monoclonal antibody against E-cadherin, rr1, was kindly donated by B. Gumbiner (Memorial Sloan-Kettering, New York, NY). Rabbit polyclonal anti-syntaxin antibodies were generated against bacterially expressed syntaxin isoforms as described previously (Bennett *et al.*, 1992; Bennett *et al.*, 1993; Hackam *et al.*, 1996). All syntaxin antibodies were affinity purified before use. All syntaxin antibodies were isotype specific, except for the syntaxin 2 antibody which showed a weak cross-reactivity with syntaxin 1 by Western blot (Gaisano *et al.*, 1996). 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 Bio-Rad Laboratories (Hercules, CA). Dog tissues were purchased from Pel-Freez Biologicals (Rogers, AR). All other chemicals and reagents unless otherwise stated were from Sigma (St. Louis, MO).

DNA and Vectors

cDNAs encoding syntaxins 1A, 1B, 2, 3, and 4 have been described previously (Bennett *et al.*, 1992, 1993). All except syntaxins 1B and 3 were modified by the addition of a nine- amino acid influenza HA epitope tag at the N-terminus. These constructs were inserted into the pCB7 expression vector which contains a hygromycin resistance marker (Brewer and Roth, 1991).

Western Blots

Frozen dog and rat tissues were weighed, finely minced, and suspended in an equal volume of water containing a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml chymostatin, and 10 μg/ml antipain; all obtained from Chemicon). The tissues were homogenized in a Potter homogenizer and boiled for 10 min after the addition of SDS to a final concentration of 6%. The DNA was sheared by repeated passage through a 22-gauge needle. Debris was removed by centrifugation and the protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL). Protein A-Sepharose beads coupled to affinity-purified anti-syntaxin antibodies were incubated with 360 μg of protein in 500 μl of 0.1% Triton X-100 in phosphate-buffered saline. After an overnight incubation at 4°C, the beads were washed three times with mixed micelle buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5 mM EDTA, 8% sucrose, 0.1% NaN_3, 10 U/ml Trasylol, and 20 mM triethanolamine-HCl, pH 8.6) and once with final wash buffer (mixed micelle buffer without detergent). The immunoprecipitated proteins were eluted and separated on an 8% SDS-polyacrylamide gel, and the proteins were transferred onto Protran nitrocellulose filters (Schleicher and Schuell, Keene, NH). The protein bands were visualized by sequential incubations with the appropriate anti-syntaxin antibody followed by horseradish peroxidase-conjugated goat anti-rabbit IgG and an enhanced chemiluminescence system (ECL, Amersham Life Science, Arlington Heights, IL).

Cell Culture and Transfection

MDCK strain II cells were maintained in minimal essential medium, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2/95% air. For all experiments, the cells were cultured on 12-mm, 0.4-μm pore size Transwells. MDCK cells expressing the wild-type rabbit polymeric immunoglobulin (Ig) receptor (Breitfeld *et al.*, 1989) were transfected with the pCB7 constructs using the calcium phosphate method, followed by selection in media containing 250 μg/ml hygromycin as described in Breitfeld *et al.* (1989).

Clones were screened for syntaxin expression by Western blot and immunofluorescence microscopy. The polarity of all clones was verified by 1) their ability to form a regular monolayer of cells connected by undisturbed tight junctions as judged by confocal immunofluorescence microscopy with an antibody directed against the tight junction protein ZO-1 (Stevenson *et al.*, 1986); 2) the ability of a confluent cell layer grown on 12-mm Transwell filters to withstand the hydrostatic pressure of approximately 1 cm in an overnight "leak test"; and 3) measuring of the polarized, preferentially apical, secretion of the endogenous soluble protein gp80 in a pulse-chase experiment as described by Okamoto *et al.* (1992). Only those clones that passed these tests were investigated further.

Confocal Immunofluorescence Microscopy

Samples were fixed with 4% paraformaldehyde followed by sequential incubations with primary antibodies and fluorescein isothiocyanate- and/or Texas red-conjugated secondary antibodies. The samples were analyzed using a krypton-argon laser coupled with a Bio-Rad MRC600 confocal head attached to an Optiphot II Nikon microscope with a Plan Apo 60× 1.4 NA objective lens. For thick cryosections, the cells were grown on collagen filters, fixed in 4% paraformaldehyde, and cryosectioned longitudinally at −100°C using a Leica Ultracut E microtome equipped with an FC4E cryoattachment. Sections (0.5-μm thick) were stained with the appropriate antibodies and viewed by confocal microscopy.

RESULTS

Syntaxins 2, 3, and 4 Are Endogenously Expressed in MDCK Cells

It has been shown previously by Northern blot analysis that the syntaxin isoforms 2, 3, and 4 are all expressed in kidney (Bennett *et al.*, 1993). This raised the possibility that two or more of these proteins are expressed in a single, polarized cell type and might be involved in vesicle targeting to apical versus basolateral plasma membrane domains. Kidney-derived MDCK cells are a very well-characterized model cell line for a polarized epithelium. Virtually all possible polarized epithelial traffic pathways have been identified and analyzed in these cells (Mostov *et al.*, 1992; Mostov and Cardone, 1995; Mostov *et al.*, 1995). Therefore, we first determined whether different isoforms of the plasma membrane syntaxins are expressed in MDCK cells. Polyclonal antibodies raised against rat syntaxins (Bennett *et al.*, 1993) were tested for their ability to recognize their counterparts in canine cells. To maximize detection sensitivity and specificity, MDCK cell and tissue homogenates of liver, kidney, and brain from both rat and dog were solubilized with detergent, immunoprecipitated with each antibody, and analyzed by SDS-PAGE followed by immunoblotting with the immunoprecipitating antibody (Figure 1).

An antibody against syntaxin 1A/1B was able to recognize both dog and rat syntaxins in brain. As expected, the neuron-specific syntaxin 1 could not be

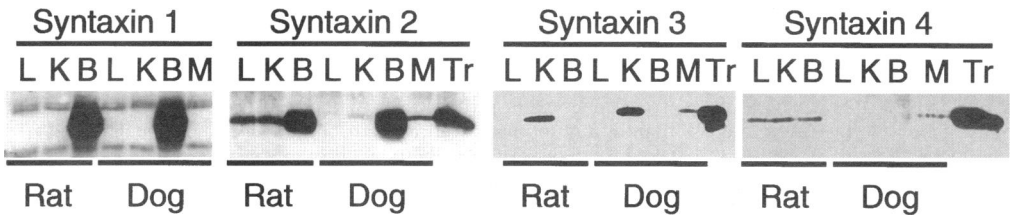

Figure 1. Analysis of endogenous syntaxin isoforms in MDCK cells. Total protein extracts from rat or dog liver (L), kidney (K), brain (B) and from MDCK cells (M) or MDCK cells transfected with syntaxins 2, 3, or 4 (Tr) were prepared. Equal amounts of protein were subjected to immunoprecipitation with antibodies directed against the rat syntaxins 1A/1B, 2, 3, or 4. The precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and detected with the appropriate antibody. Syntaxins 2, 3, and 4 could be detected in nontransfected MDCK cells. The signal of syntaxin 2, which can be seen for rat and dog brain, is due to a weak cross-reactivity of the antibody toward syntaxin 1 which is very abundant in brain (see MATERIALS AND METHODS).

detected in other tissues nor in MDCK cells. The syntaxin 3 antibody also strongly cross-reacted with the canine protein, whereas the antibodies against rat syntaxins 2 and 4 reacted only weakly with the canine homologues. Nevertheless, syntaxins 2, 3, and 4 could still be detected in MDCK cells and these isoforms were also present in kidney. The observed signal of syntaxin 2 in brain is due to a cross-reactivity of the antibody with syntaxin 1 (see MATERIALS AND METHODS). Syntaxins 2 and 4 but not syntaxin 3 were also expressed in liver. These data fit well with the previously reported tissue distribution based on Northern blot analysis where it has been shown that syntaxins 2 and 4 are widely expressed whereas syntaxin 3 expression was restricted to several tissues including spleen, lung, and kidney (Bennett *et al.*, 1993).

MDCK cells therefore endogenously express syntaxins 2, 3, and 4 or closely related homologues of these t-SNAREs. The presence of three different syntaxins in a single, clonal cell population is highly suggestive that these syntaxins may serve different vesicular trafficking pathways.

Syntaxin Isoforms Are Differentially Localized within Polarized MDCK Cells

Although syntaxins 2, 3, and 4 were detected in MDCK cells, their endogenous level of expression was too low to allow reliable localization studies by confocal microscopy. Constructs for the expression of syntaxins 2, 3, and 4 were therefore transfected individually into MDCK cells and stable clones isolated. Because the apical/basolateral polarity of epithelial cells in many ways parallels the axonal/somatodendritic polarity of neurons (Rodriguez-Boulan and Powell, 1992), we were interested in how the axonal syntaxins 1A and 1B would be distributed in MDCK cells. Therefore, we also transfected MDCK cells with cDNAs encoding these syntaxins. Several independent cell lines expressing each syntaxin isoform in various amounts were analyzed as a precaution against clonal variation. The polarity of each individual clone was verified by analyzing the polarized secretion of the endogenous soluble protein gp80, and the integrity of the monolayers was confirmed microscopically and by investigating their tightness against hydrostatic pressure (see MATERIALS AND METHODS).

The syntaxins were localized by confocal immunofluorescence microscopy using isotype-specific antisera. Since the cDNA constructs encoding syntaxins 1A, 2, and 4 were engineered to include a nine-amino acid influenza HA epitope tag (Bennett *et al.*, 1993), detection of these proteins was also possible utilizing the monoclonal antibody 12CA5. Studies using the epitope tag for detection gave results that were indistinguishable from experiments using the isotype-specific antibodies. No signal was observed in nontransfected cells. These results confirm that the observed immunofluorescent staining is specific. Cells were grown on permeable filters under conditions where they differentiate into a highly polarized columnar epithelium. For a better definition of the intracellular location of each syntaxin, the cells were costained for ZO-1, a marker for the tight junction, which separates the apical from the basolateral plasma membrane. The nuclei were visualized by propidium iodide staining.

Syntaxin 1A was associated mainly with intracellular vesicular structures distributed throughout the cytoplasm of the cell (Figure 2). Only cells expressing a very high amount of syntaxin 1A showed some staining on the basolateral plasma membrane. Identical results were obtained with syntaxin 1B. Each of the three endogenously expressed syntaxins displayed distinct localization patterns (Figure 2). Syntaxin 2 appeared almost exclusively on both the apical and basolateral plasma membranes, with very little intracellular staining. In contrast, the majority of syntaxin 3 was localized to the apical plasma membrane, but was not evident at the basolateral plasma membrane (compare also with Figures 3, 5, and 6). In addition, some syntaxin 3 staining was detected on vesicular structures throughout the cytoplasm (see below). Finally, syntaxin 4 was almost exclusively detected on the basolateral plasma membrane, with little or no intracellular

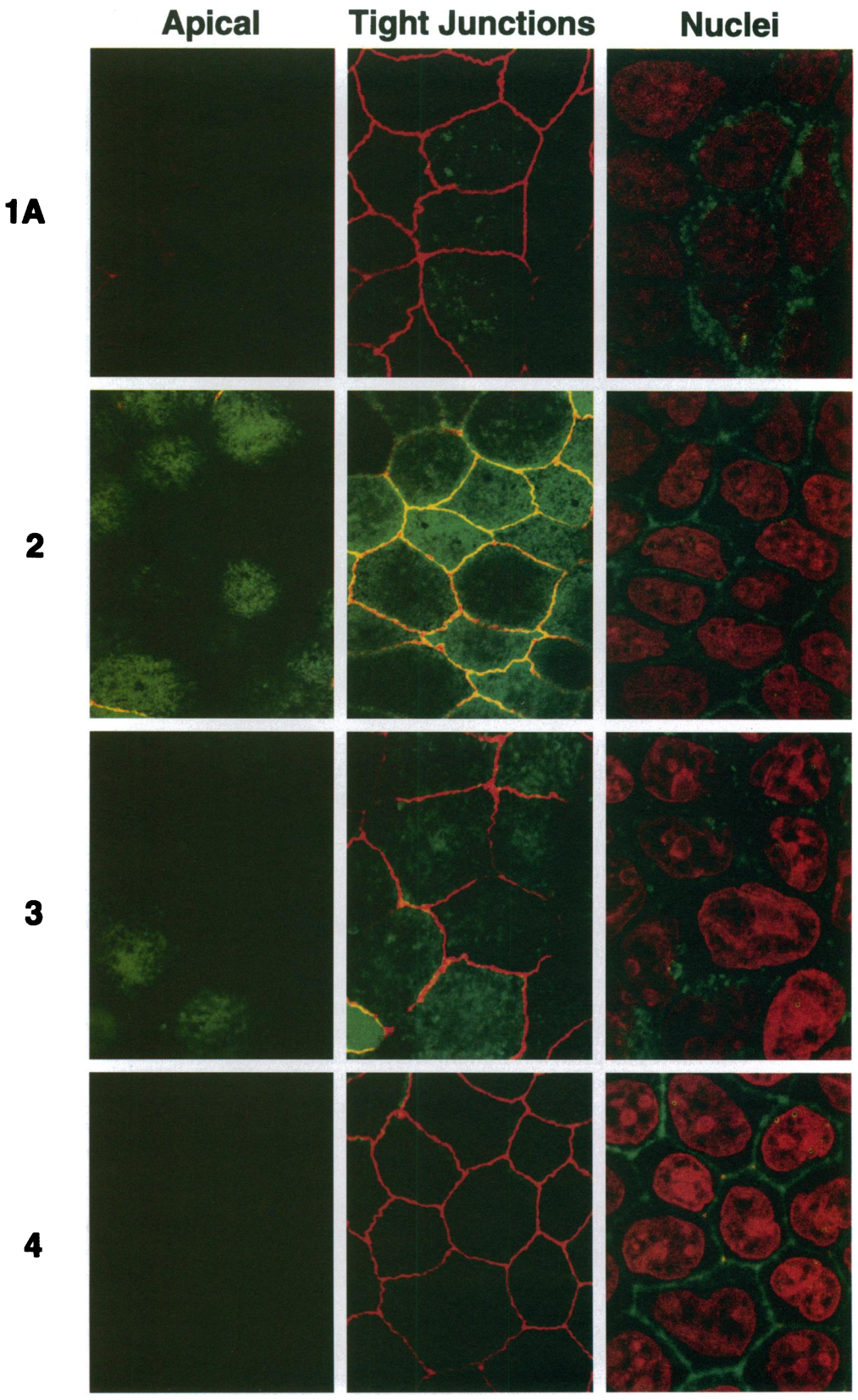

Figure 2. Localization of transfected syntaxin isoforms in MDCK cells: horizontal optical sections. MDCK cells expressing syntaxins 1A, 2, 3, or 4, as indicated on the left, were grown on filters to form a polarized monolayer, fixed, and stained immunocytochemically for the appropriate syntaxin isoform (green). For a better definition of the apical versus basolateral plasma membrane, the cells were costained for the tight junction protein ZO-1 (red) and the nuclei were stained with propidium iodide (red). The cells were investigated by confocal fluorescence microscopy and three consecutive optical sections are shown for each clone. The panels on the left show optical sections through the very apical region of the cells just above the tight junctions. The middle and right panels represent optical sections at the levels of the tight junctions and the nuclei, respectively.

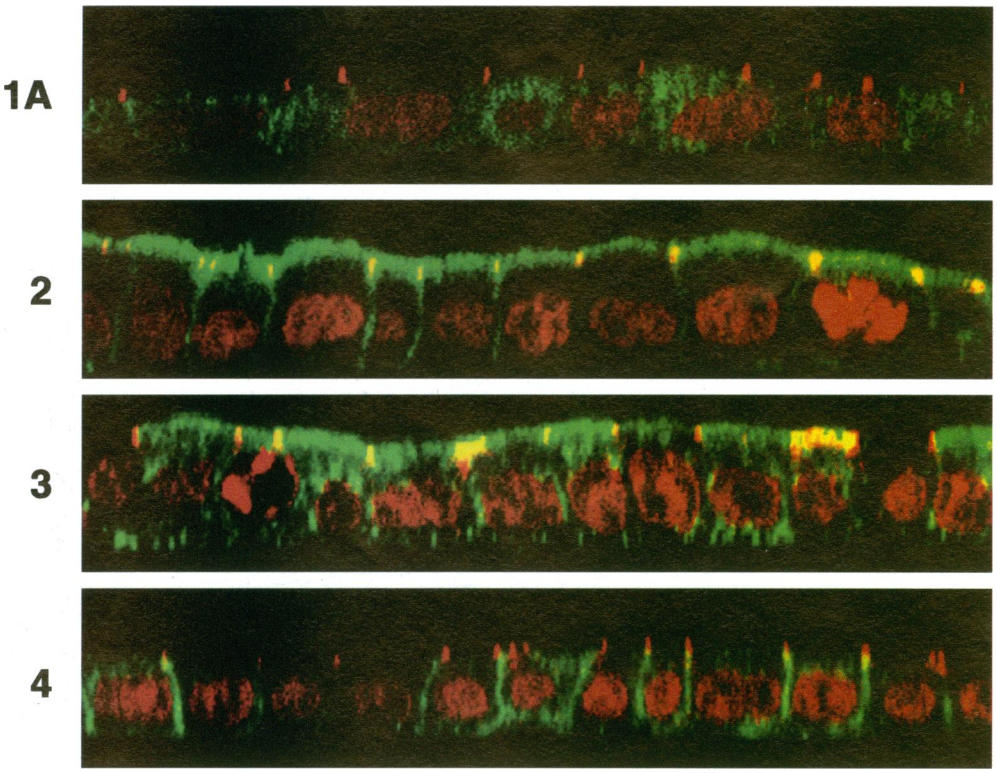

Figure 3. Localization of transfected syntaxin isoforms in MDCK cells: vertical optical sections. Syntaxin-expressing MDCK cells were treated and stained exactly as in Figure 2. Confocal sections in the vertical plane are shown with the apical side of the cells at the top. The tight junctions can be seen as red or yellow dots between neighboring cells.

staining. For comparison, Figure 3 shows vertical optical sections through four MDCK cell lines, each expressing a different syntaxin isoform. It is very clear that each syntaxin is uniquely localized in polarized MDCK cells. Most strikingly, syntaxins 3 and 4 have virtually nonoverlapping distributions.

Colocalization Studies with Basolateral and Apical Plasma Membrane Markers

The localization of syntaxins 2 and 4 on the basolateral plasma membrane was confirmed by double-label immunofluorescence with the basolateral membrane marker E-cadherin. Cells grown on filters were fixed, permeabilized, and incubated with polyclonal rabbit antibody against syntaxin and mouse anti-E-cadherin monoclonal antibody. As seen in Figure 4, the staining for syntaxins 2 and 4 colocalized precisely with that of E-cadherin.

To verify the apical plasma membrane localization of syntaxins 2 and 3, the cells were double labeled with an antibody against gp135, a 135-kDa glycoprotein that is associated with the MDCK apical plasma membrane (Ojakian and Schwimmer, 1988). However, the resolution of the confocal microscope is only approximately 0.6 μm in the vertical (i.e., Z) axis perpendicular to the plane of the monolayer, but much better (~0.2 μm) in the horizontal (i.e., X-Y) plane of the monolayer. This, coupled with the microvilli found on the apical plasma membrane, makes it difficult to distinguish between true plasma membrane localization and localization in vesicles immediately beneath the apical plasma membrane. We have, for instance, previously reported that IgA being transcytosed by the polymeric Ig receptor can accumulate in the apical recycling compartment, which consists of vesicles and tubules often lying within 1 μm of the apical plasma membrane (Apodaca *et al.*, 1994). To overcome this limitation, 0.5-μm-thick frozen sections were cut perpendicular to the plane of the monolayer. These physically cut sections were then examined in a single X-Y plane by confocal immunofluorescence microscopy. This technique circumvents the normally poor Z-axis resolution when using conventional confocal microscopy with intact filters. Figure 5 shows that the staining for syntaxins 2 and 3 coincided significantly with the apical plasma membrane marker gp135. The fuzzy appearance of the apical surface is due to the presence of microvilli on the apical surface of MDCK cells.

These colocalization studies demonstrate that the observed apical or basolateral staining of syntaxins 2, 3, and 4 is indeed due to a plasma membrane localization for these t-SNAREs.

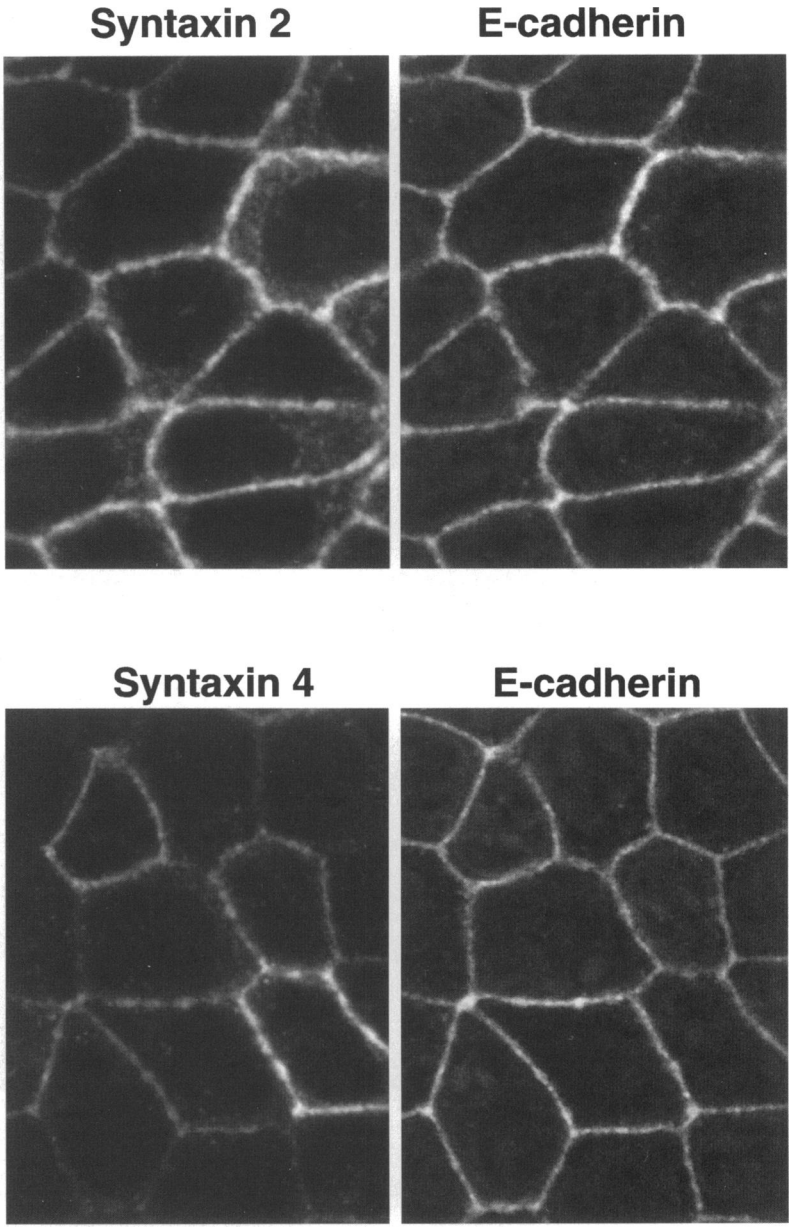

Figure 4. Syntaxins 2 and 4 colocalize with E-cadherin at the lateral plasma membrane. MDCK cells expressing syntaxin 2 or 4 were stained for the respective syntaxin (left panels) and costained for the lateral plasma membrane marker E-cadherin (right panels). The cells were examined by confocal fluorescence microscopy, and optical sections at the level of the nuclei are shown.

Identification of the Intracellular Structures Stained by Syntaxins 1A and 3

Although syntaxin 3 staining was concentrated on the apical plasma membrane, a clearly detectable fraction of syntaxin 3 was also observed on relatively large intracellular vesicles well below the tight junctions and surrounding the nucleus. The relative amount of this intracellular staining was higher in clones expressing high levels of syntaxin 3. To identify these intracellular vesicular structures, the syntaxin 3-overexpressing cells were colabeled with various subcellular markers. No significant colocalization was detected when the cells were costained for either the polymeric Ig receptor (Figure 6A), which is present mostly on endosomes and the basolateral plasma membrane, or the transferrin receptor (Figure 6B), an endosomal marker protein. Syntaxin 3 also failed to colocalize with internalized IgA or transferrin. However, when the syntaxin 3-expressing cells were colabeled with AC17, an antibody against a lysosomal membrane glycoprotein (Nabi *et al.*, 1991), the syntaxin 3 staining in the cytoplasm surrounding the nucleus overlapped extensively (Figure 6, C and E). This suggests that an overexpression of syntaxin 3 might lead to a mislocalization to lysosomes whereas the typical localiza-

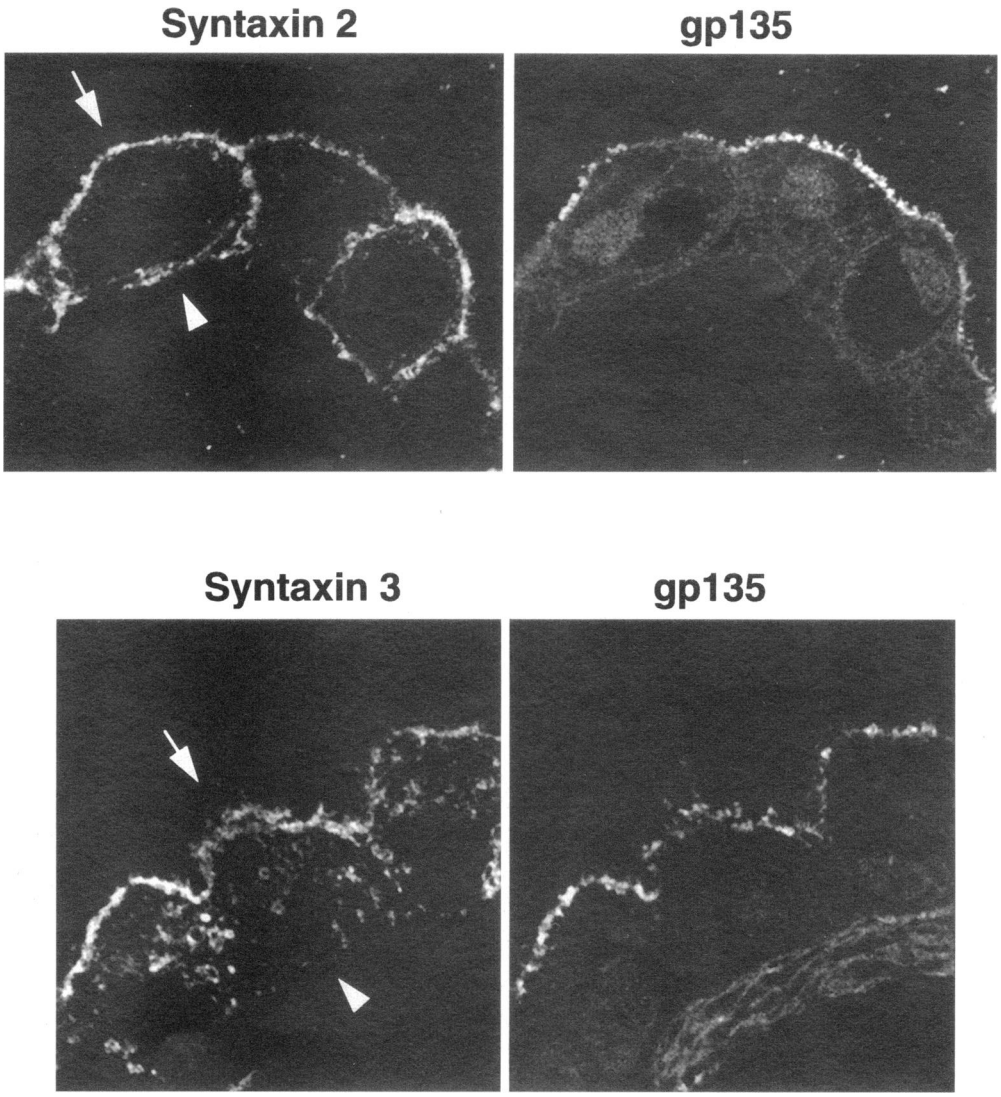

Figure 5. Syntaxins 2 and 3 colocalize with gp135 at the apical plasma membrane. Thick cryosections of MDCK cells expressing syntaxin 2 or 3 were prepared, stained for the respective syntaxin (left panels), and costained for the apical plasma membrane marker gp135 (right panels). The sections were investigated by confocal fluorescence microscopy. This technique allows the investigation of the apical-basal axis of the cells at a higher resolution than with whole cells on filters. The colocalization of the syntaxins and gp135 at the apical surface (arrows) can be clearly seen. The fuzziness of the apical surface results from the numerous microvilli. The arrowheads mark the basal cell surface.

tion, as observed with lower expressing clones, is the apical plasma membrane.

Syntaxin 1 was found to localize entirely to intracellular structures. To define these structures, we performed the same costaining analysis as described above. Syntaxin 1A did not significantly colocalize with any of the endosomal marker proteins. However, we found again an almost complete overlap with the lysosomal marker AC17 (Figure 6D). This result suggests that syntaxin 1A, which is not normally expressed in MDCK cells, cannot be correctly targeted in these cells and is instead delivered to the lysosomes.

DISCUSSION

We have examined the expression and localization of several syntaxin isoforms in a single, polarized epithelial cell line. We chose MDCK cells because they have been the most widely used model for analyzing polarized epithelial cell membrane trafficking. We found that three broadly expressed syntaxins (syntaxins 2, 3, and 4) are endogenously present in MDCK cells, raising the possibility that each may selectively serve one or more of the multiple pathways leading to the apical or basolateral plasma membrane. Consistent with this possibility, each of the syntaxins had a distinct pattern of localization in MDCK cells. Syntaxin 2 was found on the apical and basolateral surfaces, whereas syntaxin 4 and the plasma membrane fraction of syntaxin 3 were present exclusively on the basolateral or apical surface, respectively. Surprisingly, both syntaxins 1A and 1B, neither of which is endogenously expressed in MDCK cells, were almost entirely intracellular in dis-

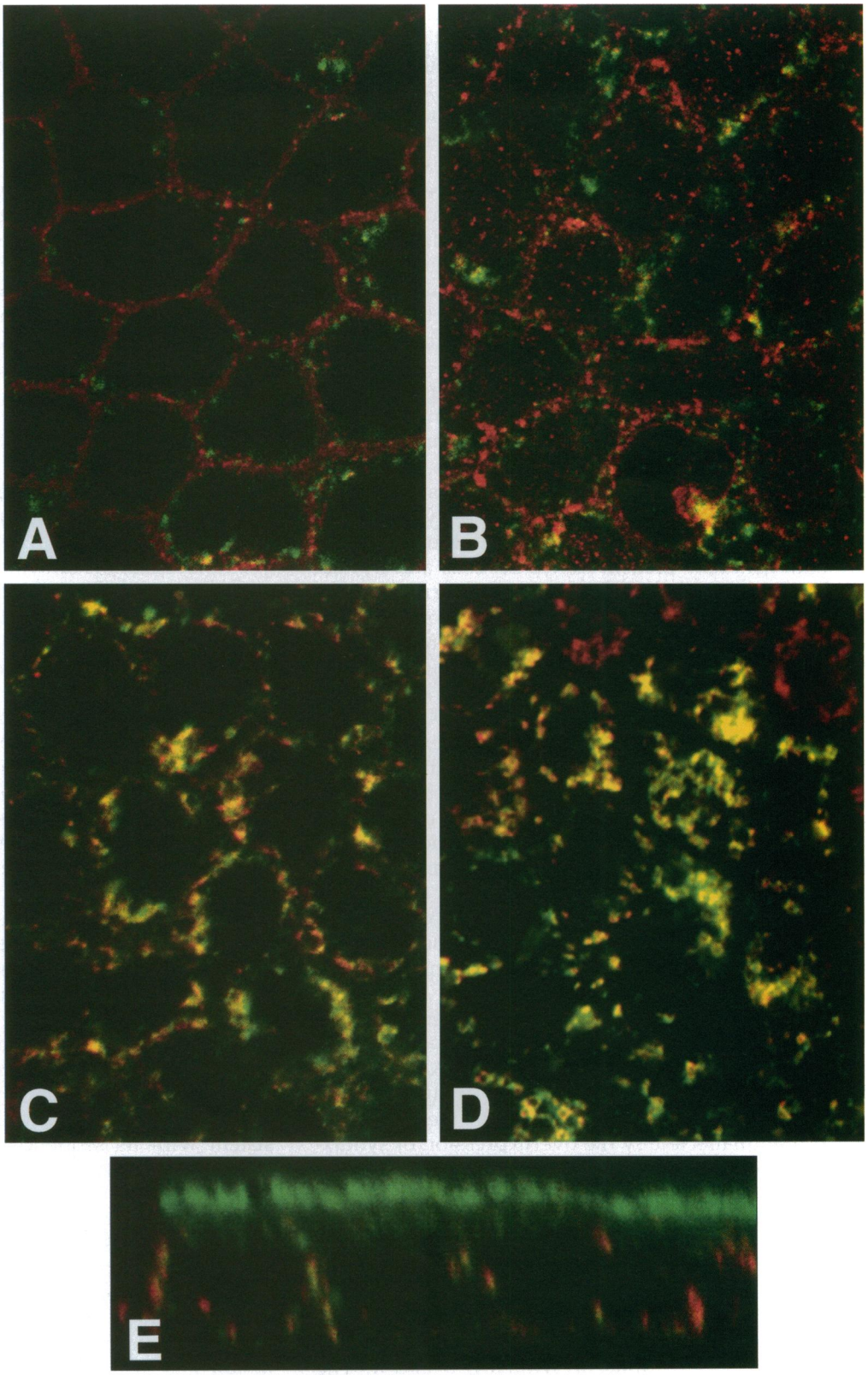

Figure 6. The intracellular component of syntaxin 3 and syntaxin 1A colocalize with lysosomes. MDCK cells expressing syntaxin 3 (A–C and E) or syntaxin 1A (D) were stained for syntaxin 3 or 1A, respectively (green), and costained for either the polymeric Ig receptor (A, red), the transferrin receptor (B, red), or the lysosomal protein AC17 (C–E, red) and investigated by confocal microscopy. Horizontal optical sections through the level of the nuclei are shown in A–D and a vertical section is shown in E. Neither the pIgR nor the TfR, which reside in endosomes and at the basolateral plasma membrane, colocalize significantly with syntaxin 3. However, the AC17 staining overlapped extensively with the staining for syntaxin 3 as well as syntaxin 1A inside the cell. On the contrary, the syntaxin 3 staining at the apical surface is devoid of any lysosomal signal, confirming the localization of syntaxin 3 at the apical plasma membrane.

tribution. These observations, in particular the mutually exclusive plasma membrane distributions of syntaxins 3 and 4, strongly support the possibility that the different syntaxin isoforms participate in different plasma membrane-directed vesicular trafficking pathways. These results are in good agreement with the central prediction of the SNARE hypothesis that different membrane compartments should be characterized by a distinct subset of t-SNAREs. Moreover, the finding that syntaxins 2 and 3 are localized to the apical surface demonstrates that this membrane indeed does possess at least this component of the SNARE machinery and makes it unlikely that vesicular fusion with this surface is only mediated by an alternative machinery.

Experimental expression of a protein in a cell always carries a risk that the protein will be mislocalized. However, several reasons make it likely that the localization data presented in the present study reflects the bona fide localization of the endogenous syntaxins in MDCK cells. First, with the exception of syntaxins 1A and 1B (considered below), the three other syntaxins are endogenously present in MDCK cells and it must be assumed that the machinery for the correct sorting of these proteins exists in these cells. Second, MDCK cells have been extensively used to express a wide variety of both epithelial and nonepithelial proteins. These exogenous proteins have generally been found to localize and function correctly in MDCK cells. Even a variety of neuronal proteins have been expressed in MDCK cells where their localization relative to neurons has demonstrated a correspondence between localization to axons and the apical surface of epithelia, and between a somatodendritic localization and the basolateral surface (Rodriguez-Boulan and Powell, 1992). Finally, and most important, each of the syntaxin isoforms gave a distinct localization pattern. This is precisely what one would expect for proteins that are localized by highly specific mechanisms and serve correspondingly specific roles.

It should also be pointed out that the overexpression of these syntaxin isoforms did not grossly disrupt cell polarity, as is clear from Figures 2 and 3. Although we have not performed a comprehensive analysis of each step in membrane traffic, two pathways were found to be largely unaffected by the overexpression of syntaxins 2, 3, and 4: the polarized, mostly apical, secretion of the endogenous protein gp80 and the transcytosis of IgA mediated by the polymeric Ig receptor. Therefore, it is unlikely that the overexpression of the syntaxin isoforms caused a major disturbance in the polarity of the MDCK cells.

We do not know how the syntaxins achieve their distinct polarized localizations, particularly the nonoverlapping localizations of syntaxins 3 and 4. The simplest explanation for the basolateral localization of syntaxin 4 is that its cytoplasmic domain contains a basolateral targeting signal (Mostov and Cardone, 1995). Several mechanisms could account for the sorting of syntaxin 3 to the apical plasma membrane. The apical localization of syntaxin 3 may be due to its lack of a basolateral targeting signal, leading perhaps to apical targeting by default. A second possibility is that syntaxin 3 is included in detergent-insoluble glycosphingolipid rafts that have been proposed to be involved in apical targeting (Simons and Wandinger-Ness, 1990). However, we have been unable to find any evidence for the partitioning of the apical syntaxins 2 and 3 into these glycosphingolipid domains. A third mechanism involving a lumenal carbohydrate signal (Fiedler and Simons, 1995) is implausible, since syntaxins have virtually no lumenal domain and are not glycosylated. Alternatively, since syntaxins may be a fundamental part of the machinery responsible for establishing and maintaining cell polarity, they might be sorted to their appropriate plasma membrane domain by a different mechanism, e.g., by retention due to association to cytoskeletal elements (Drubin and Nelson, 1996).

The localization of syntaxin 1 was found to be exclusively intracellular. Although early work on syntaxin 1 demonstrated that it is a plasma membrane protein (Bennett *et al.*, 1992, 1993), some endogenous syntaxin 1 is also present on synaptic vesicles and chromaffin granules (Koh *et al.*, 1993; Schulze *et al.*, 1995; Walch-Solimena *et al.*, 1995). Thus, it is reasonable to assume that syntaxin 1 normally recycles between the plasma membrane and endosomes. We were, however, unable to identify the syntaxin 1A-positive compartments as endosomes. Instead, we found that syntaxin 1A colocalizes almost completely with the lysosomal marker protein AC17. Since syntaxin 1 is not normally expressed in MDCK cells, this finding therefore suggests that these cells lack the proper machinery to target syntaxin 1 to the plasma membrane, either by an inefficient plasma membrane delivery, retention, or recycling. We do not know whether syntaxin 1A is directly targeted to lysosomes in MDCK cells or whether it is delivered to the plasma membrane first and then transported to lysosomes.

Our results represent the first demonstration that syntaxin 3 is localized to the plasma membrane, consistent with its sequence similarity to other plasma membrane syntaxins. However, unlike syntaxins 2 and 4, syntaxin 3 was also detectable on intracellular membranes that colocalized with a lysosomal membrane marker, especially in clones with high syntaxin 3 expression levels. This localization may simply be the result of overexpression and saturation of the cellular machinery responsible for syntaxin 3 sorting or retention. Alternatively, syntaxin 3 may participate in fusion among intracellular vesicles. Consistent with this possibility, syntaxin 3 (but not coexpressed syntaxin 2 or 4) has been localized to zymogen granules in

pancreatic acinar cells (Gaisano *et al.*, 1996), recycling tubulovesicles in gastric parietal cells (Bennett, manuscript in preparation) and secretory granules in eosinophils (Bennett, unpublished data). Recent data indicate that under certain circumstances, NSF acts solely on transport vesicles, prior to docking or fusion of these vesicles with their target (Mayer *et al.*, 1996; Steel *et al.*, 1996). This raises the possibility that v-SNAREs and t-SNAREs may interact on the same transport vesicle. This novel hypothesis is consistent with our finding of syntaxins 1A and 1B and a fraction of syntaxin 3 in intracellular compartments.

The plasma membrane localization of syntaxin isoforms 2, 3, and 4 in MDCK cells suggests that their normal function may lie, at least in part, in the plasma membrane fusion of transport vesicles. Syntaxin 2, which is present at both the apical and basolateral surface, might act as a t-SNARE for vesicles which are targeted in a nonpolarized way. In contrast, the presence of syntaxins 3 and 4 on either the apical or basolateral surface, respectively, strongly suggests that they are involved in polarized vesicular transport pathways to these surfaces. It is interesting that syntaxin 3 is not expressed in adipocytes (Volchuk *et al.*, 1996), which do not possess an apical surface, or in hepatocytes (Figure 1; Bennett *et al.*, 1993), which have an apical plasma membrane but lack a direct pathway from the TGN to this surface (Hubbard, 1991). Together, this might suggest that syntaxin 3 functions at the apical surface of polarized cells and could be potentially involved in the biosynthetic pathway.

Recent studies have found that TGN to apical plasma membrane delivery is insensitive to toxins that cleave VAMP (Ikonen *et al.*, 1995). However, the role of t-SNAREs in this transport step was not examined. It is therefore possible that an apical syntaxin, such as syntaxin 2 or 3, plays a role in apical vesicle targeting. In this case, a so far unidentified v-SNARE, one that is insensitive to the highly specific VAMP cleaving toxins used by Ikonen *et al.* (1995), would be the syntaxin 2 or 3 binding partner. Note that VAMP has been shown to selectively interact in vitro with syntaxin 4 but not with syntaxins 2 and 3 (Calakos *et al.*, 1994). The latter observation may explain the lack of VAMP involvement in apical transport, whereas the former observation is consistent with a role for the basolaterally localized syntaxin 4 in TGN to basolateral surface delivery. TGN to apical surface transport was also found to be insensitive to NSF and αSNAP (Ikonen *et al.*, 1995). It is possible that the putative SNARE complex containing the apical syntaxin 2 or 3 recruits an NSF homologue, such as p97 (Acharya *et al.*, 1995; Rabouille *et al.*, 1995), to promote membrane fusion. This would certainly not rule out the proposed involvement of annexin 13b in apical vesicle delivery (Fiedler *et al.*, 1995). Transcytosis to the apical surface has been shown to be dependent, at least in part, on NSF and a substrate that is cleaved by botulinum E toxin, most likely a homologue of SNAP-25 (Apodaca *et al.*, 1996). It therefore seems reasonable that apical transcytosis will depend on a syntaxin, although not necessarily the same syntaxin utilized for TGN to apical transport.

Syntaxins represent the first family of molecules that are part of the membrane fusion machinery and whose isoforms are differentially localized to the apical and basolateral surfaces of polarized cells. This observation is consistent with the prospect that syntaxins function in different membrane trafficking pathways and may contribute to the specificity of transport vesicle targeting in accordance with the SNARE hypothesis. The extensive base of information and tools for studying membrane traffic in MDCK cells makes this an ideal system to investigate this possibility.

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