

# Syntaxins 3 and 4 Are Concentrated in Separate Clusters on the Plasma Membrane before the Establishment of Cell Polarity

Seng Hui Low,<sup>\*†</sup> Amit Vasanthi,<sup>‡</sup> Jayasri Nanduri,<sup>§||</sup> Min He,<sup>§</sup> Nikunj Sharma,<sup>\*¶</sup> Michelle Koo,<sup>§</sup> Judith Drazba,<sup>‡</sup> and Thomas Weimbs<sup>\*†</sup>

<sup>\*</sup>Department of Molecular, Cellular, and Developmental Biology and <sup>†</sup>Neuroscience Research Institute, University of California, Santa Barbara, Santa Barbara, CA 93106-9610; <sup>‡</sup>Imaging Facility and <sup>§</sup>Department of Cell Biology, Lerner Research Institute, The Cleveland Clinic, Cleveland, OH 44195; and <sup>¶</sup>Department of Biological, Geological, and Environmental Sciences, Cleveland State University, Cleveland, OH 44115

Submitted May 31, 2005; Revised October 27, 2005; Accepted November 29, 2005  
Monitoring Editor: Sean Munro

**Syntaxins 3 and 4 localize to the apical and basolateral plasma membrane, respectively, of epithelial cells where they mediate vesicle fusion. Here, we report that before establishment of cell polarity, syntaxins 3 and 4 are confined to mutually exclusive, submicron-sized clusters. Syntaxin clusters are remarkably uniform in size, independent of expression levels, and are distinct from caveolae and clathrin-coated pits. SNAP-23 partially colocalizes with both syntaxin 3 and 4 clusters. Deletion of the apical targeting signal of syntaxin 3 does not prevent sorting into clusters away from syntaxin 4. Syntaxin 3 and 4 cluster formation depends on different mechanisms because the integrity of syntaxin 3 clusters depends on intact microtubules, whereas syntaxin 4 clusters depend on intact actin filaments. Cholesterol depletion causes dispersion of syntaxin 3 but not syntaxin 4 clusters. In migrating cells, syntaxin clusters polarize to the leading edge, suggesting a role in polarized exocytosis. These results suggest that exocytosis occurs at small fusion sites exhibiting high local concentrations of SNARE proteins that may be required for efficient membrane fusion. The establishment of separate clusters for each syntaxin suggests that the plasma membrane is inherently polarized on an ultrastructural level even before the establishment of true cell polarity.**

## INTRODUCTION

Polarity is a hallmark of most eukaryotic cells in both single and multicellular organisms. Epithelial cells are prime examples of cell types whose function critically depends on the polarized distribution of membrane proteins and lipids (Mostov *et al.*, 2003; Spiliotis and Nelson, 2003; Rodriguez-Boulan *et al.*, 2005). The plasma membrane of epithelial cells is subdivided into apical and basolateral domains, each of which typically faces very different environments and carries out different functions. Membrane trafficking pathways are used to sort plasma membrane proteins and to target them to their respective domain, thereby establishing and maintaining epithelial cell polarity. A critical component of all membrane trafficking pathways is the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) membrane fusion machinery that accomplishes the final fusion of a transport carrier with its target membrane (Chen and Scheller, 2001; Jahn *et al.*, 2003; Ungar and Hughson, 2003). SNARE proteins seem to have a dual function: they carry out the mechanics of membrane lipid bilayer

fusion and play a role in the determination of the specificity of fusion reactions. The latter is because of the property that only appropriate combinations of SNARE proteins on vesicle and target membranes leads to successful fusion (McNew *et al.*, 2000). Indeed, different SNAREs localize specifically to only certain organelles or classes of transport carriers.

The apical and basolateral plasma membrane domains of polarized epithelial cells contain two different members of the syntaxin family of SNARE proteins. Syntaxin 3 localizes to the apical and syntaxin 4 to the basolateral domain of the Madin-Darby canine kidney (MDCK) renal epithelial cell line (Low *et al.*, 1996). The same polarity also is observed in a number of other epithelial cell lines and in epithelial cells in vivo (Gaisano *et al.*, 1996; Weimbs *et al.*, 1997; Li *et al.*, 2002; Low *et al.*, 2002). The highly conserved polarity of syntaxins 3 and 4 indicates that their functions are important for polarized trafficking and epithelial polarity in general. Syntaxin 3 functions in trafficking of newly synthesized proteins from the *trans*-Golgi network (TGN) to the apical membrane as well as in apical recycling (Low *et al.*, 1998a). Conversely, syntaxin 4 functions in TGN-to-basolateral trafficking (Lafont *et al.*, 1999).

An important question is how epithelial cell polarity is established or lost. The former occurs during development and wound healing, whereas the latter is a critical feature of carcinogenesis. Carcinomas, epithelial-derived malignant tumors, undergo loss of cell polarity during progression to an invasive and metastatic phenotype. We have previously reported that syntaxins 3 and 4 relocalize to separate intra-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-05-0462>) on December 7, 2005.

<sup>||</sup> Present address: Department of Pathology, Case Western Reserve University, Cleveland, OH 44106.

Address correspondence to: Thomas Weimbs (weimbs@lifesci.ucsb.edu).

cellular vacuoles under conditions in which epithelial cells are prevented from establishing a polarized morphology (Low *et al.*, 2000). Other apical proteins are redirected into syntaxin 3-containing vacuoles, whereas basolateral proteins are redirected into syntaxin 4-containing vacuoles. Importantly, even during the complete absence of cell polarity, syntaxins 3 and 4 maintain a mutually exclusive intracellular distribution. It was previously shown that even nonpolarized, nonepithelial cells have the capability of sorting cargo proteins containing apical and basolateral targeting signals in the TGN and delivering them on separate routes to the plasma membrane (Musch *et al.*, 1996; Yoshimori *et al.*, 1996; Keller *et al.*, 2001). This suggests that "apical" and "basolateral" sorting mechanisms are not only of importance in epithelial cells but are also used by other cell types, even nonpolarized cells.

In our previous analysis, we found that even in completely nonpolarized MDCK cells, a fraction of syntaxins 3 and 4 remains on the plasma membrane (Low *et al.*, 2000). Furthermore, delivery of newly synthesized membrane and secretory proteins to the plasma membrane is ongoing under these conditions at approximately the same level as in polarized cells (Low *et al.*, 2000). We have now investigated this fraction of syntaxins 3 and 4 on the plasma membrane of nonpolarized cells and report that both syntaxins localize to submicron-sized, uniform clusters on the membrane. The syntaxin 3 and 4 clusters are mutually exclusive, which suggests that even nonpolarized cells maintain a "polarity" of their fusion machineries on an ultrastructural level. Cluster size and density is remarkably uniform and independent of syntaxin expression levels, suggesting that they are maintained by cellular mechanisms. We found that the integrity of syntaxin 3 clusters depends on an intact microtubule network and can be disrupted by cholesterol depletion. In contrast, syntaxin 4 clusters are independent of microtubules or cholesterol but depend on an intact actin cytoskeleton. In migrating fibroblasts, both syntaxin 3 and 4 clusters are polarized toward the leading edge, suggesting that fusion sites can be established and restricted at particular sites of the plasma membrane during cell polarization.

## MATERIALS AND METHODS

### Reagents and Antibodies

Affinity-purified polyclonal antibodies against the cytoplasmic domains of syntaxins and against a C-terminal peptide of human SNAP-23 have been described previously (Low *et al.*, 2000). The 9E10 anti-myc monoclonal antibody (mAb) was obtained from American Type Culture Collection (Manassas, VA). The anti-caveolin antibody was purchased from BD Biosciences (Billerica, MA). Antibodies against the clathrin heavy chain were a kind gift from Frances Brodsky (Hooper Foundation, University of California, San Francisco, San Francisco, CA). Alexa Fluor 594-phalloidin was from Molecular Probes (Eugene, OR). Secondary antibodies, cross-absorbed against multiple species and conjugated to fluorescein isothiocyanate (FITC) or Texas Red were from Jackson ImmunoResearch Laboratories (West Grove, PA).

### Expression of Epitope-tagged Syntaxin 3 and 4

Two myc epitope-tags in tandem followed by a His-tag were added to the C termini of human syntaxin 3 and 4 to study the surface expression of these proteins. The rationale for the tandem tag is that the first tag will serve as a spacer for the second to prevent possible steric interference to antibody binding too close to the membrane. We have modified the vector pcDNA4/TO/myc-His (Invitrogen, Carlsbad, CA) for tetracycline-regulatable mammalian expression to include a second myc-tag. The construction of adenoviruses encoding myc-tagged syntaxin 3 and 4 was done as described previously (He *et al.*, 1998). For virus infection, the cells were plated on coverslips and allowed to adhere for 2 h. They were then washed once with Opti-MEM and incubated with the virus diluted in Opti-MEM for 2 h. An equal volume of minimal essential medium (MEM) containing 20% fetal bovine serum (FBS) was added, and the infection was allowed to continue for 9 h. Surface-staining was then performed as described below.

### Cell Culture and Transfection of MDCK Cells

MDCK clones stably expressing hemagglutinin (HA)-tagged syntaxin 2 (Low *et al.*, 1996), SNAP-23 (Low *et al.*, 1998b), or myc-tagged syntaxins 3 or 4 (Kreitzer *et al.*, 2003) have been described previously. For all experiments, the cells were plated at low density on coverslips in 12-well tissue culture plates and grown for 16 h to study cells in a nonpolarized state. Expression of the SNAREs was induced by the addition of doxycycline for 16 h. For transient transfection experiments, the cDNAs encoding syntaxin 3 or 4 were transfected using Exgen 500 (Fermentas, Hanover, MD). For infection of MDCK cells with adenovirus expressing p75NTR and LDL receptor (LDLR), stable MDCK cells expressing syntaxin 3 or 4 were plated at low density on coverslips for 2 h. The cells were then infected with either p75NTR or LDLR expressing adenoviruses (kindly provided by Drs. A. Musch and E. Rodriguez-Boulant (Department of Cell Biology, Cornell University Medical School, New York, NY) diluted in Opti-MEM for 2 h. An equal volume of MEM containing 20% FBS was then added, and the cells were incubated for an additional 12 h. Syntaxin 4 cells were surface stained with 9E10, permeabilized, and followed by a rabbit anti-LDLR antibody. Syntaxin 3 cells were permeabilized and stained with a rabbit anti-syntaxin 3 antibody and a monoclonal anti-p75NTR antibody.

### Immunofluorescence Microscopy

Cells were plated on 12-mm coverslips and cultured for 16 h in the presence of doxycycline to induce expression of myc-tagged syntaxins. Cells were washed twice with medium and incubated with the anti-myc mAb on ice for 1 h with gentle shaking. Excess unbound antibody was washed away, and the cells were fixed with 4% formaldehyde for 30 min on ice, followed by permeabilization with 0.2% Triton X-100 and secondary antibody staining. For localizing simultaneously two proteins recognized by mouse primary antibodies (caveolin and myc), FITC-labeled F<sub>ab</sub> fragments of the secondary antibody (Jackson ImmunoResearch Laboratories) were used after incubation with the first mouse primary antibody. The cells were briefly fixed again with 4% paraformaldehyde and then incubated with the second primary antibody, followed by Texas Red-labeled secondary antibody.

Clusters were visualized using a PL Fluotar 100×, 1.3 numerical aperture oil immersion lens on a Leica DMLM fluorescence microscope. Digital images were captured using an Optronics MagnaFire digital camera and a 1.5 or 2× magnifying lens. Using Adobe Photoshop software (Adobe System, Mountain View, CA), histograms were linearly adjusted for optimal representation of the 8-bit signals, individual channels were overlaid in RGB images, and composites of panels were made for final figures.

### Nocodazole Treatment to Depolymerize Microtubules

MDCK cells stably expressing syntaxin 3 or 4 were cultured on coverslips for 16 h and incubated in bicarbonate-free MEM supplemented with 5% FBS and 20 mM HEPES on ice for 30 min to depolymerize the cold labile microtubule population. Nocodazole was then added to a final concentration of 20  $\mu$ M, and the cells were incubated for another 30 min on ice, followed by 1 h at 37°C in the continued presence of nocodazole. Cells were surface stained for syntaxins as described above. Complete disruption of microtubules was verified by immunostaining with a polyclonal antibody against tubulin (Cytoskeleton, Denver, CO).

### Cholesterol Depletion

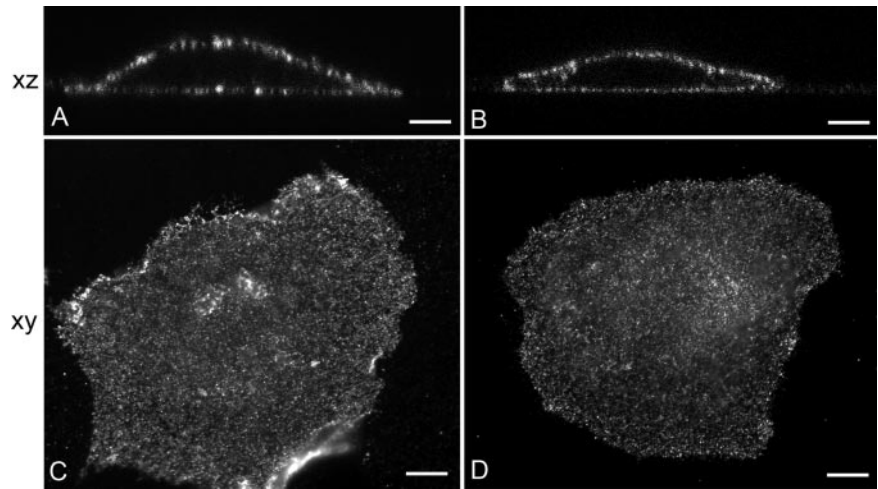
MDCK cells expressing syntaxin 3 or 4 were seeded on coverslips. After 8 h, fresh medium was added in the presence or absence of 4  $\mu$ M lovastatin and 0.25 mM mevalonate for 16 h. The cells were washed with phosphate-buffered saline (PBS) twice, treated for 30 min at 37°C with 15 mM methyl- $\beta$ -cyclodextrin in MEM medium, and then surface stained for syntaxins with the myc mAb.

### Latrunculin Treatment to Depolymerize Actin Filaments

MDCK cells expressing syntaxin 3 or 4 were cultured on coverslips for 16 h and treated with latrunculin B (0.5  $\mu$ g/ml) for 30 min and surface stained as described above. To prevent repolarization of actin fibers, latrunculin B was also added during the primary antibody incubation.

### Analysis of Syntaxin 3/4 Cluster Size

For the analysis of syntaxin cluster sizes, regions of interest (ROIs) were traced around MDCK cells (avoiding nuclear regions) stably expressing syntaxin 3/4. An adaptive neighborhood equalization filter from the Image Processing Toolkit (Reindeer Graphics, Asheville, NC) was applied to each ROI for image contrast expansion and noise reduction. Finally, syntaxin clusters in each ROI were segmented, and their diameters were measured using Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD). Because clusters seemed enlarged because of the diffraction-limited resolution of the objective, a point spread function (PSF) was generated for the imaging system by measuring the size of 200 nm fluorescent beads (Molecular Probes) and applying the same adaptive neighborhood equalization values. The actual



**Figure 1.** Syntaxin 3 and 4 localize to sub-micron-sized clusters on the plasma membrane of nonpolarized MDCK cells. MDCK cells stably expressing myc-tagged syntaxin 3 (A and C) and 4 (B and D) were surface labeled with monoclonal myc antibody followed by fluorescently labeled secondary antibody. (A and B) The xz optical sections of cells imaged by confocal microscopy. (C and D) The xy images of cells imaged by epifluorescence microscopy focusing on the basal membrane that is in contact with the coverslip. Bars, 5  $\mu$ m.

size of the clusters was then calculated by applying this PSF correction factor (Lang *et al.*, 2001).

#### Analysis of Syntaxin Cluster Size, Density, and Intensity

In immunostained MDCK cells expressing myc-tagged syntaxin 3, cluster diameter, density, and intensity were measured in  $100 \times 100$  pixel ROIs using Image-Pro Plus 5.0. Before measurement, images were processed using spectral and equalization filters that reduced the size of clusters to PSF-corrected dimensions. The resulting contrast expansion and cluster separation allowed straightforward segmentation for cluster counting and diameter measurement in both bright (high expression-level) and dim (low expression-level) cells. Finally, for intensity measurements images generated after filter application were binarized and applied as masks to corresponding unprocessed images to accurately segment clusters without manipulating image intensity.

#### Quantification of Colocalization between Various Syntaxins and SNAP-23

Using Image-Pro Plus, images of immunostained, stably transfected MDCK cells expressing combinations of syntaxin 2, 3, and 4 or SNAP-23 were separated into their respective channels; processed using spectral and equalization filters as described above; and aligned using Fourier correlation filters before merging into RGB color space (correction for minor pixel shift because of filter cube rotation). Cells in processed images were then divided into  $100 \times 100$  pixel ROIs and segmented for red, green, and yellow clusters using color profiles that were kept constant for each of the expression groups (syntaxin3/4, syntaxin2/3, syntaxin2/4, syntaxin3/SNAP-23, or syntaxin4/SNAP-23). Clusters of each color were then counted and filtered by size to conform to dimensions reported previously (eliminated partially overlapping clusters). Percentage of green clusters overlapping with red clusters and the inverse, percentage of red clusters overlapping with green clusters, were calculated. To establish thresholds for colocalization, cells expressing syntaxin 3 or 4 were double labeled with anti-myc/FITC (before permeabilization) and anti-syntaxin/Texas Red (after permeabilization) antibodies, and images were processed as mentioned above. Percentage of overlap calculated from ROIs in these images corresponded to maximum achievable levels of colocalization. To establish a baseline of random, nonspecific colocalization, percentage of overlap was recalculated for ROIs in which green clusters were intentionally shifted by 10 pixels. All overlap calculations were then normalized using these thresholds.

#### Analysis of Loss of Syntaxin Clusters after Cytoskeletal Disruption

Images of MDCK cells expressing either syntaxin 3 or syntaxin 4 and treated with latrunculin B or nocodazole were divided into  $50 \times 50$  pixel ROIs (7–12 ROIs/cell). Using Image-Pro Plus, spectral and equalization filters were applied to each ROI, and individual clusters were segmented using aspect ratio and size discriminators. This resulted in the rejection of clusters that did not conform to previously determined size ranges and circular morphology. Finally, segmented clusters in each ROI were counted and averaged with cluster counts from other ROIs within the same treatment group.

#### Syntaxin Localization in Migrating NIH 3T3 Cells

NIH 3T3 cells were maintained in DMEM medium containing 10% FBS, penicillin, and streptomycin. The cells were grown to a confluent monolayer on coverslips in a 12-well plate. They were then transiently transfected for

16 h with myc-tagged syntaxin 3 or 4 constructs using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. The cell monolayer was then scraped using the pointed edge of a sterile cell scraper (Corning Glassworks, Corning, NY), washed with medium once, and incubated with fresh medium. The cells were allowed to migrate for 9 h and then surface stained as described above.

## RESULTS

### Syntaxin 3 and 4 Localize to Submicron-sized Clusters in Nonpolarized Cells

We have previously shown that a large fraction of syntaxins 3 and 4 relocates to intracellular vacuoles in nonpolarized epithelial cells (Low *et al.*, 2000). To be able to investigate the fraction of these syntaxins that remains localized to the plasma membrane, we generated syntaxin 3 and 4 fusion proteins containing two C-terminal myc-epitope tags in tandem. These tags are predicted to be extracytoplasmic and accessible to anti-myc antibody added to the culture medium of intact cells. We have previously shown that these tagged syntaxins target properly to the apical and basolateral plasma membrane, respectively, of stably transfected, polarized MDCK cells and that the epitope tags are accessible for surface staining of intact cells (Kreitzer *et al.*, 2003).

Using these stable cell lines, we investigated the distribution of syntaxins 3 and 4 under conditions that do not allow the cells to polarize. The cells were cultured at low density on glass coverslips for 16 h during which time they proliferate and establish some initial cell–cell contacts but cannot yet establish a fully polarized morphology. Surface immunostaining using anti-myc antibody revealed that indeed both syntaxins are distributed throughout the plasma membrane, both at the surface in contact with the coverslip (which we term the basal membrane domain) and at the free membrane domain that is exposed to the culture medium (Figure 1, A and B). Because of the surface staining technique, no interfering intracellular immune signal is visible as expected.

The basal membrane of these cells lends itself to further investigation because the entire “footprint” of the cells can be visualized at high resolution in a single focal plane. We found that neither syntaxin is distributed homogeneously throughout the basal membrane but rather each localizes to small, remarkably uniform clusters (Figure 1, C and D). It is unlikely that these clusters are artifacts of the surface staining technique or the added epitope tags for the following reasons. The initial incubation with anti-myc antibody was

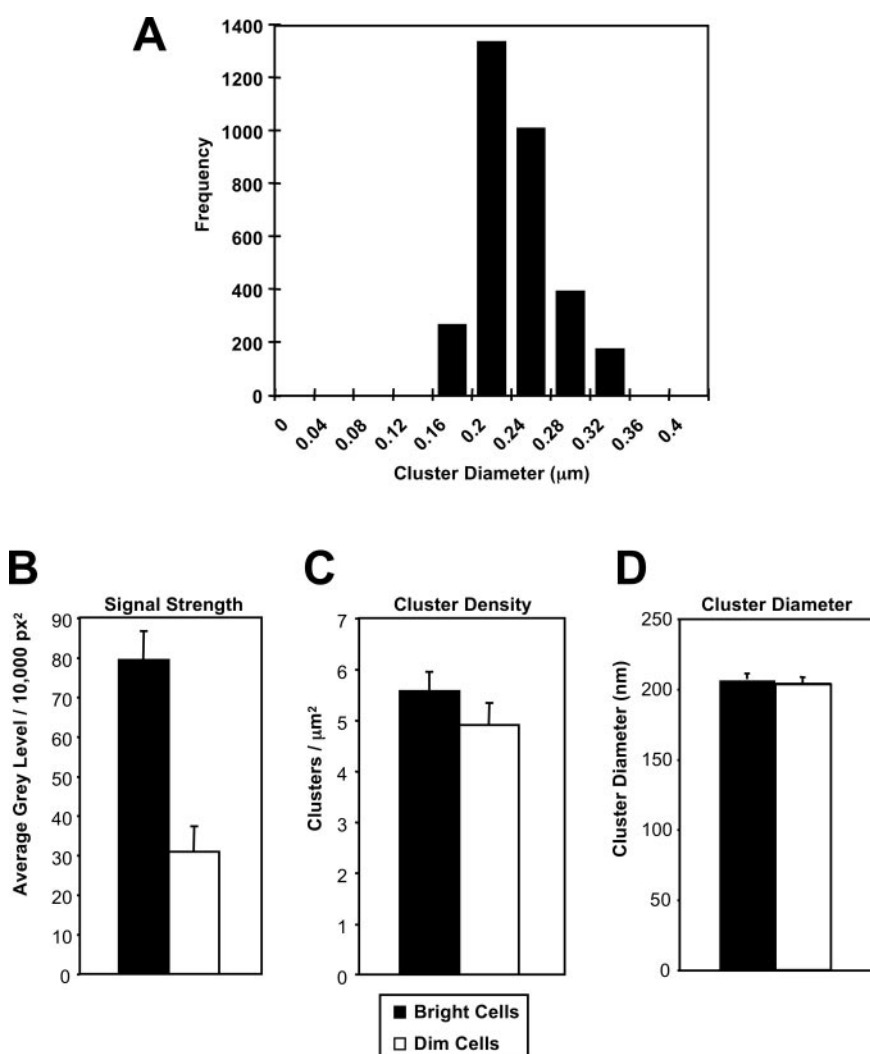
**Table 1.** Size estimation of syntaxin 3 and 4 clusters

Cell	Syntaxin 3		Syntaxin 4	
	n	Size ( $\mu\text{m}$ )	n	Size ( $\mu\text{m}$ )
1	536	$0.23 \pm 0.03$	609	$0.17 \pm 0.02$
2	585	$0.22 \pm 0.03$	646	$0.18 \pm 0.02$
3	527	$0.22 \pm 0.03$	625	$0.17 \pm 0.02$

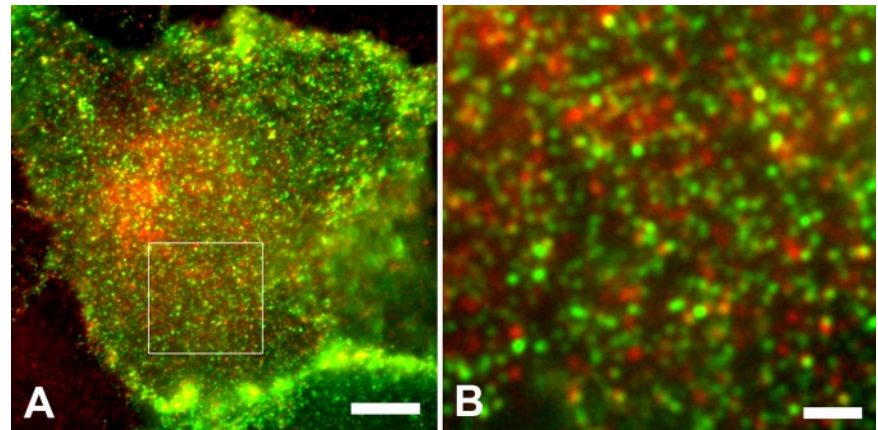
Syntaxin cluster diameters were measured in ROIs drawn around three MDCK cells stably expressing either syntaxin 3 or syntaxin 4. Each ROI was processed using adaptive equalization filters before segmentation and measurement as described in *Materials and Methods*. Values are the means  $\pm$  SEM,  $n \approx 500$ –650 clusters.

carried out at  $0^\circ\text{C}$  during which membrane fluidity is extremely low. This is followed by formaldehyde fixation and staining with fluorescently labeled secondary antibody. Both intact secondary antibodies and  $F_{ab}$  fragments (Figures 3 and 7) yielded indistinguishable results, indicating that the observed staining is not because of secondary antibody-induced clustering. The same clusters were also seen when the cells were first fixed and permeabilized and then incu-

bated with primary/secondary antibodies (our unpublished data), but, as expected, a high background from intracellular syntaxins is visible under these conditions. Furthermore, staining for endogenous syntaxin 4 (Figure 3) as well as N-terminally HA-tagged syntaxin 4 and untagged syntaxin 3 (our unpublished data) resulted in indistinguishable clusters indicating the C-terminal myc-tags do not contribute to or affect clustering. Syntaxin clusters can be disrupted, leading to smooth surface staining (see below), which indicates that our staining conditions result in a faithful representation of syntaxin localization. Culturing cells on Matrigel-coated coverslips (vs. uncoated) did not result in any noticeable change in appearance of syntaxin clusters (our unpublished observations), suggesting that cluster characteristics are not influenced by interactions with the substrate. Finally, these syntaxin 3 and 4 clusters are similar to clusters of syntaxin 1 that have been previously observed on the surface of the neuroendocrine PC12 cell line (Lang *et al.*, 2001, 2002) and pancreatic  $\beta$  cells (Ohara-Imaizumi *et al.*, 2004b). Evidence suggests that the syntaxin 1 clusters in PC12 cells are docking/fusion sites for neurotransmitter secretory granules (Lang *et al.*, 2001, 2002), whereas syntaxin 1 clusters in  $\beta$  cells are fusion sites for insulin granules (Ohara-Imaizumi *et al.*, 2004b). Both of these are regulated



**Figure 2.** Syntaxin cluster diameter and density does not vary with expression level. MDCK cells expressing myc-tagged syntaxin 3 were subjected to surface immunofluorescence microscopy using anti-myc antibody. In three fields consisting of four bright cells (high expression of syntaxin 3) and four dim cells (low expression of syntaxin 3), three to seven  $100 \times 100$  pixel ROIs were selected per cell (number of regions was dependent on cell size). Clusters from all ROIs in both bright and dim cells were binned by diameter to evaluate the cluster size distribution (A). Signal intensity (B), cluster density (C), and cluster diameter (D) were measured in each ROI after image equalization and noise reduction and are expressed as mean  $\pm$  SEM ( $n \approx 20$ ).

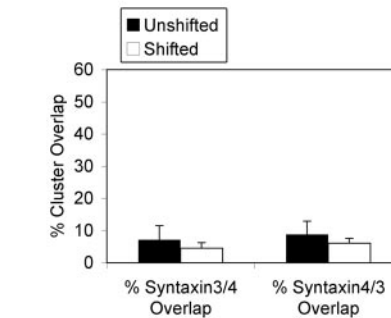


**Figure 3.** Syntaxin 3 and 4 clusters do not significantly colocalize. MDCK cells were transiently transfected for myc-tagged syntaxin 3. Syntaxin 3 (green) was first surface labeled with the myc mAb, fixed, and visualized with FITC-labeled  $F_{ab}$  fragments (green). (A) The cells were briefly fixed again and then incubated with a monoclonal anti-syntaxin 4 antibody against endogenous syntaxin 4, followed by a Texas Red-labeled secondary antibody (red). Bar, 5  $\mu$ m. (B) Magnified image of the square shown in A. Bar, 1  $\mu$ m. (C) The degree of cluster overlap between syntaxin 3 and 4 was quantified as described in *Materials and Methods*. “Unshifted” refers to actual colocalization between syntaxin 3 and 4 clusters. “Shifted” represents random, nonspecific colocalization in which green clusters were intentionally shifted by 10 pixels. The difference between actual and random colocalization of the syntaxin 3 and 4 clusters is not statistically significant.

secretory pathways in contrast to the constitutive exocytic pathways that involve syntaxins 3 and 4 in MDCK cells.

The syntaxin 3/4 clusters are of a submicron dimension that makes accurate size determination by light microscopy difficult. To estimate their size, we measured defined 200-nm-diameter fluorescent beads using identical optics and determined a correction factor for the point spread function (Lang *et al.*, 2001). The sizes of the syntaxin clusters were then estimated using this correction factor. Table 1 shows that the average size of syntaxin 4 clusters (170 nm) is consistently slightly smaller than that for syntaxin 3 clusters (220 nm). The size distribution of syntaxin clusters is remarkably narrow (Figure 2A), suggesting that cellular mechanisms must exist for the maintenance of cluster integrity at these dimensions.

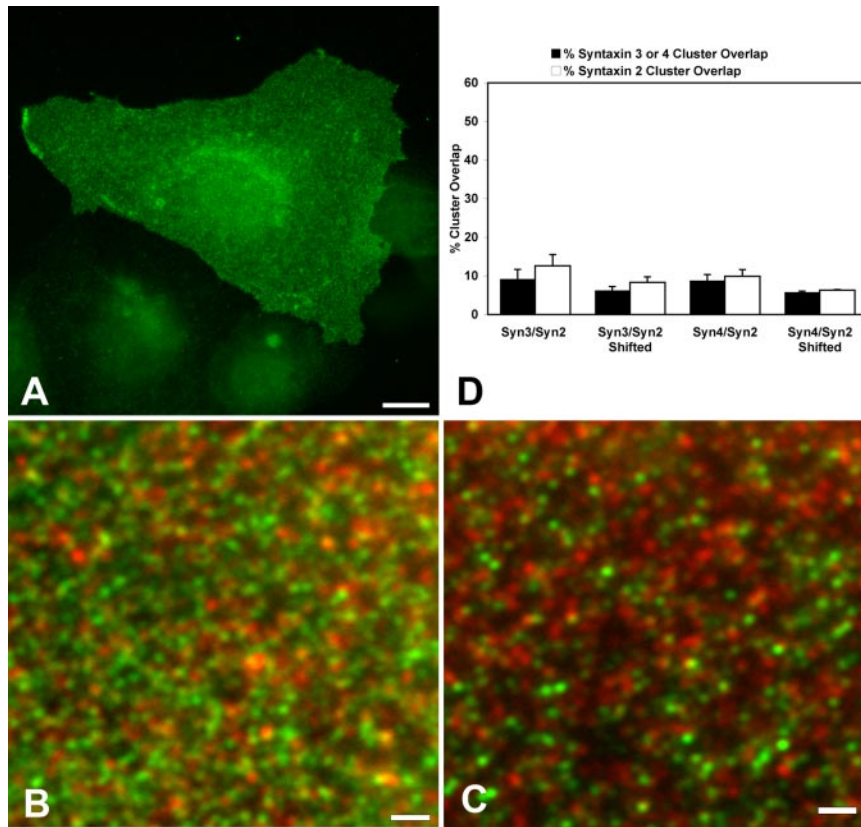
We tested whether the expression level of syntaxin 3/4 may affect the cluster diameter or the cluster density. Even though we used stably transfected MDCK cell clones, the expression levels varied considerably from cell to cell. We therefore analyzed four “bright” cells and four “dim” cells in three microscopic fields of cells expressing tagged syntaxin 3. Syntaxin clusters were analyzed in a total of 44 representative areas of 10,000 square-pixels each (Figure 2, B–D). Whereas the signal intensity differed by 2.6-fold between high- and low-expressing cells (Figure 2B), neither the cluster density (Figure 2C) nor the cluster diameter (Figure 2D) changed significantly. This result indicates that at the range of expression levels used in these experiments, there are no observable saturation effects, and it further supports the notion that the cells use molecular mechanisms to keep the syntaxin cluster size and density at a defined level. This also suggests that there is a biological reason for SNAREs to localize to membrane clusters of certain dimensions.



#### *Syntaxin 3 and 4 Clusters Are Mutually Exclusive and Do Not Colocalize Together or with Syntaxin 2 on the Plasma Membrane*

Next, we asked whether syntaxin 3 and 4 would colocalize in the same clusters. Cells were immunostained for endogenous syntaxin 4 and myc-tagged syntaxin 3. Figure 3 shows that there is no significant overlap between syntaxin 3 and 4 clusters. Quantitation of the percentage of syntaxin 3 clusters overlapping with syntaxin 4 was carried out as described in *Materials and Methods* and was 7%. Conversely, syntaxin 4 clusters overlapping with syntaxin 3 was found to be 8.7% (Figure 3B). To determine the random chance for clusters to colocalize, the green image was intentionally shifted by 10 pixels, and the incidence of overlap became 4.5 and 6.7%, respectively, which is not significantly different from the actual colocalization values. This indicates that the syntaxin 3 and 4 clusters reside in mutually exclusive domains on the plasma membrane. This result suggests that even when these two syntaxins are forced to reside in the same membrane domain, they nevertheless maintain a mutually exclusive localization just as they would in polarized epithelial cells.

We have previously shown that renal epithelial cells in vivo contain a third plasma membrane syntaxin, syntaxin 2, which localizes to either the apical or basolateral plasma membrane domain depending on the cell type along the renal tubule (Li *et al.*, 2002). In transfected, polarized MDCK cells syntaxin 2 targets to both domains (Low *et al.*, 1996). To test whether syntaxin 2 may form mixed clusters with syntaxin 3 and/or syntaxin 4, we expressed myc-tagged syntaxin 3 or 4 in stably transfected MDCK cells expressing HA-tagged syntaxin 2. Syntaxin 2 localizes to small clusters that are very similar to syntaxin 3/4 clusters (Figure 4A). However, there is no significant colocalization between



**Figure 4.** Syntaxin 2 also localizes to submicron-sized clusters on the surface of nonpolarized MDCK cells, but it does not colocalize with syntaxin 3 or syntaxin 4. MDCK cells stably expressing HA-tagged syntaxin 2 were fixed, permeabilized, and stained with a syntaxin 2 antibody (A). To colocalize syntaxin 2 with syntaxin 3 or 4, stable MDCK cells expressing HA-syntaxin 2 were transiently transfected with myc-tagged syntaxin 3 or syntaxin 4 plasmids. Syntaxins 3 (B) and 4 (C) were visualized by surface staining with anti-myc antibody (green) and syntaxin 2 with a polyclonal antibody (red). Bars, 5  $\mu\text{m}$  (A); 1  $\mu\text{m}$  (B and C). (D) Quantitation of the extent of syntaxin 2 overlap with syntaxin 3 or 4 was done as described in *MATERIALS AND METHODS*. "Shifted" represents random, nonspecific colocalization in which green clusters were intentionally shifted by 10 pixels. The difference between actual and random colocalization of the syntaxin 2 with syntaxin 3 or 4 clusters is not statistically significant.

these clusters (Figure 4, B and C). Quantitation of the percentage of overlap is shown in Figure 4D. This result indicates that even syntaxins that normally target to the same plasma membrane domain in polarized cells do not form mixed clusters, suggesting that they are formed by different mechanisms and likely serve in the exocytosis of distinct classes of exocytic vesicles.

#### *Syntaxins 3 and 4 Form Clusters in the Nonepithelial Jurkat Cell Line*

The ability of syntaxins 3 and 4 to form defined clusters may be a specific property of epithelial cells, or it may be a more general phenomenon. To address this question, we transfected Jurkat cells, a nonpolarized T-cell leukemia cell line, with the myc-tagged syntaxins 3 or 4 and performed surface immunostaining as described above. As shown in Figure 5, both syntaxins localize to clusters in Jurkat cells that seem identical to those seen in MDCK cells. Hence, the property of forming clusters is conserved across various cell types, both epithelial and nonepithelial. This suggests that the maintenance of clusters has an important biological function that is required by all cell types.

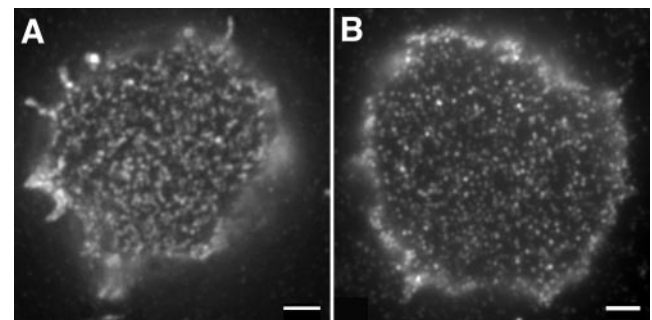
#### *Apical and Basolateral Cargo Proteins Do Not Localize to Syntaxin Clusters*

Next, we tested whether apical and basolateral proteins whose plasma membrane delivery depends on syntaxins 3/4 may localize to syntaxin clusters. We have previously shown that syntaxin 3 is required for the delivery of the p75 neurotrophin receptor (p75) (Kreitzer *et al.*, 2003). The LDLR is basolaterally delivered in MDCK cells (Matter *et al.*, 1993) and likely requires syntaxin 4. Even though p75 and LDLR localized to clusters on the plasma membrane of nonpolar-

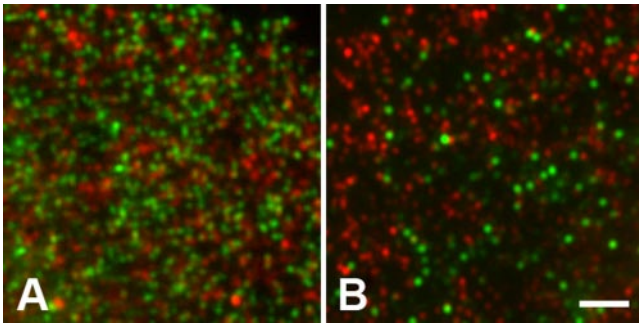
ized MDCK cells, coimmunostaining revealed no significant overlap between syntaxin 3 and p75 or between syntaxin 4 and LDLR (Figure 6). This result suggests that cargo proteins do not remain concentrated at the site of exocytosis. This is consistent with previous findings that apical and basolateral cargo proteins diffuse rapidly away from the initial fusion site after exocytosis (Keller *et al.*, 2001; Kreitzer *et al.*, 2003).

#### *Syntaxin Clusters Are Distinct from Caveolae and Clathrin-coated Pits*

Because the syntaxin clusters are of similar dimensions as caveolae and clathrin-coated pits, we asked whether syn-



**Figure 5.** Syntaxins 3 and 4 localize to small clusters in nonepithelial cells. Myc-tagged syntaxin 3 (A) or syntaxin 4 (B) were transiently transfected into Jurkat cells. The cells were plated on fibronectin-coated cover slips and surface-stained with anti-myc antibody. Micrographs show small syntaxin clusters on the basal membrane in contact with the coverslip that are of similar dimensions as syntaxin clusters in MDCK cells. Bars, 1  $\mu\text{m}$ .



**Figure 6.** Syntaxins 3 and 4 do not colocalize with p75 and LDLR, respectively. MDCK cells stably expressing syntaxin 3 or 4 were plated at low density on coverslips and either p75 or LDLR were expressed using adenovirus vectors. (A) Syntaxin 3 cells were permeabilized and stained with a rabbit anti-syntaxin 3 antibody (red) and an anti-p75 antibody (green). (B) Syntaxin 4 cells were surface stained with 9E10 (red) followed by an anti-LDLR antibody (green).

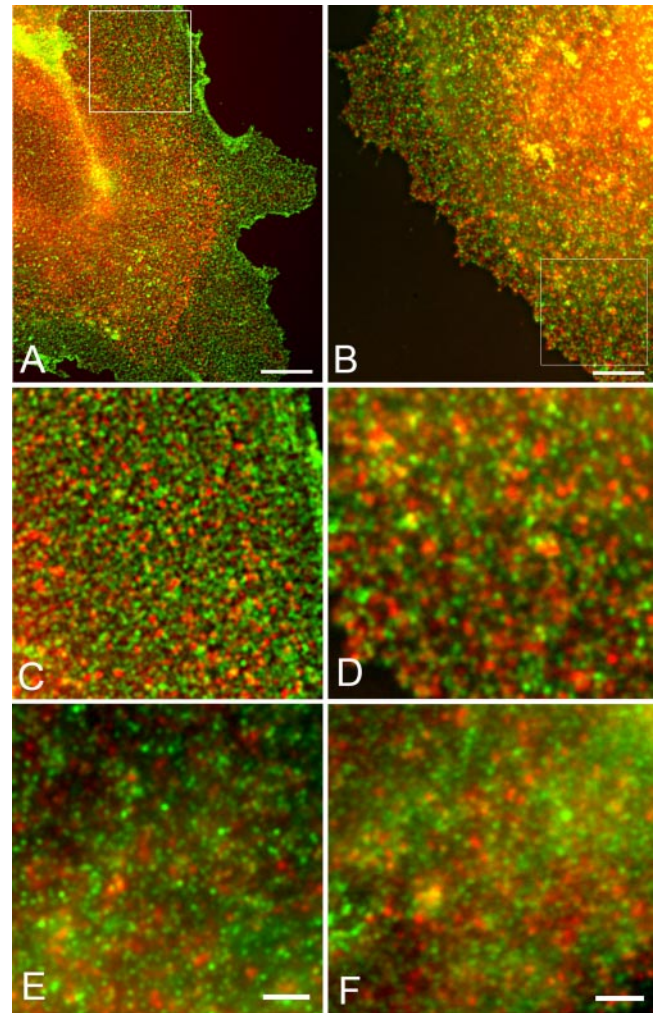
taxin 3 or 4 might localize to these well-defined structures. Coimmunostaining between myc-tagged syntaxin 3 or 4 and caveolin (Figure 7, A–D) or clathrin heavy chain (Figure 7, E and F), revealed no significant overlap. This result indicates that syntaxin clusters are not likely to be endocytic structures.

#### *Syntaxin 3 Clusters Require Intact Microtubules and Syntaxin 4 Clusters Require Actin for Their Integrity*

We next asked whether cytoskeletal elements are required for the maintenance of defined syntaxin 3 and 4 clusters. Microtubules were disrupted by cold treatment and treatment with the tubulin polymerization inhibitor nocodazole. As shown in Figure 8, disruption of microtubules had no effect on syntaxin 4 clusters, but it caused a loss of well-defined syntaxin 3 clusters. Microtubule disruption resulted in a more homogeneous distribution of syntaxin 3 throughout the basal membrane (Figure 8C). Quantitation of the cluster dispersion is shown in Figure 8E, where there was an almost fourfold decrease in syntaxin 3 clusters after microtubule disruption. Conversely, disruption of the actin cytoskeleton with the actin polymerization inhibitor latrunculin B had no effect on syntaxin 3 clusters, but it caused the partial loss of organization of syntaxin 4 clusters (Figure 9). This led to a more diffuse distribution of syntaxin 4 throughout the basal membrane (Figure 9D). These results indicate that the organization of syntaxin 3 and 4 clusters depends on different mechanisms involving microtubules and actin filaments, respectively.

#### *Syntaxin 3 Clusters Are Sensitive to Cholesterol Depletion*

We investigated the possibility that the cholesterol content of the plasma membrane may be important for syntaxin cluster organization. It has been previously reported that syntaxin 3 but not syntaxin 4 partially fractionates with detergent-insoluble membrane preparations (Lafont *et al.*, 1999). We treated MDCK cells expressing syntaxin 3 or 4 with methyl  $\beta$ -cyclodextrin, which depletes cholesterol from the plasma membrane and is thought to cause disruption of membrane microdomains in live cells. As shown in Figure 10, this treatment had no effect on syntaxin 4 clusters, but it caused very extensive disruption of syntaxin 3 clusters. Again, syntaxin 3 becomes homogeneously distributed throughout the basal membrane under these conditions (Figure 10C). This result indicates that syntaxin 3 cluster

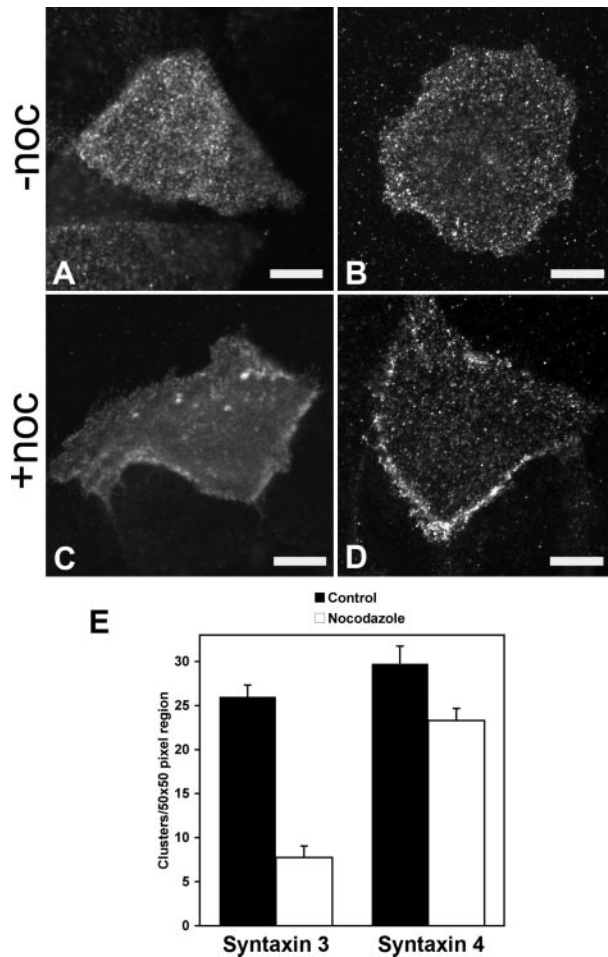


**Figure 7.** Syntaxin 3 and 4 clusters are distinct from caveolae and clathrin-coated pits. MDCK cells expressing myc-tagged syntaxin 3 (A, C, and E) or 4 (B, D, and F) were surface stained with monoclonal anti-myc antibody, fixed, permeabilized with 0.2% Triton X-100, and followed by FITC-labeled  $F_{ab}$  fragment secondary antibody (green). The cells were fixed once again and stained with anti-caveolin 1 antibody (A–D) or anti-clathrin heavy chain antibody (E and F) in red. Bars, 5  $\mu$ m (A and B). (C and D) Magnified images of the squares shown in A and B, respectively. Bars 1  $\mu$ m (C–F).

formation is dependent on the cholesterol content of the plasma membrane, whereas the mechanism of syntaxin 4 cluster formation is cholesterol independent.

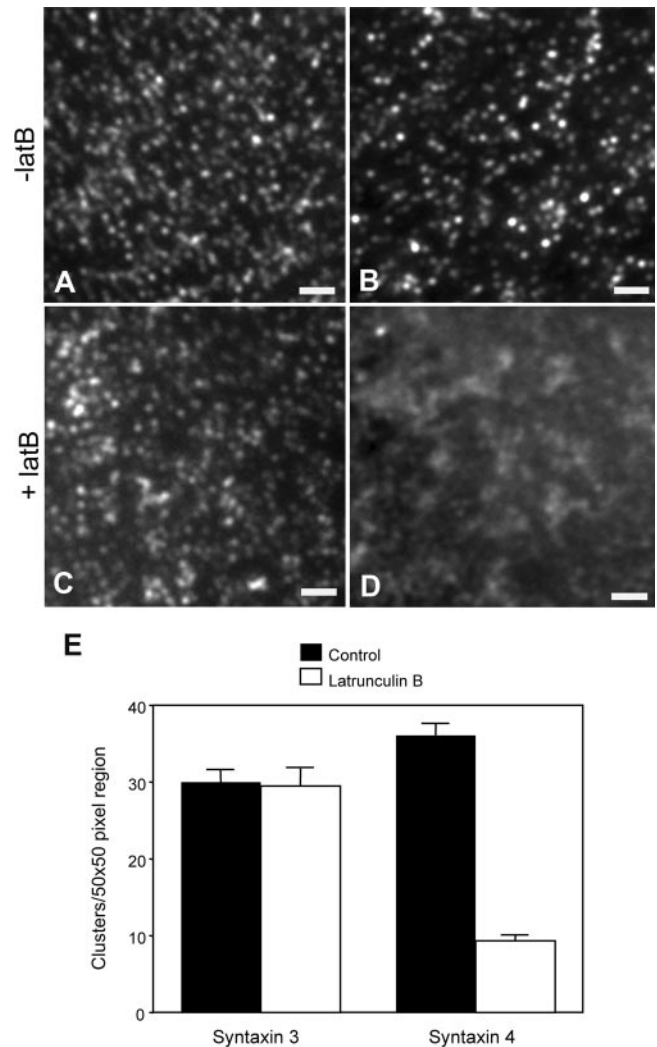
#### *Syntaxin 3 and 4 Clusters Partially Overlap with SNAP-23 Clusters*

Both syntaxin 3 and 4 have been shown to be able to bind to the t-SNARE SNAP-23 (Ravichandran *et al.*, 1996). In polarized MDCK cells, SNAP-23 localizes to both the apical and basolateral plasma membrane (Low *et al.*, 1998b), and its inactivation by the botulinum neurotoxin E causes inhibition of both apical and basolateral trafficking pathways (Low *et al.*, 1998a). This indicates that SNAP-23 is a common binding partner to both syntaxins and is required to form functional, heterodimeric t-SNAREs at the apical and basolateral domains of epithelial cells. If the syntaxin clusters in nonpo-



**Figure 8.** Disruption of the microtubule network with nocodazole causes dispersion of syntaxin 3 clusters but not of syntaxin 4 clusters. MDCK cells expressing myc-tagged syntaxin 3 (A and C) or syntaxin 4 (B and D) were untreated (A and B) or treated with nocodazole (C and D) as described in *Materials and Methods* and surface stained with monoclonal anti-myc antibody. Bars, 5  $\mu$ m. The quantitation of the clusters was carried out as described in *Materials and Methods* and is shown in E.

larized cells are functional docking and fusion sites for exocytic vesicles, they would be expected to contain SNAP-23. To test this, we used stably transfected MDCK cells expressing human SNAP-23 and expressed myc-tagged syntaxin 3 or 4 using adenoviral vectors. Syntaxin 3/4 clusters were labeled by surface staining that was followed by immunostaining using an anti-human SNAP-23 antibody. Like the syntaxins, SNAP-23 is also present as clusters and partially colocalizes with syntaxin 3 and 4 as evidenced by yellow clusters (Figure 11, A–D). Quantification of the extent of overlap between the syntaxin and SNAP-23 clusters revealed that ~30% of syntaxin 3 clusters overlapped with SNAP-23, and conversely 37% of SNAP-23 clusters colocalized with syntaxin 3 (Figure 11E). The overlap between syntaxin 4 and SNAP-23 was slightly higher and ~44% of syntaxin 4 clusters overlapped with SNAP-23 and 51% of SNAP-23 clusters overlapped with syntaxin 4. This result indicates that a significant fraction of syntaxin 3/4 clusters also contain SNAP-23, suggesting that they are functional fusion sites.

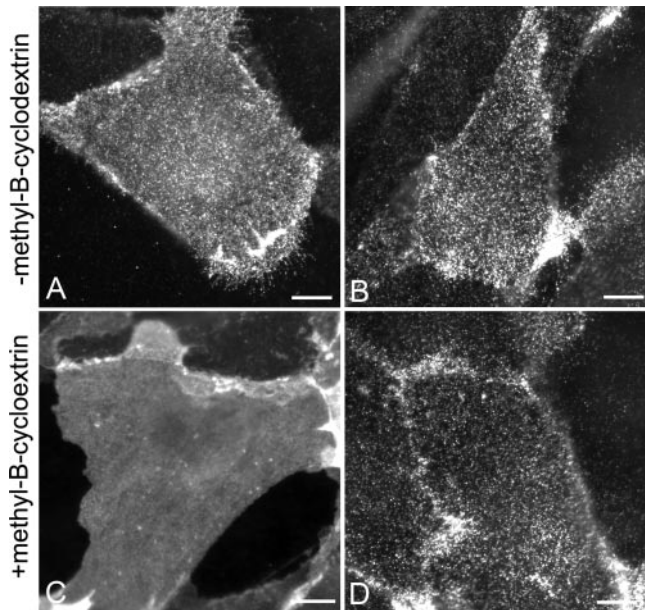


**Figure 9.** Depolymerization of the actin cytoskeleton disrupts the integrity of syntaxin 4 clusters. MDCK cells stably expressing syntaxin 3 (A and C) or syntaxin 4 (B and D) were untreated (A and B) or treated for 30 min with latrunculin B (0.5  $\mu$ g/ml) at 37°C (C and D). Cells were immediately surface stained for syntaxins followed by secondary antibody. Bars, 1  $\mu$ m. Quantitation of the cluster density was done as described in *Materials and Methods* and is shown in E.

#### *Syntaxin 3 and 4 Clusters Polarize to the Leading Edge of Migrating Cells*

Cell migration involves polarized membrane traffic toward the leading edge of the cell brought about by the fusion of transport vesicles at this site (Schmoranzler *et al.*, 2003). We asked whether syntaxin clusters may exist in migrating cells and whether they may exhibit polarity. NIH 3T3 cells were used in this study because they are well characterized in migration studies. The cells were plated on coverslips at high density, transfected with either syntaxin 3 or 4, and allowed to form a confluent monolayer. Once confluent, the monolayer was wounded by scraping, and the cells were allowed to migrate into the cleared space for 9 h. The localization of the syntaxin clusters was visualized by surface staining. To identify the leading edge, the cells were costained for filamentous actin that is known to prominently accumulate there (Figure 12, A and D). Strikingly, both





**Figure 10.** Cholesterol depletion causes dispersion of syntaxin 3 but not syntaxin 4 clusters. MDCK cells stably expressing myc-tagged syntaxin 3 (A and C) or 4 (B and D) were untreated (A and B) or treated with 4  $\mu$ M lovastatin and 0.25 mM mevalonate (C and D). After 16 h, the cells were washed twice with PBS and treated with 15 mM methyl- $\beta$ -cyclodextrin for 30 min and then surface immunostained for syntaxin 3/4 using the anti-myc antibody. Bars, 5  $\mu$ m.

syntaxin 3 (Figure 12, B and C) and syntaxin 4 (Figure 12, E and F) clusters are significantly concentrated at the leading edge of migrating cells and colocalize with F-actin. This suggests that exocytosis can occur at the very edge of the lamellipodium. Furthermore, this suggests that cargo from at least two different pathways, involving syntaxin 3 and 4, are preferentially exocytosed at the leading edge in migrating cells.

#### **The Mislocalized $\Delta$ 38 Mutant of Syntaxin 3 Forms Clusters That Do Not Colocalize with Syntaxin 4**

We have found that syntaxin 3 contains an apical targeting signal that is centered around a conserved FMDE motif in the N-terminal three-helix bundle (Sharma, Low, Misra, Pallavi, and Weimbs, unpublished data). Deletion of the first 38 amino acids of syntaxin 3 results in random targeting of the mutant protein to both the apical and basolateral plasma membranes in polarized MDCK cells. We therefore asked whether the mistargeted syntaxin 3 mutant may now have access to syntaxin 4 clusters. We subjected MDCK cells stably expressing the myc-tagged  $\Delta$ 38 mutant or wild-type syntaxin 3 to anti-myc surface staining followed by immunostaining for endogenous syntaxin 4. Wild-type syntaxin 3 clusters do not overlap with syntaxin 4 (Figure 13A) as shown above. Similarly, the  $\Delta$ 38 syntaxin 3 mutant is also found in clusters on the plasma membrane that are separate from syntaxin 4 clusters (Figure 13B). Quantitation of the extent of overlap between the mutant syntaxin 3 and syntaxin 4 clusters is shown in Figure 13C. This result indicates that syntaxin 3 does not require an intact apical targeting signal for its ability to form clusters. Furthermore, it indicates that the mechanism of the segregation of different syntaxins in clusters must differ from the mechanisms used to segregate them during polarized targeting.

## DISCUSSION

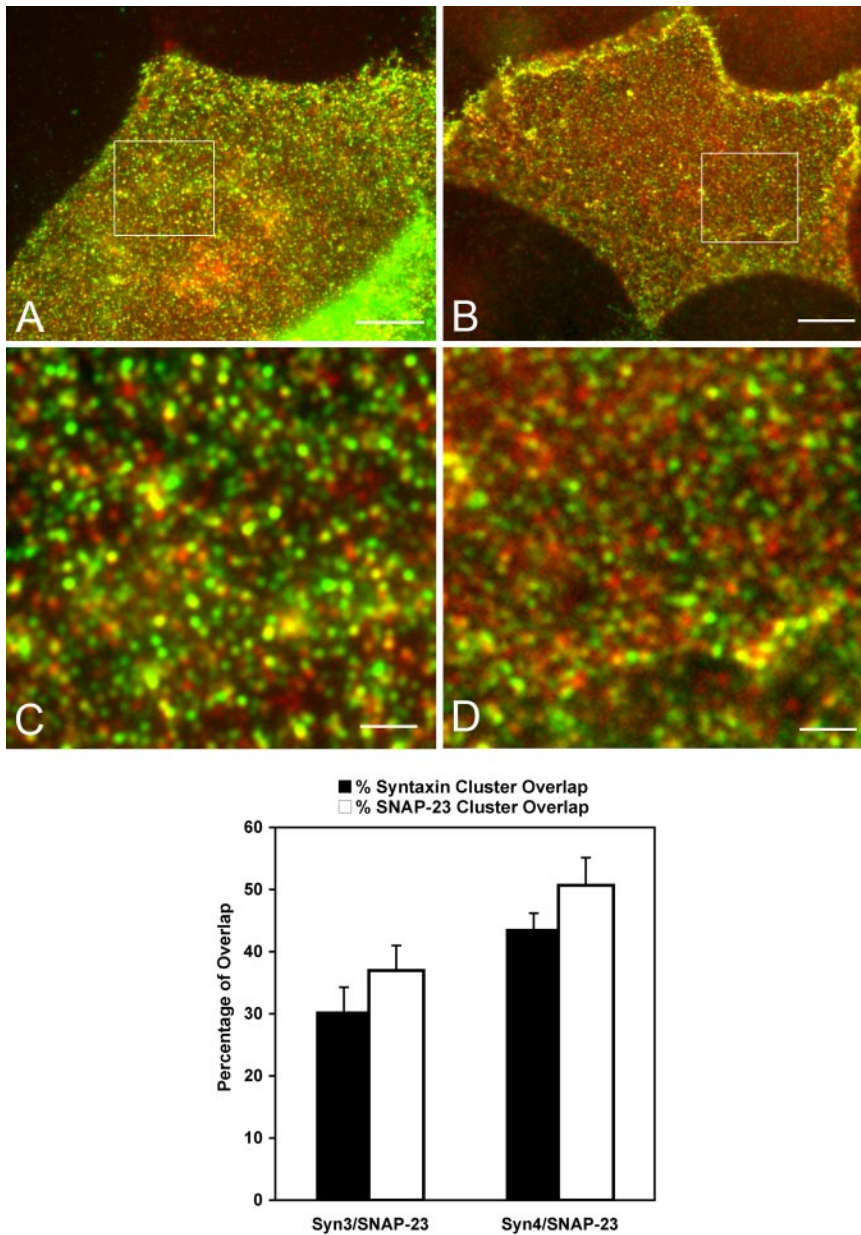
### *Mechanism of Syntaxin Clustering*

In this study, we show that syntaxins 3 and 4, which exhibit mutually exclusive, polarized localizations in polarized epithelial cells, also maintain mutually exclusive localizations in nonpolarized cells. They are present as discreet, uniform clusters on the plasma membrane. The uniformity of these clusters and the finding that their dimensions and quantity are independent of the syntaxin expression level strongly suggest that the size of syntaxin clusters is of functional importance and that mechanisms must exist to maintain this size. Our results indicate that syntaxin 3 and 4 cluster integrity is maintained by different mechanisms. Microtubule disruption resulted in disintegration of syntaxin 3 clusters without effect on syntaxin 4 clusters. Similarly, cholesterol extraction only affected syntaxin 3 but not syntaxin 4 clusters. In contrast, disruption of actin polymerization affected only syntaxin 4 but not syntaxin 3 clusters.

Clusters of very similar dimensions have previously been reported for syntaxin 1 in both a neuroendocrine cell line and in pancreatic beta cells. Together with our findings of syntaxin 2, 3, and 4 clusters, this suggests that cluster formation is a common feature of all plasma membrane syntaxins, and possibly all syntaxins in general. The previously described syntaxin 1 clusters serve in regulated secretory pathways that are characterized by the presence of docked vesicles at the site of the syntaxin 1 clusters. It was therefore a possibility that the formation of syntaxin 1 clusters could be induced by secretory vesicles during docking. However, the syntaxins that we describe here in MDCK cells serve constitutive secretory pathways, and at steady-state there is not a significant number of docked vesicles present at the plasma membrane in these cells. This strongly argues that syntaxin clusters are formed independently of vesicle interactions and that they preexist before docking.

It is presently unclear how microtubules and actin filaments may contribute to clustering of syntaxin 3 and 4, respectively. It is plausible that syntaxin clusters contain proteins that interact with these cytoskeletal elements. We have previously found that microtubule disruption causes partial basolateral mislocalization of syntaxin 3 in polarized MDCK cells (Kreitzer *et al.*, 2003). It is therefore possible that both clustering and apical targeting of syntaxin 3 involve the same microtubule-dependent mechanism. However, our syntaxin 3 mutant with a deletion of its apical targeting signal is still able to cluster (Figure 13B), suggesting that clustering is not sufficient for apical targeting of syntaxin 3. Interestingly, syntaxin 4 has been reported to interact with actin filaments in *in vitro* sedimentation assays, whereas syntaxins 2 and 3 did not interact (Band *et al.*, 2002). This suggests that F-actin may play a role in syntaxin 4 clustering by direct interaction.

Our finding that syntaxin 3 clusters are disrupted by cholesterol depletion is similar to the previously reported effect on syntaxin 1 clusters in PC12 cells (Lang *et al.*, 2001) and  $\beta$  cells (Ohara-Imaizumi *et al.*, 2004a), which suggests that the clustering of both syntaxins may depend on a similar mechanism. Only a small fraction of syntaxin 3 partitions in detergent-insoluble, floating membrane fractions (Lafont *et al.*, 1999; Sharma, Low, Misra, Pallavi, and Weimbs, unpublished data). Cholesterol depletion may have effects that indirectly affect syntaxin clustering. Alternatively, it is possible that clustering is mediated either by partitioning into cholesterol-enriched membrane microdomains or by exclusion from them. Either way, cholesterol depletion would be expected to result in the same disruptive



**Figure 11.** SNAP-23 colocalizes significantly with both syntaxin 3 and 4 clusters. MDCK cells stably expressing SNAP-23 were transiently transfected with myc-tagged syntaxin 3 (A and C) or syntaxin 4 (B and D). The cells were surface stained for syntaxins (green) followed by a polyclonal SNAP-23 (red) antibody. Magnified images of the squares in A and B are shown in C and D, respectively. Bars, 5  $\mu\text{m}$  (A and B); 1  $\mu\text{m}$  (C and D). The extent of syntaxin3/SNAP-23 and syntaxin4/SNAP-23 cluster colocalization was analyzed in nine different cells per group (E). The overlap between SNAP-23 and syntaxin 3/4 clusters was quantified as described in *Materials and Methods*. Values shown represent percentage of syntaxin 3/4 clusters that colocalize with SNAP-23 clusters or percentage of SNAP-23 clusters that colocalize with syntaxin 3/4 clusters (mean  $\pm$  SEM,  $n = 45$ ).

effect. In vitro, syntaxin 1 has been found to form homooligomers, which may suggest a mechanism contributing to cluster formation (Lerman *et al.*, 2000).

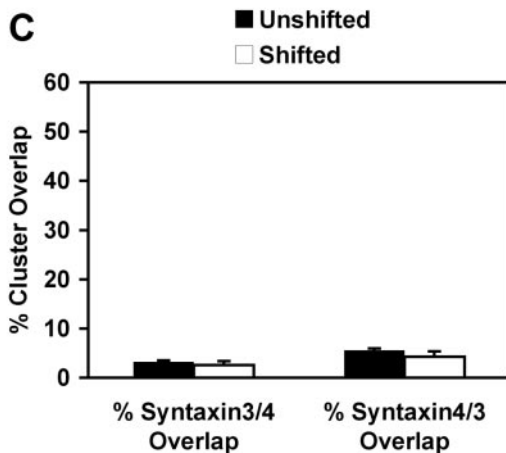
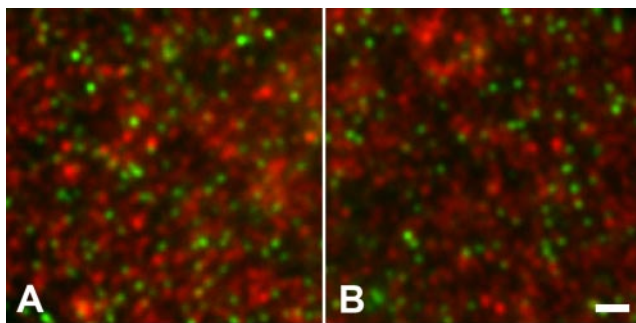
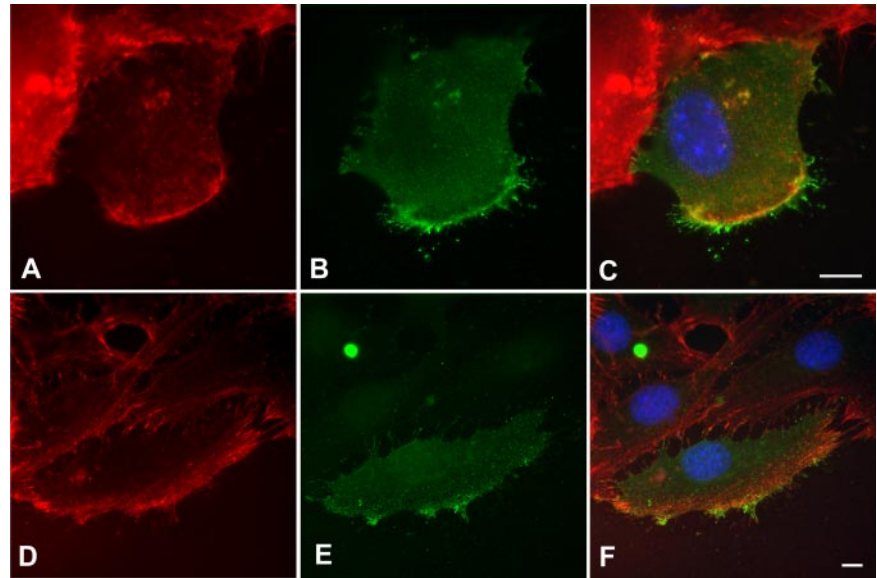
Our finding that different mechanisms are involved in cluster formation of syntaxin 3 and 4 probably explains why these two syntaxins are sorted into mutually exclusive plasma membrane clusters. It is possible that the same mechanisms play a role in the sorting of syntaxins 3 and 4 during their polarized targeting to the apical and basolateral membrane, respectively, in epithelial cells. It is currently unknown how these syntaxins are sorted during polarized targeting, but it is possible that syntaxin clustering already occurs along the secretory pathway.

#### *Implications for Membrane Fusion Mechanism*

Using SNARE proteins incorporated into artificial liposomes, Rothman and colleagues have previously shown that mixing of liposomes containing cognate SNAREs results in

membrane fusion (Weber *et al.*, 1998; McNew *et al.*, 2000). These results demonstrated that SNAREs are the minimal machinery required for fusion. Using different methodology, another group observed fusion between synaptic vesicles and artificial liposomes containing syntaxin 1 and SNAP-25 only when calcium was added (Hu *et al.*, 2002). Based on this difference, it was argued (Rizo, 2003) that the higher protein:lipid ratios used by Rothman's group resulted in artifactual membrane fusion, whereas the lower protein:lipid ratios used by Hu *et al.* (2002) represent more physiologically relevant conditions. However, our results presented here and those by others show that SNAREs are very significantly concentrated in small clusters in vivo. Because of the small dimensions of these clusters, it is difficult to calculate the actual densities of SNAREs contained within them. Nevertheless, one can argue that the high protein:lipid ratios used by Rothman *et al.* may actually represent the more physiologically relevant conditions.

**Figure 12.** Syntaxin 3 and 4 polarized to the leading edge of migrating NIH 3T3 cells. NIH 3T3 cells were cultured to a confluent monolayer on coverslips and transiently transfected with myc-tagged syntaxin 3 or 4 plasmids. The cell monolayer was then scraped, and the cells were allowed to migrate into the wound area for 9 h. Syntaxin 3 (B) or syntaxin 4 (E) was visualized by surface staining with the anti-myc antibody (green) and F-actin (A and D) was stained with Alexa Fluor 594-phalloidin. Bars, 5  $\mu\text{m}$ .



**Figure 13.** The mislocalized  $\Delta 38$ -syntaxin 3 mutant also forms clusters, but it does not colocalize with syntaxin 4. MDCK cells stably transfected with myc-tagged wild-type syntaxin 3 (A) or the  $\Delta 38$  syntaxin 3 mutant (B) were surface stained with a polyclonal anti myc antibody (red) followed by immunostaining for endogenous syntaxin 4 (green). Bar, 1  $\mu\text{m}$ . The degree of  $\Delta 38$ -syntaxin 3 and 4 cluster overlap has been calculated and is shown in C. The difference between actual (“Unshifted”) and random (“Shifted”) colocalization of the syntaxin 3 and 4 clusters is not statistically significant.

Given the dependence of syntaxin clustering on intact microtubules or filamentous actin shown here, it is unlikely that syntaxin clustering will occur in artificial liposomes. Therefore, choosing high protein:lipid ratios may overcome this limitation and generate similar SNARE densities to those that are locally induced by clustering in vivo.

We can currently only speculate why high local concentrations of SNAREs may be required for membrane fusion. It is plausible that a threshold level of SNARE density is required for fusion. If very low concentrations of SNAREs could lead to vesicle fusion, then it would be difficult to prevent unwanted fusion at inappropriate locations. For example, the trafficking of small amounts of newly synthesized SNAREs to their final destinations would provide inappropriate fusion sites on organelles of the secretory pathway.

It is also possible that high local SNARE concentrations may be required to sustain a fusion event after it has been initiated. This view may be supported, albeit indirectly, by results reported by zharvSchmoranzler *et al.* (2003). These authors found that disruption of microtubules with nocodazole causes a significantly higher frequency of “partial” fusion events at the plasma membrane of migrating fibroblasts. Partial fusion events were those in which a post-Golgi transport vesicle delivers only part of its cargo before dissociating again from the plasma membrane. Because we found that similar nocodazole treatment causes the dispersion of syntaxin 3 clusters (Figure 8C), a possible interpretation is that “complete” fusion events require a high density of SNARE molecules such as in a cluster. The lower syntaxin density after cluster disruption may still allow initial docking and fusion. However, a large number of assembled SNARE complexes may be required to maintain the fusion pore for some time. If not enough SNARE complexes are present, NSF may be able to disassemble them faster than fusion can be completed, which would result in partial fusion events.

#### Implications for Cell Polarity

It has previously been shown that even nonpolarized cell lines are capable of sorting newly synthesized “apical” and

“basolateral” cargo proteins in the TGN and delivering them on separate pathways to the plasma membrane (Müsch *et al.*, 1996; Yoshimori *et al.*, 1996; Keller *et al.*, 2001). Our finding of separate syntaxin 3 and 4 clusters in nonpolarized cells suggests that these pathways terminate by fusion at their “cognate” syntaxin clusters. This suggests that nonpolarized cells have all mechanisms in place that are required for the polarized delivery of membrane and secretory proteins. In a sense, nonpolarized cells are capable of polarized exocytosis, albeit at an ultrastructural level. It is plausible that these preexisting mechanisms enable cells to rapidly acquire polarized morphologies by spatial rearrangement of their fusion sites. This is supported by our finding that fibroblasts that were induced to migrate by the wounding of a cell monolayer localize syntaxin 3 and 4 to the leading edge (Figure 12). It has previously been shown that fusion of post-Golgi vesicles containing the LDLR preferentially occurs at the leading edge of migrating fibroblasts (Schmoranzer *et al.*, 2003).

An unexpected result was that disruption of the actin cytoskeleton leads to the disassembly of syntaxin 4 clusters. This suggests that actin filaments may play a role in the establishment of basolateral fusion sites. Previously, disruption of actin filaments by treatment with latrunculin B in MDCK cells has been shown to lead to partial apical mislocalization of the normally basolateral transferrin and LDL receptors. Furthermore, the recycling of transferrin, which normally recycles via the basolateral plasma membrane domain became unpolarized (Sheff *et al.*, 2002). Together with our findings, this suggests that actin disruption may result in the inability of epithelial cells to establish and maintain syntaxin 4 fusion sites at the basolateral membrane.

The actin cytoskeleton and actin-binding proteins have also been shown to be involved in the establishment of the immunological synapse between T-cells and antigen-presenting cells (Spiliotis and Nelson, 2003). Exocytosis preferentially polarizes to this region. Interestingly, syntaxin 4 and SNAP-23 have recently been shown to accumulate in T-cells at the site of the immunological synapse (Das *et al.*, 2004). Again, our results may suggest that actin is also directly involved in establishing syntaxin 4 fusion sites in this example of cell polarity.

## ACKNOWLEDGMENTS

We thank Heike Fölsch (Northwestern University, Chicago, IL) for help with the construction of adenovirus vectors for syntaxin expression; Frances Brodsky for gifts of antibodies; and Anne Musch, Enrique Rodriguez-Boulant, and Moses Chao (New York University, New York, NY) for the p75 and LDLR adenoviruses. This work was supported by National Institutes of Health Grant R01DK62338 (to T. W.), a Scientist Development Grant from the American Heart Association (to S.H.L.), and a Predoctoral Fellowship from the American Heart Association (to N. S.).

## REFERENCES

Band, A. M., Ali, H., Vartiainen, M. K., Welti, S., Lappalainen, P., Olkkonen, V. M., and Kuismanen, E. (2002). Endogenous plasma membrane t-SNARE syntaxin 4 is present in rab11 positive endosomal membranes and associates with cortical actin cytoskeleton. *FEBS Lett.* *531*, 513–519.

Chen, Y. A., and Scheller, R. H. (2001). SNARE-mediated membrane fusion. *Nat. Rev. Mol. Cell Biol.* *2*, 98–106.

Das, V., Nal, B., Dujecourt, A., Thoulouze, M. I., Galli, T., Roux, P., Dautry-Varsat, A., and Alcover, A. (2004). Activation-induced polarized recycling targets T cell antigen receptors to the immunological synapse; involvement of SNARE complexes. *Immunity* *20*, 577–588.

Gaisano, H. Y., Ghai, M., Malkus, P. N., Sheu, L., Bouquillon, A., Bennett, M. K., and Trimble, W. S. (1996). Distinct cellular locations and protein-protein interactions of the syntaxin family of proteins in rat pancreatic acinar cells. *Mol. Biol. Cell* *7*, 2019–2027.

He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* *95*, 2509–2514.

Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A., and Davletov, B. (2002). Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. *Nature* *415*, 646–650.

Jahn, R., Lang, T., and Sudhof, T. C. (2003). Membrane fusion. *Cell* *112*, 519–533.

Keller, P., Toomre, D., Diaz, E., White, J., and Simons, K. (2001). Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat. Cell Biol.* *3*, 140–149.

Kreitzer, G., Schmoranzer, J., Low, S. H., Li, X., Gan, Y., Weimbs, T., Simon, S. M., and Rodriguez-Boulant, E. (2003). Three-dimensional analysis of post-Golgi carrier exocytosis in epithelial cells. *Nat. Cell Biol.* *5*, 126–136.

Lafont, F., Verkade, P., Galli, T., Wimmer, C., Louvard, D., and Simons, K. (1999). Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells. *Proc. Natl. Acad. Sci. USA* *96*, 3734–3738.

Lang, T., Bruns, D., Wenzel, D., Riedel, D., Holroyd, P., Thiele, C., and Jahn, R. (2001). SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. *EMBO J.* *20*, 2202–2213.

Lang, T., Margittai, M., Holzler, H., and Jahn, R. (2002). SNAREs in native plasma membranes are active and readily form core complexes with endogenous and exogenous SNAREs. *J. Cell Biol.* *158*, 751–760.

Lerman, J. C., Robblee, J., Fairman, R., and Hughson, F. M. (2000). Structural analysis of the neuronal SNARE protein syntaxin-1A. *Biochemistry* *39*, 8470–8479.

Li, X., Low, S. H., Miura, M., and Weimbs, T. (2002). SNARE expression and localization in renal epithelial cells suggest mechanism for variability of trafficking phenotypes. *Am. J. Physiol.* *283*, F1111–F1122.

Low, S. H., Chapin, S. J., Weimbs, T., Kömüves, L. G., Bennett, M. K., and Mostov, K. E. (1996). Differential localization of syntaxin isoforms in polarized MDCK cells. *Mol. Biol. Cell* *7*, 2007–2018.

Low, S. H., Chapin, S. J., Wimmer, C., Whiteheart, S. W., Kömüves, L. K., Mostov, K. E., and Weimbs, T. (1998a). The SNARE machinery is involved in apical plasma membrane trafficking in MDCK cells. *J. Cell Biol.* *141*, 1503–1513.

Low, S. H., Marmorstein, L. Y., Miura, M., Li, X., Kudo, N., Marmorstein, A. D., and Weimbs, T. (2002). Retinal pigment epithelial cells exhibit unique expression and localization of plasma membrane syntaxins which may contribute to their trafficking phenotype. *J. Cell Sci.* *115*, 4545–4553.

Low, S. H., Miura, M., Roche, P. A., Valdez, A. C., Mostov, K. E., and Weimbs, T. (2000). Intracellular Redirection of Plasma Membrane Trafficking after Loss of Epithelial Cell Polarity. *Mol. Biol. Cell* *11*, 3045–3060.

Low, S. H., Roche, P. A., Anderson, H. A., van IJzendoorn, S.C.D., Zhang, M., Mostov, K. E., and Weimbs, T. (1998b). Targeting of SNAP-23 and SNAP-25 in Polarized Epithelial Cells. *J. Biol. Chem.* *273*, 3422–3430.

Matter, K., Whitney, J. A., Yamamoto, E. M., and Mellman, I. (1993). Common signals control low density lipoprotein receptor sorting in endosomes and the Golgi complex of MDCK cells. *Cell* *74*, 1053–1064.

McNew, J. A., Parlati, F., Fukuda, R., Johnston, R. J., Paz, K., Paumet, F., Sollner, T. H., and Rothman, J. E. (2000). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature* *407*, 153–159.

Mostov, K., Su, T., and ter Beest, M. (2003). Polarized epithelial membrane traffic: conservation and plasticity. *Nat. Cell Biol.* *5*, 287–293.

Müsch, A., Xu, H., Shields, D., and Rodriguez-Boulant, E. (1996). Transport of vesicular stomatitis virus G protein to the cell surface is signal mediated in polarized and nonpolarized cells. *J. Cell Biol.* *133*, 543–558.

Ohara-Imaizumi, M., Nishiwaki, C., Kikuta, T., Kumakura, K., Nakamichi, Y., and Nagamatsu, S. (2004a). Site of docking and fusion of insulin secretory granules in live MIN6 beta cells analyzed by TAT-conjugated anti-syntaxin 1 antibody and total internal reflection fluorescence microscopy. *J. Biol. Chem.* *279*, 8403–8408.

Ohara-Imaizumi, M., Nishiwaki, C., Kikuta, T., Nagai, S., Nakamichi, Y., and Nagamatsu, S. (2004b). TIRF imaging of docking and fusion of single insulin granule motion in primary rat pancreatic beta-cells: different behaviour of granule motion between normal and Goto-Kakizaki diabetic rat beta-cells. *Biochem. J.* *381*, 13–18.

Ravichandran, V., Chawla, A., and Roche, P. A. (1996). Identification of a novel syntaxin- and synaptobrevin/VAMP-binding protein, SNAP-23, expressed in non-neuronal tissues. *J. Biol. Chem.* *271*, 13300–13303.

Rizo, J. (2003). SNARE function revisited. *Nat. Struct. Biol.* *10*, 417–419.

- Rodriguez-Boulan, E., Kreitzer, G., and Musch, A. (2005). Organization of vesicular trafficking in epithelia. *Nat. Rev. Mol. Cell Biol.* 6, 233–247.
- Schmoranzler, J., Kreitzer, G., and Simon, S. M. (2003). Migrating fibroblasts perform polarized, microtubule-dependent exocytosis towards the leading edge. *J. Cell Sci.* 116, 4513–4519.
- Sheff, D. R., Kroschewski, R., and Mellman, I. (2002). Actin dependence of polarized receptor recycling in Madin-Darby canine kidney cell endosomes. *Mol. Biol. Cell* 13, 262–275.
- Spiliotis, E. T., and Nelson, W. J. (2003). Spatial control of exocytosis. *Curr. Opin. Cell Biol.* 15, 430–437.
- Ungar, D., and Hughson, F. M. (2003). SNARE protein structure and function. *Annu. Rev. Cell Dev. Biol.* 19, 493–517.
- Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* 92, 759–772.
- Weimbs, T., Low, S. H., Chapin, S. J., and Mostov, K. E. (1997). Apical targeting in polarized epithelial cells: there's more afloat than rafts. *Trends Cell Biol.* 7, 393–399.
- Yoshimori, T., Keller, P., Roth, M. G., and Simons, K. (1996). Different biosynthetic transport routes to the plasma membrane in BHK and CHO cells. *J. Cell Biol.* 133, 247–256.