The Role of Syntaxins in the Specificity of Vesicle Targeting in Polarized Epithelial Cells[□]

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In polarized epithelial cells syntaxin 3 is at the apical plasma membrane and is involved in delivery of proteins from the *trans*-Golgi network to the apical surface. The highly related syntaxin 4 is at the basolateral surface. The complementary distribution of these syntaxins suggests that they play a role in the specificity of membrane traffic to the two surfaces. We constructed a chimeric syntaxin where we removed the N-terminal 29 residues of syntaxin 3 and replaced it with the corresponding portion of syntaxin 4. When expressed in polarized epithelial cells, this chimera was exclusively localized to the basolateral surface. This indicates that the N-terminal domain of syntaxin 3 contains information for its polarized localization. In contrast to the apical localization of syntaxin 3, the basolateral localization of syntaxin 4 was not dependent on its N-terminal domain. Syntaxin 3 normally binds to Munc18b, but not to the related Munc18c. Overexpression of the chimera together with overexpression of Munc18b caused membrane and secretory proteins that are normally sent primarily to the apical surface to exhibit increased delivery to the basolateral surface. We suggest that syntaxins may play a role in determining the specificity of membrane targeting by permitting fusion with only certain target membranes.

INTRODUCTION

Eukaryotic cells contain numerous intracellular membranous compartments that are connected by vesicular traffic. After vesicles bud off from a membrane, they must be targeted to and fuse with the correct target membrane. How vesicles are specifically targeted to the correct membrane and avoid fusion with the incorrect membrane remains a paramount question (Mostov *et al.*, 2003; Nelson, 2003; Rodriguez-Boulant *et al.*, 2005). Several classes of molecules may contribute to this specificity and it is possible that specificity is conferred by multiple layers of molecular machinery, which may act sequentially. For instance, at least in larger animal cells, vesicles may first reach the vicinity of their target membrane by using motor proteins and cytoskeletal filaments. Thus, some of the specificity may be achieved by binding and activation of the correct motor protein to the vesicle. In many trafficking steps, vesicles are then brought closer to the membrane by tethering complexes. For in-

stance, the yeast exocyst and its homologous mammalian sec6/8 complex tether vesicles to the specific locations on the plasma membrane (Lipschutz and Mostov, 2002; Novick and Guo, 2002).

SNARE proteins act later and may catalyze fusion itself (Sollner, 2003; Ungar and Hughson, 2003; Jahn, 2004). The original formulation of the SNARE hypothesis postulated that specific v-SNAREs on the vesicle paired with cognate t-SNAREs on the target, thereby providing specificity to fusion (Sollner *et al.*, 1993). This premise was challenged when SNARE proteins, lacking their membrane anchors, were produced. Soluble versions of v- and t-SNAREs paired almost completely promiscuously, suggesting that pairing of specific SNAREs did not contribute to specificity (Fasshauer *et al.*, 1999; Yang *et al.*, 1999). More recently, full-length SNAREs have been embedded into liposomes. This has been used to reconstitute membrane fusion between liposomes (or other lipid bilayers) containing v-SNAREs and those containing t-SNAREs (Weber *et al.*, 1998; McNew *et al.*, 2000). In this *in vitro* system, substantial specificity was observed, which largely mirrored the specificity of fusion events known *in vivo*. Nevertheless, interpretation of the liposome fusion results have been controversial, e.g., because fusion appears to be substantially slower than *in vivo*. *In vivo*, especially in yeast, some SNAREs can act in multiple pathways, suggesting that SNAREs cannot provide all of the specificity (Pelham, 2001). So far, a direct test of the role of syntaxins in the specificity of fusion *in vivo* has not been reported.

Here we studied the role of syntaxins, a subunit of the t-SNARE, in the specificity of fusion in polarized epithelial cells. The plasma membrane of these cells is divided into separate apical (AP) and basolateral (BL) domains, which are separated by tight junctions. These surfaces have largely nonoverlapping protein compositions. Proteins are sent to the correct surface either by direct delivery from the TGN, or

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Abbreviations used: Adv, adenovirus; AP, apical; BL, basolateral; dox, doxycycline; EGFP, enhanced green fluorescent protein; MDCK, Madin-Darby Canine Kidney; NTD, N-terminal domain; S/M, Sec1/Munc18; pIgR, poly-immunoglobulin receptor; syx3, syntaxin 3; syx4, syntaxin 4; syx1A, syntaxin 1A; TGN, *trans*-Golgi network; TM, transmembrane domain.

by delivery to one surface and then endocytosis followed by transcytosis to the other surface. Syntaxin 3 (syx3) is located exclusively at the AP domain, as well as intracellularly in endosomes and lysosomes (Gaisano *et al.*, 1996; Low *et al.*, 1996; Fujita *et al.*, 1998). In contrast, the highly homologous syntaxin 4 (syx4) is located entirely at the BL surface. This pattern has been observed in numerous epithelial cell types, suggesting that it may reflect a fundamental aspect of the roles of syx3 and 4 in specific traffic. Syx3 is involved in TGN to AP traffic, whereas syx4 has been implicated in TGN to BL traffic (Low *et al.*, 1998; Lafont *et al.*, 1999). The existence of branching pathways from the TGN to AP or BL surface makes epithelial cells a particularly fortuitous system for studying the role of syntaxins in the specificity of vesicle fusion.

We took advantage of the homology between syx3 and syx4 to construct chimeras between the two syntaxins. We found that the N-terminal 29 amino acids of syx4, when substituted into the N terminus of syx3, were sufficient to redirect the chimeric syx from the AP to the BL surface. We then examined the effect of expression of this chimera on polarized traffic and showed that some cargo proteins were partially redirected from the AP to the BL surface, directly showing that syntaxins play a role in the specificity of membrane fusion *in vivo*.

MATERIALS AND METHODS

Generation of Syntaxin Chimeras and Mutants and Other Constructs

Plasmids containing syx3 and HA-tagged syx4 have been described previously (Low *et al.*, 1996). Syx3 was HA-tagged by subcloning. We checked the effect of the presence of HA-tag on localization of syx3 and 4 and could not find any differences from the nontagged versions. Chimeras were made by introducing restriction sites (*EcoRI* for syntaxin 3/4(1–38), *ScaI* for syntaxin 3/4(1–29) and *BssHII* for syntaxin 3/4-TM) via silent mutations in the sequence using PCR. The products were digested and ligated to form the chimeras. Mutations were made using the Quickchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA). All sequences were verified at the UCSF core sequence facility.

The constructs with mouse Munc18b and Munc18c were a kind gift of Dr. J. Pessin (University of Iowa, Iowa City, IA). Munc18b already had an N-terminal FLAG-tag and in Munc18c a FLAG-tag was introduced by PCR. Both Munc18b and Munc18c were subcloned in pADtet (Verges *et al.*, 2004).

For making the EGFP constructs, the N-terminal regions and the TMDs of syx3 and 4 were generated with the appropriate restriction sites using PCR and cloned into pEGFP-N1 (Clontech, Palo Alto, CA) and this new construct was subcloned into pCB7. The vector for expressing p75NTR was a kind gift of Dr. M. Chao (New York University Medical Center, New York, NY).

The GST constructs were made by subcloning the different constructs in one of the pGEX-4T vectors (Amersham Biosciences, Piscataway, NJ) depending on the reading frame.

Cell Culture, Transfections, and Generation of Cell Lines

MDCK cells were grown in MEM supplemented with penicillin/streptomycin (pen/strep) and 10% fetal bovine serum (FBS). COS-7, Cre8, and 293 cells were grown in DMEM with 4.5 g/ml glucose supplemented with pen/strep and 10% FBS. Fisher rat thyroid (FRT) cells were grown in Coon's modified Ham's-F12 medium supplemented with glutamine, pen/strep, and 10% FBS.

Transient transfections on MDCK cells, grown for 2 d on filters, were done with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. Stable transfections were done with the calcium/phosphate precipitation method. Clones were selected using hygromycin (pCB7) or G418 (pCDNA3).

In case of tet-repressible MDCK cell lines, a MDCK cell line with the transactivator present (T23; Verges *et al.*, 2004) was stably transfected with a vector (pADtet) containing the gene of interest and cotransfected with pCDNA6/V5-His (Invitrogen). Clones were selected using blasticidin (Calbiochem, La Jolla, CA). Clones were chosen for nonleaky inducible expression and polarization state of noninduced cells.

Adenovirus Preparation and Cellular Infection

Adenovirus (Adv) were made according to published methods (Verges *et al.*, 2004) using Cre8 and 293 cells. The p75NTR virus was a kind gift of Dr. E.

Rodriguez-Boulant (Cornell University Medical College, New York). For infections of MDCK cells, titers of 3–4 pfu/cell were used. In case of coinfection of transactivator (tta) virus a titer of 4 pfu/cell was used. MDCK cells were infected with Adv as described previously (Verges *et al.*, 2004). In some cases during infection 20 ng/ml doxycycline (dox) was present to repress expression of the proteins. After incubation, fresh medium with or without dox was added, and the cells were incubated for another 18 h.

Immunofluorescence Microscopy

Mouse antibodies to gp135, E-cadherin, and rabbit polyclonal antibodies to syx3 and 4 have been described previously (Low *et al.*, 1996). The syx3 and 4 antibodies were affinity-purified according to standard protocols. Polyclonal antibody to HA-tag and goat antibodies to Munc18b and c were from Santa Cruz Biotechnology (Santa Cruz, CA), FLAG-tag antibodies (M2 and M5) were from Sigma (St. Louis, MO), and the antibody to GFP was from Roche (Indianapolis, IN). The polyclonal antibody to p75NTR was a generous gift from Dr. M. Chao.

Cells were processed for immunofluorescence as described (Low *et al.*, 1996) using Alexa-dye antibodies (Alexa-488 or -555, Molecular Probes, Eugene, OR) as secondary antibodies. In some cases actin was visualized by using fluorescently labeled phalloidin: Alexa-594 (Figure 1) or -546 (Figure 4) (Molecular Probes). Confocal microscopy was done on a Bio-Rad MRC1024 (Hercules, CA) with a Nikon eclipse TE300 inverted microscope (Melville, NY), Plan Apo 63 $\times$ oil objective (Figure 1) or a Zeiss LSM5 Pascal confocal microscope (Thornwood, NY), Planapochromat 63 $\times$ oil objective (Figure 4). Images were converted to Tiff format using NIH image (Figure 1) or Image J (Figure 4) and final composite figures were created using Adobe Photoshop 5.5 (Mac; San Jose, CA).

Delivery Assays

The delivery of p75NTR was done according to methods described previously (Yeaman *et al.*, 1997). Briefly, cells expressing p75NTR were pulsed for 20 min and chased for 2 h with ³⁵S-L-cysteine (NEN, Boston, MA). The surface was biotinylated (Pierce, Rockford, IL) at the apical or basolateral side and subsequently the total p75NTR was immunoprecipitated. p75NTR was eluted from the immunoprecipitates by boiling in SDS. Part of the immunoprecipitate was analyzed for total p75NTR and the remaining part was incubated with streptavidin (Pierce) to recover the biotinylated p75NTR. Samples were run on SDS-PAA gels and analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

The basolateral delivery of pIgR was done as described (Aroeti *et al.*, 1993). In this case cells infected with chimera with or without dox were pulse-chase labeled with ³⁵S-L-cysteine (NEN) and the amount of basolateral delivered pIgR was determined by reduction of labeled pIgR from cells treated with V8 protease at the basolateral side compared with nontreated cells.

Basolateral to apical transcytosis was done as described previously (Cardone *et al.*, 1994) on T23 infected with syx3/4(1–29) chimera.

The delivery of gp80 was done by pulse-labeling cells for 15 min with ³⁵S-L-cysteine/methionine at 37°C (Amersham Biosciences), let gp80 accumulate at 18°C in the Golgi for 2 h and chase for 1 h at 37°C (Hansen *et al.*, 1995). Samples were taken from apical and basal side and run on SDS-PAA gel under reducing conditions. In this case gp80 gives three bands whose intensity was quantified by phosphorimager (Molecular Dynamics).

Immunoprecipitations

For immunoprecipitations, cells grown on filter were lysed for 30 min in buffer containing HEPES (25 mM) pH 7.4, NaCl (150 mM), EDTA (5 mM) and Triton-X-100 (1%). This buffer was supplemented with a protease inhibitor cocktail and the phosphatase inhibitors NaF (10 mM) and Na₃VO₄ (100 mM). The lysate was centrifuged for 15 min and the supernatant was precleared by incubating 15 min with CL-2B beads. The lysate was first incubated for 30 min with the anti-FLAG antibody (M2, Sigma) and after this incubation protein-G was added and incubation was continued for 2 h. The beads were washed five times with lysis buffer. All steps were done at 4°C. The immunoprecipitate complexes were analyzed by SDS-PAA gel electrophoresis and Western.

GST-pulldown Experiments

GST-syx proteins were made using standard procedures. Quantification of GST constructs was done by comparing with different bovine serum albumin concentrations on SDS-PAA gel. For the pulldown of Munc18b or c using the different GST constructs, COS-7 cells were transiently transfected with Munc18b or c expression vectors. After 2 d of expression, a lysate was made using the above described lysis buffer. The lysate was centrifuged at 100,000 $\times$ g for 1 h at 4°C and 200 μ g of supernatant was incubated for 2 h with 15 μ g of the different GST constructs bound to glutathione beads with rotation at 4°C. After the incubation the beads were extensively washed with lysis buffer and pulled down proteins were analyzed by SDS-PAA gel electrophoresis and Western blotting.

RESULTS

To identify the sorting signals responsible for polarized localization of syntaxins, we made chimeras by exchanging structural domains of syx3 and 4 (Figure 1, A and B). Exchange of the transmembrane domain (TMD) of syx4 into the backbone of syx3 (syx3/4-TMD, construct *a*) did not change the apical localization of syx3. Putting the H3 helix of syx4 into syx3 (syx3/4-H3, construct *b*) yielded very poor expression and intracellular localization, presumably due to misfolding.

Strikingly, when we exchanged the N-terminal domain (NTD, residues 1–29) of syx4 onto the remainder of syx3 (syx3/4-(1–29), construct *c*), the chimera was completely redirected from the AP to the BL surface. This result was robustly obtained with several expression methods (transient transfection, recombinant adenovirus, or stable cell lines with Tet-repressible expression), and was also obtained with FRT cells, which localize some GPI-anchored proteins differently from MDCK cells (Supplementary Figure S1A).

When EGFP (enhanced green fluorescent protein) was anchored to the membrane by the syx4-TMD, (EGFP/syx4-TMD, Figure 1, A and B, construct *e*), it was mostly apical, with some basolateral. Adding the syx3-NTD (syx3-(1–34)/EGFP/4-TMD), construct *f*) did not affect the localization. In contrast, adding the syx4-NTD (syx4-(1–40)/EGFP/syx4-TMD, Figure 1, A and B, construct *g*) was sufficient to redirect the chimera entirely to the BL surface. These findings indicate that the syx4-NTD contains a cytoplasmic BL targeting signal, similar to the BL signals found in many BL proteins.

Consistent with this idea, the NTDs of syx3 and syx4 are each very highly conserved in vertebrates. Furthermore, the NTD of the related plasma membrane syntaxin 1A (syx1A) is unstructured in the crystallographic structure of syx1A paired with Munc18a (Misura *et al.*, 2000), suggesting that the NTD could be free to interact with the basolateral targeting machinery.

We systematically mutated the NTD residues that differ between syx3 and syx4, changing groups of 2–4 residues at a time, and identified L25, V26 as amino acids involved in the basolateral localization of the chimera. Mutation of these residues in the chimera into alanine gave a random plasma membrane localization of the chimera (syx3/4-(1–38)-L25A, V26A, Supplementary Figure S1, B and C, construct *e*). Interestingly, introducing a leucine and valine in the syntaxin 3 NTD, (I24L, A25V Supplementary Figure S1, B and C, construct *f*) resulted in a nonpolarized localization of syntaxin 3.

Deletion of the NTD from syx3 (syx3-Δ(1–34), Figure 1, A and B, construct *d*) caused localization to both surfaces, suggesting that the syx3-NTD plays some role in apical targeting (Figure 1B and Supplementary Figure S1, B and C). Interestingly, another deletion of fewer residues (syx3-Δ(1–28), construct *n* in Supplementary Figure S1, B and C) did not result in mistargeting, suggesting a role for residues 29–34 in the apical targeting of syntaxin 3. Also, mutating residues 24–27 (constructs *k–m* in Supplementary Figure S1, B and C) resulted in mistargeting of syntaxin 3, suggesting multiple regions in the N-terminal region of syntaxin 3 important for its apical localization.

However, we could not redirect syx4 to the AP surface by substituting in any single domain of syx3, including the syx3-NTD (Figure 1A, constructs *h–j*).

Proteins of the Sec1/Munc18 (S/M) family bind to syntaxins and may act as chaperones to regulate SNARE complex assembly (Gallwitz and Jahn, 2003; Gerst, 2003; Toonen

A

B

Figure 1. The NTD of syx3 is important for its apical localization. (A) This is a schematic representation, showing the organization of the different domains of syx3 and 4, the chimeras of both syntaxins, the fusions of EGFP linked to different domains of syx3 and 4, and a summary of the localization results. In the diagram blue is syx3, red is syx4, and green is EGFP. (B) Vertical (X-Z) sections of localization of syntaxins, chimeras, and fusions in MDCK cells, visualized by immunofluorescent confocal microscopy. The top panel is syx3 wild-type (stained green with anti-syx3), which is entirely apical. The boundaries of the cell are visualized using fluorescent phalloidin (red). Syx3/4-(1–29) has a clear basolateral localization, whereas syx3/4-TMD and syx3-Δ(1–34) are mainly apical (though with a small amount of basolateral signal). The bottom three panels show localization of the EGFP fusions, which were stained green with anti-EGFP. EGFP/syx4-NTD and syx3-(1–34)/EGFP/syx4-TMD were mainly apical, with some basolateral. Use of the NTD of syx4 (syx4-(1–40)/EGFP/syx4-TMD) directed the EGFP entirely to the basolateral surface. These images were from experiments where the different constructs were transiently transfected into MDCK cells, so only a fraction of the cells in the monolayer express the transfected gene. Equivalent localizations were obtained with other expression methods, such as selection of stable cell lines under tet-repressible control, or recombinant adenovirus. Bar, 10 μ m.

Figure 2. Syx3/4(1-29) chimera binds both Munc18b and c. (A) MDCK cell lines were established that express FLAG-tagged Munc18b or c under tet-repressible control; removal of doxycycline (dox) induces expression. These cells were incubated with or without dox for 4 d. Lysates of these cells were made and analyzed by immunoblotting for the presence of Munc18b (top row) or Munc18c (bottom row), using antibodies specific for either protein. Left lane shows the parental T23 cell line. Munc18b was detected by its antibody only in the Munc18b-expressing cell line grown in the absence of dox; endogenous Munc18b was not detected under these conditions. Endogenous Munc18c was detected by its antibody in all cells, though the level of Munc18c was much higher in the Munc18c-expressing cells grown in the absence of dox. (B) Cells were grown for 4 d + or - dox, and the FLAG-tagged Munc18b or c were immunoprecipitated using anti-FLAG antibody. In the top row the immunoprecipitates were analyzed for the presence of FLAG-tagged Munc18b or c by Western blotting with anti-FLAG. In the bottom row the immunoprecipitates were analyzed for the presence of coimmunoprecipitating endogenous syx3 or 4 by Western blotting with syx3- or syx4-specific antibodies. A fraction (25 μ l) of total cell lysate (1 ml) was also analyzed (lysate). (C) MDCK cells with Munc18b or c under tet-repressible control were grown for 4 d without dox and infected with Adv encoding syx3, 4 or the syx3/4(1-29), as indicated below the panel. The cells were incubated for 18 h to allow expression of the syntaxins. The FLAG-tagged Munc18b or c were then immunoprecipitated. The immunoprecipitates were analyzed by Western blotting for the presence of FLAG-tagged Munc18 (top row). In the bottom row coimmunoprecipitated, exogenously expressed syx3 or 3/4(1-29) were detected with anti-syx3, whereas exogenously expressed syx4 was detected with anti-syx4. Control lanes (con) are from cells not infected with syntaxin expressing Adv and shows immunoprecipitation of the FLAG-tagged Munc18b or c. In these controls, no endogenous coimmunoprecipitating syntaxins were detected with antibody to syx3, due to the small number of cells used.

and Verhage, 2003). As the S/M proteins Munc18b and c bind specifically to syx3 and 4, respectively, we investigated the effects of our chimeras on interaction with Munc18s (Hata and Sudhof, 1995; Tellam *et al.*, 1995; Riento *et al.*, 1996).

We stably expressed FLAG-tagged Munc18b or c under tet-repressible control (Figure 2A). Expression of Munc18b or c alone had no effect on polarization, as determined by IF

using gp135, p58 and ZO-1 as markers for respectively AP, BL, and tight junctions (unpublished data). We also detected no effect on transcytosis or recycling of pIgR (unpublished data), suggesting that overexpression of Munc18b or c did not affect these pathways, which are known to be syx3-independent. As expected, when FLAG-tagged Munc18b was immunoprecipitated, endogenous syx3 was present in the immunoprecipitate, but endogenous syx4 was not detected (Figure 2B). Conversely, endogenous syx4, but not endogenous syx3, was coimmunoprecipitated with FLAG-tagged Munc18c (Figure 2B).

To study the interaction of syx3/4(1-29) with Munc18, we infected the FLAG-tagged Munc18-expressing cells with recombinant adenovirus (Adv) encoding syx3/4(1-29) or wild-type syntaxins as controls. FLAG-tagged Munc18b or c were immunoprecipitated and analyzed for the presence of coimmunoprecipitating exogenously expressed syntaxins. As shown in Figure 2C, the recombinant syx3 and 4 maintained their specificity for binding only to Munc18b or c, respectively. Surprisingly, syx3/4(1-29) could be coimmunoprecipitated with either Munc18b or c.

To understand why syx3/4(1-29) can bind to both Munc18b and c, we reconstituted this interaction using purified syntaxins fused to GST, which were incubated with lysates from COS-7 cells transiently transfected with Munc18b or c. As expected, syx3 bound to Munc18b, syx4 bound to Munc18c, and syx3/4(1-29) bound to both Munc18b and c (Figure 3A). Deletion of the NTD of syx3 (syx3- Δ (1-34)) or the NTD of syx4 (syx4- Δ (1-40)) did not alter the specific binding of these syntaxins to Munc18b or c, respectively. This indicates that the specific binding of the syntaxins to their cognate Munc18 can be mediated by a portion of the syntaxin other than the NTD.

To test the role of the NTDs alone, we fused just the NTDs of syx3 or syx4 to GST and used these to pull down Munc18s. We found that the syx4-NTD bound specifically to Munc18c, whereas the syx3-NTD did not bind to either Munc18 (Figure 3B). To confirm this interaction *in vivo*, we used the chimeras described in Figure 1 of the syntaxin NTDs fused to EGFP and anchored by the syx4-TMD (Figure 1A, constructs f and g). These were stably expressed and immunoprecipitated using anti-GFP. The chimera with the syx4-NTD could specifically coimmunoprecipitate Munc18c, but not b, whereas the chimera with the syx3-NTD did not detectably coimmunoprecipitate either Munc18 (Figure 3C).

We next examined how the interaction of syx3/4(1-29) influenced the localization of Munc18s. Although we could not reliably localize endogenous Munc18b or c in MDCK cells, overexpressed Munc18b (Figure 4, A-C) and Munc18c (D-F) were diffusely cytosolic, consistent with previous observations. Overexpression of syx3 caused recruitment of Munc18b (Figure 4, G-I) but not Munc18c (J-L), to the apical plasma membrane. Note the yellow color at the apical surface in panels G and I, where Munc18b is coexpressed with syx3, indicating the colocalization of the two proteins.

In contrast, where syx3 is coexpressed with Munc18c, the apical surface is mainly green, showing that syx3, but not Munc18c, is at the apical surface (Figure 4, J and L). Conversely, overexpression of syx4 caused recruitment of Munc18c to the basolateral plasma membrane (Figure 4, P-R). Note the yellow basolateral outline of the cells' basolateral surface in panel Q and the yellow lateral borders in the X-Z section in panel R, indicating the colocalization of syx4 and Munc18c. In panels Q and R, the diffuse cytosolic staining of Munc18c was reduced in the cells that expressed high levels of syx4, which also supports the idea that Munc18c is recruited from the cytosol to the plasma mem-

Figure 3. N-terminal regions of syx3 and 4 are essential for specificity in Munc18 binding. (A) FLAG-tagged Munc18b or c were transiently transfected into cells. After 2 d of expression, cells were lysed. Lysates were incubated with 15 μ g of different GST-syntaxin fusion proteins (lacking the TMD of the syntaxin), as indicated above the panel. After incubation, GST-constructs were pulled down with glutathione-beads and extensively washed. Pulled down proteins were analyzed by Western blotting for the presence of FLAG-tagged Munc18b or c. Note that syx3/4-(1-29) is uniquely able to bind to both Munc18b and c. GST alone bound to glutathione beads did not pull down Munc18b or c (gst), and lysate shows the expression levels of Munc18b or c in transfected cells. (B) FLAG-tagged Munc18b or c were transiently in cells for 2 d and the cells were lysed, as in Figure 3A. Lysates were incubated with 15 μ g of fusion proteins containing just the NTD of syx3 (residues 1-34) or syx4 (residues 1-40) fused to GST. Note that syx4 NTD binds specifically to Munc18c, whereas the syx3 NTD shows no detectable binding to either Munc18b and c. GST alone bound to glutathione beads, while lanes labeled "lysate" is lysate of cells expressing Munc18b or c. In panels a and b, the FLAG-tagged Munc18b and c were transiently expressed in COS-7 cells to produce large amounts of protein easily. Equivalent results, though with weaker signals, were obtained using MDCK cells expressing FLAG-tagged Munc18b or c (unpublished results). (C) MDCK cell lines were made stably expressing EGFP fused with the NTD of syx3 or syx4 and the TMD of syx4 (syx3-(1-34)/EGFP-syx4-TMD and syx4-(1-40)/EGFP-syx4-TMD, respectively; schematics of their structures are shown Figure 1A, constructs f and g). These cells were transiently transfected with FLAG-tagged Munc18b or c, and the syntaxin NTD-EGFP fusions were immunoprecipitated with anti-EGFP. The immunoprecipitates were analyzed by Western blotting for the presence of Munc18b or c using the FLAG-antibody.

brane. In contrast, overexpression of syx4 largely did not recruit Munc18b to the basolateral surface. Note the green basolateral outline of the cells in panel N. (There is a small amount of yellow staining in panels N and O, suggesting a low level of recruitment of Munc18b by syx4; the reason for this is unknown.)

Importantly, overexpression of syx3/4-(1-29) caused the recruitment of both Munc18b and c to the basolateral sur-

Figure 4. Syx3/4-(1-29) chimera relocates Munc18b to the basolateral membrane. MDCK cells were grown for 4 d without dox. Cells in A-C, G-I, M-O, and S-U express FLAG-tagged Munc18b, whereas all other panels express FLAG-tagged Munc18c. Munc18s were detected with anti-FLAG and stained red. In controls, no Adv was used (A-F). Other cells were infected with Adv to express syx3 (G-L), syx4 (M-R), or syx4/3-(1-29) (S-X). In the no-virus control (A-F) actin was stained green to outline the cells. In virus infected HA-tagged syx3, syx4, or syx3/4-(1-29) were visualized with anti-HA; all HA-tagged syntaxins were stained green. Note that the level of expression of syntaxins is very variable between cells. Horizontal (X-Y) sections are shown at the level of the apical surface (top row in each section), and through the middle portion to show basolateral staining (middle row in each section). Vertical (X-Z) sections are shown in the bottom row of each section. Munc18b or c (red) were only visible in the induced cells and absent in cells incubated with dox (unpublished data). Bar, 20 μ m.

face. Note that the basolateral surface is yellow in both Munc18b (Figure 4, T and U) and Munc18c (Figure 4, W and X). Again, there is a reduction in diffuse cytosolic staining of Munc18b and c in these panels, consistent with recruitment from cytosol to plasma membrane. This suggests that the interaction of syx3/4-(1-29) with both Munc18s occurs *in vivo* and is capable of localizing both Munc18s to the site of this syntaxin chimera.

Finally, we examined the role of syntaxins and Munc18s in the specific targeting of proteins to the apical or basolateral surface. We asked if the ectopic localization of syx3 to the basolateral surface would cause some normally AP "cargo" proteins to be delivered instead to the BL surface.

Stable cell lines expressing Munc18s were infected with adenovirus encoding syntaxins under tet-repressible control. We quantified the polarized secretion of gp80, the major endogenous secretory protein of MDCK cells. Munc18b or c alone, or in combination with syx3 or syx4, had no effect on polarized secretion (Figure 5, A and B). In contrast, expression of syx3/4-(1-29) together with Munc18b (but not

Figure 5. Munc18b causes an increase in basolateral retargeting of gp80 and p75NTR when coexpressed with syx3/4-(1-29). Stable MDCK cell lines expressing FLAG-tagged Munc18b or c under control of tet-repressible promoter were grown in the absence of dox for 4 d to induce expression of the Munc18. Cells were then infected with Adv encoding syx3, syx4, or syx3/4-(1-29). Infected cells were incubated for 18 h with the indicated concentration of dox to allow controlled expression of the syntaxin (see Supplementary Figure S2). Previously expressed Munc18b or c remained at a nearly constant level during these 18 h. Apical and basolateral secretion of gp80 was determined by a metabolic pulse-label and 60-min chase and analyzed on SDS-PAGE gel. Apical and basolateral secretion of gp80 was normalized to gp80 secretion of uninfected cells grown in the presence of 20 ng/ml dox and expressed as a percentage of the total secreted gp80; this total did not change with increasing chimera expression. (A) MDCK cells coexpressing syx3 and Munc18b or c do not show any significant missorting of gp80 ($n = 4$). (B) Similarly MDCK cells coexpressing syx4 and Munc18b or c do not show any significant missorting of gp80 ($n = 4$). (C) MDCK cells coexpressing syx3/4-(1-29) and Munc18b show a significant increase in basolateral secretion of gp80. Under control conditions (i.e., no virus) $24 \pm 4\%$ of total secreted gp80 was basolateral. In contrast, at 0 ng/ml dox (full syx3/4-(1-29) expression), basolateral delivery of gp80 was increased to $39 \pm 4\%$ ($n = 4$, $p < 0.005$). In all quantitative experiments in this article the mean and SD are shown when significant as determined by Student's *t*-test. (D) MDCK cells with Munc18b or c under tet-repressible control were grown for 4 d without dox and coinfecting with Adv for syx3/4-(1-29) and Adv for p75NTR (In this particular cell line, p75NTR expression was constitutive, not under tet repressible control). After 18-h incubation, delivery of p75NTR to the apical and basolateral plasma membranes was determined. Delivery of p75NTR normalized to control cells (where expression of both Munc18 and chimera were completely suppressed by inclusion of dox) is shown ($n = 5$). Basolateral delivery for control + dox was $11 \pm 2\%$ of total p75NTR plasma membrane delivery and basolateral delivery when Munc18b was overexpressed together with syx3/4 was $18 \pm 3\%$ ($p < 0.001$).

Munc18c) increased BL secretion by 1.66-fold (Figure 5C). AP secretion decreased by 21%, so the ratio of basolateral/apical increased by 2.1-fold. A lesser degree of expression, produced by inclusion of 0.1 ng/ml dox, gave a smaller effect, showing that the degree of mistargeting was depen-

dent on the level of expression. We also examined a transfected apical membrane protein, p75NTR. As shown in Figure 5D, the combined expression of syx3/4-(1-29) and Munc18b increased basolateral delivery by 177% and decreased apical delivery by 16%, again giving a 2.1-fold change in the basolateral/apical ratio. These results are a minimal estimate, as expression of syx3/4-(1-29) is not uniform, with some cells having much less expression than others (Figure 4, S–X). As might be expected, much higher levels of expression begin to randomize polarity, damaging the integrity of the monolayer and thereby preventing us from accurately measuring polarized delivery (unpublished results).

Expression of syx3/4-(1-29) without overexpression of Munc18b gave smaller, but statistically significant, effects on mistargeting of gp80 and p75NTR (Supplementary Figure S2). In contrast, Munc18b overexpression without syx3/4-(1-29) gave no effect. This suggests Munc18b may not directly contribute to specificity, but the elevated level of exogenous Munc18b may help provide sufficient Munc18b at the basolateral surface to facilitate the functioning of the syx3/4-(1-29). Nonetheless, only Munc18b and not Munc18c works in combination with syx3/4-(1-29) (Figure 5, C and D). This suggests that the interaction of Munc18c with the syx4-NTD that is contained within syx3/4-(1-29) is not sufficient to promote mistargeting and that syx3/4-(1-29) and Munc18b combined have a very specific effect on the targeting machinery.

DISCUSSION

We constructed chimeras of syx3 and 4 and found that the NTD of syx4 contained a BL targeting signal that was sufficient to target a syx3 chimera (syx3/4-(1-29)) or membrane-anchored EGFP to the BL domain. Expression of syx3/4-(1-29) together with Munc18b caused the partial mistargeting of two AP cargo proteins, gp80 and p75NTR, to the BL surface. This suggests that syntaxins contribute to the specificity of vesicle targeting in polarized epithelial cells.

Our observed degree of retargeting of both secretory and apical membrane proteins to the basolateral surface is very likely to be physiologically relevant. The high degree of polarization of membrane proteins at steady state is the combined product of polarized biosynthetic targeting, selective stabilization and sorting into the recycling or transcytotic pathways after endocytosis. Polarized biosynthetic targeting is only about ~80–90% accurate, as shown here and in many previous studies (Rodríguez-Boulán *et al.*, 2005), and so a 2.1-fold shift is a significant fraction of the total available dynamic range.

Our experiments were conducted in the presence of endogenous syx3, which may compete for directing apical cargo proteins to the normal apical location. We have been unable to substantially reduce the level of endogenous syx3 by a variety of transient or stable RNAi approaches. This is consistent with the results of Schuck and Simons, (Schuck *et al.*, 2004), who were unable to substantially knock down syx3 using a lentivirus method that successfully knocked down all 13 other proteins that they tried. It may be that RNAi of syx3 has unexpected toxicity to MDCK cells, e.g., loss of the TGN to AP pathway could cause toxic accumulation of some proteins intracellularly.

Our experiments in the presence of endogenous syx3 enable the cells to maintain some polarity, which permits us to measure polarized targeting; very likely this would not be possible if syx3 and AP targeting were simply eliminated. Although the background of endogenous syx3 may reduce

the magnitude of retargeting of AP cargo to the BL surface that we can observe, our experimental design allows us to clearly show that retargeting syx3 to the BL surface is permissive for normally AP vesicles to fuse instead with the BL surface. This key observation directly shows that syntaxins play a role in specificity of targeting *in vivo*.

Although our data show that syx3 is involved in the specificity of apical membrane fusion, it is not clear what the mechanism is behind this specificity. Coimmunoprecipitation studies showed that despite the different localization of syx3 and syx3/4-(1–29) both bind VAMP-7 (Ti-VAMP) in contrast to syx4 (unpublished data). This would indicate that the specificity in SNARE pairing was not changed in the syx3/4-(1–29).

Recently it was found that at least for some yeast SNAREs the specificity is defined by the core region (Paumet *et al.*, 2004). It remains to be seen if this is also the case for the plasma membrane syntaxins. The hydrophobic residues in the core region of the plasma membrane syntaxins that interact with the other SNARE proteins are well conserved. Yet there are several residues in the core region that are different in syx3 when compared with syx4. These residues may interact with a yet to be identified other protein that could be involved in the specificity.

The interaction of the different plasma membrane syntaxins with complexin I and II is a good example that the core region can bind selectively to different proteins. Among the plasma membrane syntaxins, only syx4 is not able to bind complexin I or II and this specificity in binding involves a small stretch of amino acids (Pabst *et al.*, 2000). Complexins are thought to be involved in the late stages of membrane fusion (Reim *et al.*, 2001; Tokumaru *et al.*, 2001) so it is not likely they play a role in specificity.

We cannot exclude that the Habc domain may also play role in determining the specificity. However, in previous studies it was found that this domain has a regulatory function (Nicholson *et al.*, 1998; Dulubova *et al.*, 1999; Parlati *et al.*, 1999; Munson *et al.*, 2000).

It remains to be seen if syntaxins are similarly involved in specificity of targeting in other membrane traffic steps. Syx1A, a neuronal-specific syntaxin, is normally found at the plasma membrane. When expressed in HeLa cells, syx1A is retained in the ER and causes the mistargeting of normally plasma membrane cargo to the ER (Martinez-Arca *et al.*, 2003). Although these results are compatible with our conclusion that syntaxins are involved in the specificity of targeting, a limitation is that they are based on expression of syntaxin in a cell type that normally does not make syx1A. This ectopic syx1A was retained in the ER by an unknown mechanism, though the observation that coexpression of its cognate S/M protein, Munc18a, enabled syx1A and the mislocalized cargo to move to the plasma membrane, suggests the syx1A in the ER may have been improperly folded or Munc18a is needed for plasma membrane localization of syx1A.

Syntaxin 3/4-(1–29), and to a lesser extent chimera syx4/3-(1–32), do not exclusive bind to one munc18 isoform as their wild type syntaxin form do. This suggests that the NTDs plays a role in munc18 binding. Indeed, our data indicate that the syx4-NTD can specifically interact with Munc18c, and this can account for the ability of syx3/4-(1–29) to bind to both Munc18s. Syx4 therefore interacts with Munc18c in two ways, through its NTD and via the remainder of the syx4 molecule. S/M proteins are known to interact with syntaxins in several modes (Carr *et al.*, 1999; Misura *et al.*, 2000; Bracher and Weissenhorn, 2002; Dulubova *et al.*, 2002, 2003). As shown for closely-related Munc18a and

syx1A (Misura *et al.*, 2000), the Munc18 can wrap around the folded syntaxin; this may be how syx3 and 4 interact with their cognate Munc18s, even in the absence of the syntaxin NTDs. Alternatively in several nonplasma membrane routes, the S/M protein binds to the extreme N terminus of the syntaxin. This may be how the syx4-NTD binds to Munc18c.

Several articles have shown that the function plasma membrane syntaxins are closely connected to the presence of their interacting S/M partner. For at least two forms of munc18 (a and c) null mice have been obtained and in both cases this resulted in a reduced expression of their cognate syntaxin (Verhage *et al.*, 2000; Kanda *et al.*, 2005). Interestingly, in case of Munc18a its absence resulted in total loss of neurotransmitter release, but in the case of Munc18c an increase in GLUT4 translocation was found.

It is not clear if absence of Munc18a or c resulted in the inability of syntaxins to reach the plasma membrane, as was shown for a cell line system in which the absence of Munc18a resulted in intracellular retention of syx1A (Rowe *et al.*, 1999). However, in null mutants of *unc-18* in *Caenorhabditis elegans*, syntaxin was still found at the plasma membrane (Weimer *et al.*, 2003).

In our study we show that Munc18 may also contribute to the specificity, as only Munc18b together with syx3/4-(1–29) causes an increase in basolateral missorting of apical proteins. It is possible, though, that the contribution of Munc18b to specificity is only indirect, e.g., in enabling the syx3/4-(1–29) chimera to function properly at the basolateral surface.

Other molecules have been identified that influence polarized biosynthetic sorting at each of several steps. For example, in the TGN, protein kinase D is involved in production of vesicles that go to the basolateral, but not apical surface (Yeaman *et al.*, 2004). Interestingly, expression of kinase-dead protein kinase D gives an approximately two-fold decrease in the basolateral/apical ratio, quite similar to the magnitude of the effect in our experiments, but in the opposite direction. After leaving the TGN, vesicles can ride on microtubules to the vicinity of the plasma membrane. Depolymerization of microtubules results in delivery of apical cargo to the basolateral surface. Interestingly, syx3 is also mislocalized to the basolateral surface under these conditions and the misdelivery of apical proteins to the basolateral surface is syx3-dependent (Kreitzer *et al.*, 2003).

A likely interpretation is that one effect of microtubule depolymerization is to ectopically localize syx3 to the basolateral surface, thereby redirecting cargo to the basolateral surface. This is in agreement with our conclusions, though our ectopic localization of syx3 through genetic fusion gives a much tighter causal link between syntaxins and specificity of targeting, as compared with the pleiotropic effects of microtubule depolymerization. The exocyst is a complex involved in tethering vesicles bound for the basolateral surface (Grindstaff *et al.*, 1998). Basolaterally targeted vesicles probably fuse in the lateral region of the cell, just below the tight junction (Kreitzer *et al.*, 2003).

It is not known where apically targeted vesicles fuse, but it is possible that they fuse with the region of the apical surface closest to the tight junction. It may be that separate apically and basolaterally targeted vesicles are produced in the TGN and sent to the region of the tight junction and that syntaxins 3 and 4 control only a final proofreading or “permission” step for fusion with the correct apical or basolateral domain. This could explain how even in the presence of hierarchy of multiple steps and proteins that control polar-

ized sorting, expression of syx3/4-(1–29) and Munc18b can produce a 2.1-fold shift in targeting.

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