Transport and function of syntaxin 3 in human epithelial intestinal cells

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Breuza, Lionel, Jack Fransen, and André Le Bivic. Transport and function of syntaxin 3 in human epithelial intestinal cells. Am J Physiol Cell Physiol 279: C1239–C1248, 2000.—To follow the transport of human syntaxin (Syn) 3 to the apical surface of intestinal cells, we produced and expressed in Caco-2 cells a chimera made of the entire Syn3 coding sequence and the extracellular domain of the human transferrin receptor (TfR). This chimera (Syn3TfR) was localized to the apical membrane and was transported along the direct apical pathway, suggesting that this is also the case for endogenous Syn3. To test the potential role of Syn3 in apical transport, we overexpressed it in Caco-2 cells and measured the efficiency of apical and basolateral delivery of several endogenous markers. We observed a strong inhibition of apical delivery of sucrose-isomaltase (SI), an apical transmembrane protein, and of a-glucosidase, an apically secreted protein. No effect was observed on the basolateral delivery of Ag525, a basolateral antigen, strongly suggesting that Syn3 is necessary for efficient delivery of proteins to the apical surface of intestinal cells.

apical transport; soluble N-ethylmaleimide-sensitive factor attachment protein receptors; Caco-2 cells

The plasma membrane of epithelial cells is divided into specialized subdomains such as the apical (or luminal) domain, which faces the external medium, and the basolateral domain, which mediates contact with the surrounding cells and the basement membrane. These plasma membrane domains have different lipid and protein compositions, and their specific organization is a prerequisite for their physiological functions (for a recent review, see Ref. 41). How plasma membrane proteins are transported to their final site after biosynthesis has been investigated in several different epithelial models in the last decade (for review, see Ref. 26). Some epithelial cells such as Madin-Darby canine kidney (MDCK; Ref. 20) and Fisher rat thyroid (Ref. 42) cells favor a direct transport of apical and basolateral proteins to their respective membranes after exit from the Golgi complex. On the other hand, epithelial cells from the digestive tract such as hepatocytes (1) or enterocytes (18, 25) tend to transport apical proteins via an indirect pathway that includes a transcytotic step from the basolateral to the apical membrane. As yet, there is no mechanistic explanation for this major difference between epithelial cells.

At least two sorting steps are involved in the transport of plasma membrane proteins from the Golgi complex. The first step is the recognition of apical and basolateral proteins through intrinsic sorting signals and their packaging into distinct transport vesicles. The second step of sorting involves specific recognition between transport vesicles and their target domain in the plasma membrane. This specific fusion event could be accomplished through the specific pairing of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins located on the vesicle (v-SNAREs) and SNARE proteins located on the target membrane (t-SNAREs) (32, 37). SNARE complexes are supposed to participate in the fusion event and in the specificity of the membrane fusion (33, 38, 39). Besides SNAREs, several other proteins are involved in the regulation of membrane trafficking and fusion. Among them are N-ethylmaleimide-sensitive factor (NSF), α-soluble NSF attachment protein (α-SNAP), Sec1, and rab proteins (5, 10, 35).

Targeting of transport vesicles to apical or basolateral membranes is likely to use the same mechanisms as other transport steps, and, indeed, several t-SNAREs have been localized to the plasma membrane of epithelial cells. Syntaxins (Syn2, -3, and -4 are expressed in epithelial cells, and, although Syn2 is not polarized in MDCK cells, Syn3 is found apically in both MDCK (21) and Caco-2 cells (4) and Syn4 is found basolaterally in MDCK cells (21). Syntaxins form a SNARE complex with proteins of the SNAP-25 family (36), and it has been shown that SNAP-23 (a SNAP-25 homologue) is localized on both membranes in MDCK cells (23), suggesting that SNAP-23 may form com-
PLEXES WITH SYN3 AND SYN4. DESPITE THE ACCUMULATION OF DATA ON A POTENTIAL SNARE MACHINERY AT THE APICAL MEMBRANE, IT HAS BEEN SUGGESTED THAT APICAL TRANSPORT DOES NOT RELY ON THIS MACHINERY IN PERMEABILIZED MDCK CELLS BUT USES ANOTHER MECHANISM (12) IN WHICH Annexin XIIIs appears to be a crucial component (15). ON THE OTHER HAND, BASOLATERAL TRANSPORT IS NSF DEPENDENT AND UTILIZES RAB 8, α-SNAP, SNAP-23 (22), AND THE SEC6/8 COMPLEX ALSO FOUND IN YEAST AND IN NEURONS (8). THE FINDING THAT TRANSPORT TO APICAL MEMBRANE WAS NSF INDEPENDENT WAS RECENTLY CHALLENGED BY A STUDY ON THE ROLE OF COMPONENTS OF THE SNARE FUSION MACHINERY IN THE SAME MDCK CELLS; OVEREXPRESSION OF RAT SYN3 IN THESE CELLS CAUSED AN INHIBITION OF TRANS-GOLGI NETWORK (TGN)-TO-APICAL TRANSPORT AND OF APICAL RECYCLING (22). FURTHERMORE, TOXIN E FROM CLOSTRIDİUM BOTULİNUM (WHICH CLEAVES SNAP-23) AND ANTIBODIES AGAINST α-SNAP WERE ABLE TO INHIBIT BOTH TGN-TO-APICAL AND BASOLATERAL TRANSPORT IN PERMEABILIZED MDCK CELLS, SUGGESTING THAT THE SNARE MACHINERY IS INDEED INVOLVED IN APICAL TRANSPORT. BOTH GROUPS (12, 22), HOWEVER, FOUND NO INFLUENCE OF NSF IN DIRECT APICAL TRANSPORT, INDICATING THAT THIS PROCESS IS NSF INDEPENDENT. RECENTLY, LAFONT ET AL. (16) FOUND THAT SYN3 AND SNAP-23 WERE INVOLVED IN APICAL TRANSPORT IN MDCK CELLS TOGETHER WITH A V-SNARE CALLED TI-VAMP. THESE CONFLICTING RESULTS DEMONSTRATE THAT MORE WORK NEEDS TO BE DONE ON THE ROLE OF SNARE COMPONENTS IN APICAL FUSION. CACO-2 CELLS PROVIDE A GOOD EPITHELIAL MODEL TO TEST THE ROLE OF POTENTIAL ELEMENTS OF THE SORTING AND TRANSPORT MACHINERIES TO THE APICAL MEMBRANE.

TO IDENTIFY THE BIOSYNTHETIC PATHWAY FOLLOWED BY SYN3 IN INTESTINAL CELLS, WE HAVE PRODUCED A CHIMERA (SYN3TFR) WITH THIS PROTEIN AND THE EXTRACELLULAR DOMAIN OF THE HUMAN TFR. THIS CHIMERA WAS LOCALIZED AT THE APICAL MEMBRANE OF CACO-2 CELLS LIKE ENDGENOUS SYN3, INDICATING THAT THE CHIMERA COULD BE USED TO FOLLOW THE BIOGENETIC PATHWAYS OF SYN3. USING A COMBINATION OF METABOLIC PULSE CHASE AND SELECTIVE SURFACE BIOTINYLA TION, WE SHOWED THAT SYN3TFR WAS MAINLY TRANSPORTED ALONG THE DIRECT PATHWAY TO THE APICAL SURFACE, MOST LIKELY TO PREVENT FORMATION OF BASOLATERAL COMPLEXES AND UNWANTED FUSION EVENTS. WHEN SYN3 WAS OVEREXPRESSED IN CACO-2 CELLS, WE OBSERVED A REDUCED DELIVERY OF TWO APICAL MARKERS FOLLOWING THE DIRECT APICAL PATHWAY, I.E., SUCRASE-ISOMALTASE (SI) AND α-GLUCOSIDASE. NO EFFECT COULD BE DETECTED ON THE DELIVERY OF A BASOLATERAL MARKER. THIS IS THE FIRST REPORT OF THE TRANSPORT AND POTENTIAL FUNCTION OF A T-SNARE IN POLARIZED HUMAN INTESTINAL CELLS.

MATERIALS AND METHODS

Reagents and antibodies. Sulfo NHS-SS biotin, sulfo- NHS-LC biotin, and immobilized streptavidin were purchased from Pierce Chemical (Rockford, IL); protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Monoclonal antibodies against SI and dipeptidyl peptidase IV (DPP IV) were from Dr. A. Quaroni (Ithaca, NY), and the monoclonal antibody against Ag525 had been characterized previously (17). Polyclonal anti-placental alkaline phosphatase (PLAP) was from Accurate Chemical and Scientific (Westbury, NY). The monoclonal antibody against the extracellular domain of TFR was from Roche Diagnostics (Meylan, France), and the monoclonal antibody against the cytoplasmic domain was provided by Dr. I. Trowbridge (La Jolla, CA). The polyclonal antibody against SNAP-23 was produced as described for anti-Syn3 (4) by injecting purified histidine (His)-tagged full-length SNAP-23 into a rabbit (20 μg/boost, 1 boost every 3 wk). His-tagged SNAP-23 was also coupled to CNBr-activated Sepharose beads to purify polyclonal antibodies against SNAP-23. Monoclonal anti-syntaxin 4 (S40220) was purchased from Transduction Laboratories (Lexington, KY). Antí-α-glucosidase (118G3) was described previously (13).

Constructs, cell culture, and transfection. Human Syn3 full-length cDNA (U32315) was subcloned in bicistronic pIRE-1-neo (Clontech Laboratories, Palo Alto, CA) and resequenced entirely. A human Syn3TFR chimera was obtained by PCR using human Syn3 cDNA and human TFR as templates (16). Two sets of primers were designed for Syn3: 5’GAGCTCGAGACACCATCAGAGCAG3’ and 5’AGAATTCCAGGC- CAACGGAATGATCAAT3 for Syn3 and 5’GAATTCATGATCAATAG3’ and 5’GATCATCTTA- AAACATGTCATTGATC3’ for TFR. EcoR I restriction sites in 3’ and 5’ ends of Syn3 and TFR, respectively, allowed us to fuse in frame Syn3 and TFR cDNA, replacing the stop codon of Syn3 by a TCA codon encoding a serine. Chimeric cDNA was subcloned in pIRE1-neo. SNAP-23 cDNA was isolated by PCR on a sample of human intestine cDNA library in Agt11 (Clontech) using two designed primers, 5’GATCATGATGACAATCATGCTGATCA3’ and 5’TCTAGAT- AGCTAAATGCTT3’. PCR products were subcloned in pIRE1-neo, and one clone was entirely sequenced. His-tagged SNAP-23 was obtained by subcloning the cDNA in pQE-30 vector from Qiagen (Chatsworth, CA) and expressing it in M15 Escherichia coli according to the manufacturer’s instructions. Caco-2 cells were from Dr. A. Zweibaum (Villejuif, France) and were grown as previously described (6) with 10 mM sodium butyrate induction (10 mM, 16 h). The sequence of Syn3 in clone 33 was determined by RT-PCR using a Superscript kit (GIBCO), a high-fidelity PCR kit (Roche Diagnostics), and primers located 5’ and 3’ in the expression vector pIREs. All clones were tested for correct polarization of endogenous markers by selective surface biotinylation (34). Using the same experiments, we determined the polarity of the Syn3TFR chimera.

Immunofluorescence and confocal microscopy. Cells were grown on glass coverslips and processed as described previously (19). For Syn4, methanol fixation was performed by placing coverslips in pure methanol at −20 °C for 3 min and in PBS containing Ca2+ and Mg2+ for rehydration. Confocal microscopy analysis was performed using Zeiss confocal microscope (LSM 410 invert). Pulse-chase and transport assays. Cells were grown on Transwells (Costar Data Packaging, Cambridge, MA) to confluence and processed 10–15 days later. Filters were incubated for 20 min in DMEM without methionine and cysteine and for 30 min in the same medium supplemented with radiolabeled [35S]methionine and cysteine [Redivue Promix (58S) cell, Amersham]. Newly synthesized proteins were chased for 0.5–8 (chimera targeting), 1 (basolateral antigens), or 4 (apical antigens) h in the presence of a 100-fold excess of cold methionine and cysteine. After three washes in
ice-cold PBS containing Ca\(^{2+}\) and Mg\(^{2+}\), cells were biotinylated from the apical or basolateral side. After cell lysis, antigens were immunoprecipitated, eluted from beads, and 1/10 analyzed by SDS-PAGE (total fraction). The surface fraction of the antigen was recovered by streptavidin immunopurification from the eluate and quantified by SDS-PAGE, autoradiography, and BioImage Quantifier software (BioImage, Ann Arbor, MI) analysis. The surface appearance of the chimera on both domains of the cells was expressed as the percentage of the amount at the time of maximal expression at the cell surface. The percentage of apical or basolateral SI was expressed as the ratio of surface to total antigen. From the same experiments, autoradiographic analysis with Bio Image IQ allowed us to calculate the percentage of mature and immature SI. Chimera-processing experiments were carried out as described above. Pulse-chased cells (0–180 min) were lysed, and newly synthesized proteins were precipitated with anti-Syn3 or anti-TfR. Immunoprecipitates were analyzed by SDS-PAGE.

For \(\alpha\)-glucosidase secretion, cells were processed as described for apical delivery of SI. Apical medium was harvested, cells were lysed, and both medium and cells were submitted to immunoprecipitation in 1% Triton X-100 with monoclonal anti-\(\alpha\)-glucosidase. Antigens were eluted from beads, and an aliquot was analyzed by SDS-PAGE and autoradiography. \(\alpha\)-Glucosidase was quantified using BioImage Quantifier software. The percentage of secreted \(\alpha\)-glucosidase was expressed as the ratio of secreted to total antigen.

**RESULTS**

**Localization and transport of Syn3TfR chimera in Caco-2 cells.** To follow the intracellular transport of Syn3 after biosynthesis, we designed a chimera comprising the entire sequence of human Syn3 (4) linked to the extracellular domain of the human TfR (Fig. 1). This construct allowed us to use the selective surface biotinylation technique developed previously (19). This chimera, Syn3TfR, was expressed into Caco-2 cells by stable transfection, and several clones were selected for chimera expression by immunofluorescence using anti-Syn3 antibodies. Because endogenous Syn3 gave a weak signal with this antibody, expression of the chimera could be detected easily over the background. Localization of Syn3TfR was performed by double labeling with apical and basolateral markers in transfected cells and confocal microscopy analysis (Fig. 2). Syn3TfR labeled with anti-Syn3 antibody colocalized with SI (Fig. 2C), suggesting that it behaved like endogenous Syn3, which we localized at the apical membrane in a previous study (4). As for transfected Syn3 (Fig. 2A), we could observe some subapical staining in Syn3TfR-transfected cells. To ascertain whether the apical labeling observed with the antibodies against Syn3 was indeed caused by the chimera, we used a monoclonal antibody directed against the extracellular domain of TfR to label transfected cells. Again, a strong labeling of the apical membrane could be observed over the normal basolateral and intracellular staining usually found for the endogenous TfR. This labeling colocalized with the staining obtained with polyclonal antibodies against Syn3 or PLAP, an apical marker (Fig. 2, F and H, respectively). Conversely, with a monoclonal antibody against the cytoplasmic domain of TfR, no apical staining and no colocalization with Syn3 or PLAP was observed in transfected cells (Fig. 2, E and G), indicating that the chimera was most likely to be the protein responsible for the apical staining. This surface localization was confirmed biochemically using the selective surface labeling with sulfo-NHS-biotin followed by immunoprecipitation and peroxidase-coupled streptavidin blotting. Surface Syn3TfR was concentrated at the apical membrane (>95%; Fig. 3B), whereas endogenous TfR (84 kDa) was mainly found at the basolateral membrane (>80%). The apparent molecular mass of the chimera was 120 kDa, in good agreement with the calculated mass of 105 kDa. Because TfR contains complex N-glycans, we followed the maturation of the chimera to make sure that it was correctly processed. Both TfR and the chimera showed a concomitant increase in apparent molecular mass during a chase after a short metabolic pulse, indicating that this was indeed the case.

Syn3 has been proposed to play a role in apical delivery in MDCK cells, which mostly use the direct apical transport pathway. In Caco-2 cells, which favor the apical indirect pathway, it was of importance to determine the biogenetic pathway taken by Syn3. Because Syn3TfR and endogenous Syn3 had the same localization, we used the chimera to identify the biosynthetic route taken by newly synthesized Syn3, the extracellular domain of TfR being known to carry no crucial sorting information (14, 29). Transfected Caco-2 cells grown on filters were submitted to a short metabolic pulse and then chased for a given time. Cell surface apical or basolateral biotinylation was performed, and the biotinylated \(^{35}\)S-Syn3TfR was recovered and analyzed as described previously (19). A typical experiment is shown in Fig. 3C. Syn3TfR appeared rapidly at the apical surface (half-time of 200 min). Only a small percentage (<5%) of Syn3TfR was de-
tected in the meantime on the basolateral surface, indicating that the chimera most probably used the direct apical pathway as did SI (18, 25). To ensure that expression of Syn3TfR did not induce a change in the biogenetic pathways of apical proteins in Caco-2 cells, we measured in the same cells the kinetics of transport of DPPIV, an apical enzyme that uses both the direct and indirect pathways (7, 25). Indeed, a sizable pool of DPPIV was detected on the basolateral surface (30% after 2 h of chase) with kinetics different from Syn3TfR. Thus transfected Caco-2 cells retained the ability to use the indirect pathway, whereas the chimera used the direct pathway almost exclusively.

Selection and characterization of Caco-2 clones overexpressing Syn3. To test the potential role of Syn3 in direct apical delivery in Caco-2 cells, we chose to overexpress it in these cells because it has been shown for other syntaxins that overexpression inhibits fusion (3, 28, 40). Caco-2 cells were transfected with pRES1-neo (Clonetech) containing the human cDNA coding for Syn3 (4) and selected using 1 mg/ml of G418. This plasmid allows us to select high-expressing clones via its bicistronic organization. Several clones were obtained and tested for expression of Syn3 compared with control cells transfected with neurotrophin receptor p75 (p75NTR) (Ref. 27). We observed that strong overexpression of Syn3 prevented a normal growth of transfected cells; therefore, we used overnight sodium butyrate induction to stimulate transcription and to select clones that could only express high levels of Syn3 after stimulation. Several clones were selected that grew normally in nonstimulated conditions while expressing high levels of Syn3 after stimulation. Clones Syn3-9, -21, and -33 and control cells expressing p75NTR (75-25) were treated for 16 h with 10 mM of sodium butyrate and tested for expression of Syn3 by Western blotting on a microsomal fraction (Fig. 4A). The extent of Syn3 overexpression over control levels was 10-fold for clone Syn3-21 and 4- to 5-fold for clones Syn3-9 and Syn3-33 after butyrate stimulation. The homogeneity of the Syn3-9, -21, and -33 clones was tested by immunofluorescence using an affinity purified anti-Syn3 antibody. On average, the percentage of cells overexpressing Syn3 was between 50 and 70% (Fig. 4B). To take into account the heterogeneity of Syn3 expression, we calculated the ratio between the
level of Syn3 (determined by Western blotting) and the percentage of positive cells (determined by immunofluorescence) with clone 3-21 taken as 1 (Fig. 4C). The percentage of cells expressing SI, an apical differentiation marker, was also quantified; it was 68% in Syn3-21 and 73% in Syn3-33 cells (not shown). These percentages were typical of Caco-2 cells, which always expressed apical markers in a mosaic pattern (2).

The polarized distribution of apical and basolateral markers was examined in Syn3-overexpressing clones and control cells using selective surface biotinylation. All clones expressed apical (SI, DPPIV) and basolateral (Ag525) markers in a polarized fashion (not shown), indicating that no important change in the polarity of the clones was provoked by selection.

Cell surface delivery of newly synthesized apical and basolateral markers in cells overexpressing Syn3. To evaluate the impact of Syn3 overexpression on membrane trafficking to the apical or basolateral surfaces, cell surface delivery of an apical and a basolateral marker was studied using a combination of metabolic pulse chase and cell surface biotinylation. Cells grown on filters and treated overnight with butyrate were pulsed for 30 min with [35S]methionine and cysteine, chased for 1 (half-time of basolateral delivery of the Ag525) or 4 (half-time of apical delivery of SI) h, and biotinylated on the basolateral side for Ag525 or the apical side for SI. Four filters were used for each clone, and the amount of newly synthesized surface Ag525 or SI was quantified after immunoprecipitation and streptavidin precipitation followed by SDS-PAGE and fluorography. Relative apical (○) and basolateral (●) pool is expressed.
SI was not caused by missorting, because no increase could be detected in the basolateral delivery of this enzyme in the same cells after 4 h (Fig. 6A). A delay in apical delivery of SI could be explained by a slower processing of the immature enzyme by the Golgi apparatus. To test this hypothesis, we measured the percentage of immature SI (9) after 4 h of chase in control and Syn3-21 cells (Fig. 6B). Processing of SI was similar in both cells, indicating that the delay in apical delivery was not caused by a lack of addition of complex glycans by the Golgi complex.

Because SI principally uses the direct pathway (18), it was important to confirm the effect of overexpression of Syn3 on this pathway. We followed the secretion of α-glucosidase, an enzyme that is apically secreted in Caco-2 cells (13). Cells grown on filters and treated overnight with butyrate were pulsed for 30 min with [35S]methionine and cysteine and chased for 4 h. α-Glucosidase was precipitated from the apical me-
when the relative quantity of Syn3 per cell was calculated in each clone, clone 33 showed the lowest value, suggesting that the threshold for a dominant negative effect was close to that level. To rule out a possible mutation that might have invalidated Syn3 function after clonal selection, we checked the Syn3 sequence in clone 33 by amplification of the transgene by RT-PCR. Two cDNA clones were sequenced, and neither of them exhibited a mutation, confirming the hypothesis that the weaker inhibition of apical delivery might be caused by lower levels of expression. These data also suggest that correct levels of Syn3 could be necessary for proper protein delivery to the apical membrane of intestinal cells.

**DISCUSSION**

**Expression and transport of Syn3TfR.** We have designed a chimera comprising the entire coding sequence of Syn3 and the extracellular domain of TfR. We chose this receptor because its extracellular domain has already been used as a reporter in polarity studies, because it does not appear to contain strong sorting information (14, 29). In the case of our chimera, this seems to be true, because we could not detect any difference in subcellular localization between Syn3 (endogenous or transfected) and Syn3TfR. Syn3TfR was localized at the apical membrane by using antibodies recognizing either the Syn3 part or the extracellular domain of TfR. As expected, antibodies against the intracellular domain of TfR did not label the apical membrane. The chimera was transported along the Golgi apparatus and processed as the endogenous TfR, because we could observe a shift in mobility during maturation. Finally, it reached the apical membrane.

![Fig. 6](http://ajpcell.physiology.org/) A: basolateral delivery of SI is unaffected in cells overexpressing Syn3. Different clones of Caco-2 cells (75-25, Syn3-21, and Syn3-33) were grown on filters to allow cells to form a polarized monolayer, sodium butyrate treated, and pulse-labeled for 20 min. Newly synthesized proteins were chased for 4 h and then biotinylated on the basolateral side of the cells. Cell lysates were submitted to SI immunoprecipitation and streptavidin precipitation. Aliquots of total and biotinylated SI were analyzed by SDS-PAGE and fluorography and quantified with Biolmage IQ. In each case, basolateral to total SI ratio was determined. Data are means ± SE (n = 4 experiments) and are expressed as % of control cells (75-25). No significant difference was observed on basolateral delivery of SI. B: Syn3 overexpression does not affect SI processing. Control clone (75-25) and clone Syn3-21 of Caco-2 cells were pulse-labeled, and newly synthesized proteins were chased for 4 h. SI was immunoprecipitated and analyzed by SDS-PAGE. Respective quantities for mature (filled bars) and immature (open bars) forms of SI were determined by scanning densitometry and Biolmage IQ software. Data are means ± SE (n = 4 experiments) and are expressed as % of total SI. No significant difference was observed between control (75-25) and Syn3-21.

![Fig. 7](http://ajpcell.physiology.org/) Effect of Syn3 overexpression on α-glucosidase apical secretion. Control (75-25) and Syn3-overexpressing (Syn3-21, Syn3-9, and Syn3-33) Caco-2 clones were grown on filters to form polarized monolayers and treated with 10 mM sodium butyrate for 16 h. Cells were pulse-labeled for 20 min, and newly synthesized proteins were chased for 4 h. α-Glucosidase immunoprecipitation was performed on the apical medium and cell extracts. Immunoprecipitates were analyzed by SDS-PAGE and quantified as described in Fig. 6. Data are means ± SE (n = 4 experiments) and are expressed as % of control cells (75-25). *Significant difference (P < 0.005) from control cells using ANOVA followed by Fisher’s PLSD test.
mainly using the direct apical pathway, because very little of the chimera population was detected even transiently on the basolateral membrane whereas the endogenous apical protein DPPIV, using the indirect pathway, was easily detected on the same membrane in the meantime. It is likely that the transport pattern of the chimera reflects the actual transport of endogenous Syn3, because most apical proteins in Caco-2 cells follow the indirect pathway (18, 25) and thus entry in the direct pathway was likely to be driven by the Syn3 part of the chimera. In terms of the regulation of Syn3 activity, the use of the direct pathway could be important because we have shown that Syn3 is complexed to another t-SNARE, SNAP-23, on the apical membrane. SNAP-23 is also present on the basolateral membrane in Caco-2 cells (Fig. 2A), where it is complexed to Syn4 (not shown) also expressed on that membrane (Fig. 2A). Thus a transient expression of Syn3 on the basolateral membrane could induce the formation of potentially active complexes and provoke unwanted membrane delivery. This work is the first description of the intracellular transport of a polarized t-SNARE in epithelial cells. It is striking that Syn3 is not always expressed at the apical membrane of epithelial cells. In particular, it has been reported to localize at the basolateral membrane of renal intercalated cells (24) but to be apical in kidney-derived MDCK cells (21). These data raise the possibility that syntaxins may control the expression of a subset of plasma membrane proteins with a tight physiological regulation of their polarized expression in a tissue-specific manner. In our case, we showed that Syn3 is localized apically both in Caco-2 and in normal colonic cells in vivo (4).

Effects of Syn3 overexpression. To study the potential role of Syn3 in intestinal cells, we have transfected it in Caco-2 cells and selected clones that overexpress it after butyrate treatment. To overcome the possible additional effects of selection and butyrate treatment, as a control we used a clone transfected with wild-type p75\textsuperscript{NTR}, an apical protein, as previously described (27). Control cells were kept under the same conditions and treated during experiments like the clones overexpressing Syn3. Three different clones with various levels of expression were selected and tested for transport of one basolateral and two apical markers. We found that overexpression of Syn3 did not significantly alter delivery of Ag525 to the basolateral surface, indicating that the basolateral pathway was not dependent on the level of Syn3 present in the cells. Accordingly, overexpression of Syn3 also had no effect on the basolateral transport of the polymeric immunoglobulin receptor, confirming that in epithelial cells Syn3 is probably not involved in this pathway (22).

Overexpression of Syn3, on the other hand, caused a specific inhibition of apical delivery of a transmembrane protein, SI, and a secreted protein, \(\alpha\)-glucosidase. Apical delivery of both proteins was significantly reduced in clones 9 and 21, whereas the effect was much more reduced in clone 33, suggesting a dose-dependent effect. This could be explained by a higher ratio (0.54 vs. 0.47) of the amount of Syn3 and the percentage of cells expressing it in clone 9 vs. clone 33 or by a different balance between putative partners of Syn3 in clone 33. In clone 21, both the level of Syn3 and the percentage of overexpressing cells were higher than in clone 33. We have ruled out a possible mutation of Syn3 leading to a deficient protein in clone 33. Our efforts to obtain clones either expressing more Syn3 or exhibiting a higher percentage of overexpressing cells failed, probably because their normal growth was impaired above a certain level of expression. It is likely that in each cloned population, transport of SI to the apical surface was even more inhibited in cells overexpressing Syn3 than the average numbers (50% of inhibition) found in Fig. 5A. Conversely, in the same population, cells with normal levels of Syn3 were likely to show no effect, reducing the overall measured effect. Our assay measured cell surface delivery as an average for the population tested with a balance between normal and overexpressing cells. Apical markers most probably were transported in these cells with much slower kinetics rather than being accumulated intracellularly, because we never observed a strong accumulation of SI, for example, inside the cells (not shown).

The delay we observed in the apical delivery of both SI and \(\alpha\)-glucosidase was not caused by a change in the kinetics of processing by the Golgi enzymes, because there was no significant difference of the percentage of endoglycosidase H-resistant SI between control and clone 21 cells after 4 h of chase. Reduced apical delivery of SI was also not caused by mis-sorting of this protein to the basolateral membrane, because there was no significant increase of basolateral newly synthesized SI after 4 h of chase. Therefore, inhibition of apical transport in cells overexpressing Syn3 is most probably a post-Golgi event. The question remains as to whether Syn3 is involved in the targeting or in the fusion of apical transport vesicles. Both SI and \(\alpha\)-glucosidase use the direct apical transport route, and thus we postulate that Syn3 is a key component regulating this pathway, as also suggested by Low et al. (22). The implication of Syn3 in the indirect (or transcytotic) pathway remains to be investigated in intestinal cells, even though it was shown by the same authors that in MDCK cells the overexpression of Syn3 did not affect this pathway. The mechanism by which this inhibition is mediated is not clear, but similar effects have been observed previously in other systems. For example, in Drosophila the overexpression of Syn1 provoked a specific inhibition of synaptic vesicle fusion (40), whereas in pancreatic island cells, overexpression of Syn1A inhibited glucose-stimulated insulin secretion (28). Even in constitutive transport, such as endoplasmic reticulum to Golgi transport, overexpression of Syn5 caused an inhibition (3). In these studies, the effect was restricted to one transport pathway. This inhibitory effect of overexpression of Syn3 could be mediated by association with munc 18-2, which is also enriched in the apical membrane of Caco-2 cells (31), preventing the recycling of SNARE complexes at the apical membrane after their participation in a fusion event. Any change in the balance of apical SNAREs in epithelial
cells might lead to a perturbation in the efficiency of the vesicular targeting or fusion.

After some debate (12, 22), it now appears that in MDCK cells the apical pathway also relies on Syn3/SNAP-23 (16, 22), indicating that it might be a common feature for apical transport in epithelial cells. The results presented here strongly suggest that the direct apical pathway involves the SNARE machinery to operate in Caco-2 cells, establishing that this pathway is of the same nature as in MDCK cells even if the two cell lines show different behaviors in sorting and delivery of apical proteins.

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