Putative O-Glycosylation Sites and a Membrane Anchor Are Necessary for Apical Delivery of the Human Neurotrophin Receptor in Caco-2 Cells*

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Laure Monlauzeur, Lionel Breuza, and André Le Bivic‡

We have expressed the human neurotrophin receptor p75 (p75NTR) in the intestinal epithelial cell line Caco-2 as a model to study intracellular transport and subcellular sorting signals in intestinal cells. p75NTR was localized at the apical membrane of Caco-2 cells and reached this membrane mainly via an indirect pathway. Apical localization, intracellular routing, and basolateral to apical transcytosis were not affected by truncation of the cytoplasmic domain or replacement of the transmembrane domain by a glycosyl phosphatidylinositol anchor. Removal of membrane anchoring resulted in basolateral secretion of the ectodomain of p75NTR in Caco-2 cells but in apical secretion in Madin-Darby canine kidney (MDCK) cells. Substitution of potential O-glycosylation sites present in the stalk of p75NTR led to intracellular cleavage and secretion of the ectodomain into the basolateral medium both in Caco-2 and MDCK cells. These results suggest that the stalk of p75NTR carries an apical sorting information that is recognized efficiently by Caco-2 cells only when attached to the membrane. This apical sorting information is linked to the presence of predicted O-glycosylation sites in that region. These putative O-glycosylation sites also play a role in the regulation of p75NTR transport to the cell surface and in the prevention of rapid degradation by cleavage of the stalk domain.

How proteins are targeted to plasma membrane domains in epithelial cells remains a fundamental question. In the last decade, progress has been made by investigating both transport routes and targeting signals that deliver proteins to the apical and basolateral surfaces of epithelial cells. A great amount of work has been performed on an in vitro model, the Madin-Darby canine kidney (MDCK) cell line. In these cells, most newly synthesized plasma membrane proteins are sorted in a post-Golgi compartment, the trans-Golgi Network (TGN) and then transported to their steady state membrane domain (for reviews, see Refs. 1–3). In MDCK cells, targeting signals have been identified both for apical and basolateral proteins. Basolateral targeting signals so far identified, are localized in the cytoplasmic domain of transmembrane proteins such as pIgR (4), LDLR (5), hNGFR (6). Molecularly, these targeting signals are dependent either on a tyrosine motif (7, 8), dileucine motif (9), or hydrophobic/aromatic amino acids (10). Some of these are reminiscent of endocytic signals, but interactions with proteins from clathrin coats have not yet been firmly established. Apical signals on the other hand seem to be dependent on post-translational modifications. Addition of a glycosylphosphatidyl inositol (GPI), to a number of proteins leads to apical localization (11–13). GPI-anchored proteins are not solubilized in Triton X-100 buffers at 4 °C (14) and can be enriched in membrane structures containing high levels of cholesterol, glycosphingolipids, and signal transduction proteins (15). It has been proposed that apical sorting occurs by formation of membrane microdomains enriched in apical components in the Golgi apparatus and delivery of these domains through vesicular intermediates to the apical membrane (16). Besides GPI anchors, a role for N-glycosylations was suggested for the targeting of apically secreted proteins (17, 18).

The vast majority of these data was obtained using the kidney-derived cell line MDCK. There are hints, however, that in other cell lines, apical sorting information may be recognized differently. For example, in FRT cells derived from rat thyroid, the GPI anchor is not recognized as a dominant apical signal (19). Some viruses also bud on opposite surfaces of FRT and MDCK cells suggesting that the cellular machinery responsible for targeting may not be the same in FRT cells (20). In Caco-2 cells most of apical proteins are targeted as in MDCK cells with the exception of secreted proteins (21). The main difference between Caco-2 and MDCK cells resides in the intracellular pathway taken by apical proteins en route to the apical membrane. The indirect (or transcytotic) pathway appears predominant in intestinal (22, 23) and liver (24) cells and must be governed by targeting signals too.

One important initial step toward understanding the molecular bases of the variation in final localization and sorting pathways is to carefully compare the decoding of the known sorting signals in different epithelial cell types. With this objective, we characterized the sorting of several mutant forms of the human neurotrophin receptor (p75NTR) (25) in Caco-2 cells. We previously showed that the TGN of MDCK cells recognizes an apical sorting signal in the ectodomain of p75NTR and a basolateral signal in a mutant form of the same protein that is dominant over the luminal apical signal (6). We have now characterized the recognition of p75NTR sorting signals by Caco-2 cells. We showed that Caco-2 cells can decode apical information in p75NTR only when the molecule is bound to the membrane by a transmembrane anchor or by a GPI, a secretory form of p75NTR being discharged basolaterally. WT and all mutant forms of p75NTR reached the apical surface by transcytosis, indicating that recognition of the apical signal occurred...
only after basolateral delivery of the proteins. Importantly, our studies also revealed that basolateral sorting and transcytosis of membrane proteins does not require cytoplasmic signals, as opposed to MDCK cells. Mutation of most of the putative O-glycosylation sites present in the stalk of p75<sup>NTR</sup> has a strong effect on the transport of the resulting p75 mutant (p75<sup>ΔO</sup>). p75<sup>ΔO</sup> is cleaved intracellularly to escape endoplasmic reticulum retention and the majority of the cleaved, mature form made of the ectodomain is secreted in the basolateral medium of both Caco-2 and MDCK cells. This is the first direct evidence that putative O-glycosylation sites present in the stalk of an apical protein may play a key role in transport, stability, and targeting of an apical protein to the apical membrane of Caco-2 cells.

**MATERIALS AND METHODS**

**Reagents**—Cell culture reagents were purchased from Life Technologies, Inc. Affinity-purified antibodies (rabbit anti-mouse IgG) and TRITC-conjugated antibodies were from Biosys (Pasteur Institute, Paris). Protein A-Sepharose was from Pharmacia Fine Chemicals (Uppsala, Sweden). Sulfosuccinimidyld-6-(biotinamido) hexanoate (NHS-LC-biotin) and streptavidin-agarose were purchased from Pierce. Products for sequencing, endonuclease H, F neuraminidase, and O-glycosidase were from Boehringer Mannheim Biochemical (Mannheim, Germany). All other reagents were from Sigma.

**Cells and Antibodies**—Caco-2 cells (a gift from Dr. A. Zweibaum, Villejuif, France) were grown as described previously (26). For experiments, cells were grown on Transwells chambers (Costar) for 15 days. Mouse monoclonal antibody ME20-4 against human NGF (neurotrophin) receptor was produced as ascites and used as described in the text. Rabbit polyclonal antibody against the cytoplasmic tail of p75<sup>NTR</sup> was kindly provided by M. Chao, Cornell University Medical College, New York.

**Transfection, Clonal Selection, and Detection of p75**—Cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s instructions. Resistant colonies growing in the presence of 1 mg/ml G418 were isolated using metal cloning rings and scored for p75<sup>NTR</sup> expression by indirect immunofluorescence. Direct immunofluorescence was performed as described previously (27). For each construct, several clones expressing different amounts of p75<sup>NTR</sup> mutants were selected and characterized. Cell surface biotinylation, pulse-chase, immune, and streptavidin precipitation surface delivery experiments and transcytotic transport were carried out as in Monlaueur et al. (8) and as in Le Bivic et al. (23). Triton X-100 insoluble C-terminal fragments were described in Garcia et al. (26). Phosphoinositid stipitated phosphatase C digestion and Triton X-114 partitioning was performed according to Lisanti et al. (13). N- and O-glycan digestions were performed as in Yeaman et al. (28).

**Constructs**—Full-length (WT) and tailless mutant XI have been described previously (6). Secreted p75<sup>NTR</sup> (p75<sup>sec</sup>) was prepared by polymerase chain reaction and introduction of a stop codon at residue 219 of the ectodomain. This construct still possess a signal peptide for entry into the secretory pathway. A p75<sup>ΔO</sup>anchored to the membrane by a glycosylphosphatidyl inositol was designed by fusing in frame the ectodomain of p75<sup>NTR</sup> ending at residue 204 (GPI5) or 214 (GPI6) (both BstEII sites) with the placent al alkaline phosphatase sequence necessary for GPI transfer in the endoplasmic reticulum, i.e. residues 482–513 (29). This sequence was amplified by PCR using a primer containing a BstEII site in frame with those present in p75<sup>WT</sup>. The PCR product was inserted in a plasmid containing p75<sup>WT</sup> after a BstEII site digestion. A p75<sup>ΔO</sup> in which the main potential sites (5 serines and 7 threonines) for O-glycosylations were changed to alanines was constructed as follows. A first round of PCR was performed to amplify fragment 1–627 changing Ser177, Ser177, Ser180, Ser183, and Thr184 to A. The mutated serines and threonines had a score > 0.5 using NetOglyc Prediction Program, indicating that they are putative O-glycosylation sites. Both fragments were ligated using an introduced SacI site at position Asp194 changing it to Ghu<sup>194</sup> and the resulting construct (p75<sup>ΔO</sup>) was subcloned in pIREs expression vector (CLONTECH, Palo Alto, CA). All constructs were sequenced using the Sanger technique with the Pharmacia T7 kit.

![Fig. 1. Scheme of p75<sup>NTR</sup> cDNA mutants. Boxes represent the signal sequence and transmembrane domain (TM). Filled ovals represent cysteine-rich repeats, while open ovals represent O-glycan chains. WT, full-length cDNA; XI, tailless cDNA with only five amino acids on the cytoplasmic side (KRWNS); GPI-6 and GPI-5, chimera between residues 1 and 204 or 1 and 214 of p75<sup>NTR</sup>, respectively, and residues 452–513 of human placental alkaline phosphatase; p75<sup>sec</sup>, cDNA coding for a secreted form of p75<sup>NTR</sup> with a stop codon replacing codon for amino acid 219, p75<sup>ΔO</sup>, cDNA coding for a p75<sup>NTR</sup> in which serines 171, 177, 179, 183, and 198 and threonines 172, 180, 184, 199, 205, 206, and 216 were changed to alanines.](image-url)
After selection of several independent clones, indirect immunofluorescence localization was performed on confluent cells. A punctated apical pattern was observed for p75WT, p75XI, and p75GPI expressing cells suggesting that these three constructs were localized apically (not shown). Selective surface biotinylation was done on clones that were grown on Tranwells for at least 15 days. After immunoprecipitation of p75WT, p75XI, and p75GPI-5 with a specific monoclonal antibody, surface biotinylated p75NTR constructs were revealed by streptavidin coupled to peroxidase (Fig. 3A). Several clones were processed for each construct, and quantification of surface apical and basolateral p75NTR was performed by densitometry. 85%, 90% and 85% of surface p75WT, XI and GPI-5, respectively, was found in the apical membrane of transfected Caco-2 cells (Fig. 3B). This apical localization was confirmed by confocal microscopy after indirect immunofluorescence on cells grown on filters (not shown). Thus the cytoplasmic domain does not seem to play a role in apical targeting while the transmembrane domain can be substituted by a GPI anchor.

These data suggested that if there was apical sorting information in human p75NTR it could be present either in the ectodomain or in the transmembrane domain. To examine this hypothesis, a secreted form of p75NTR (p75sec) lacking the ectodomain or in the transmembrane domain was expressed in Caco-2 cells. Several clones were tested for the secretory capacity of each clone. The basolateral secretion of p75sec could be explained by indirect transport of membrane bound p75NTR. To test this hypothesis, cell surface delivery of p75WT, XI, and GPI-5 was monitored using a combination of [35S]cysteine pulse and chase followed by selective surface biotinylation at different times (Fig. 5A). All p75NTR constructs were detected predominantly on the basolateral surface of Caco-2 cells in the first 2 h of chase. The basolateral amount of both forms then decreased slowly, while the apical pool of p75WT, XI, and GPI-5 increased over the time of chase. After 6 h of chase all constructs were preferentially expressed on the apical membrane.

Fig. 2. Phosphoinositol phospholipase C (PIPLC) digestion of p75GPI. Triton X114 phases from Caco-2 cells expressing p75GPI-5 or 6 after a [35S]cysteine pulse were treated with (+) or without (−) PIPLC and the resulting aqueous (Aq) and detergent (Det) phases were immunoprecipitated with ME20-4 antibodies. Immunoprecipitated p75NTR was then analyzed by SDS-PAGE and revealed by fluorography. Quantification of the bands was performed by scanning densitometry and expressed as a percentage of total p75NTR.

Fig. 3. Surface expression of p75NTR mutants in transfected Caco-2 cells at steady state. A, cells were grown on filters and biotinylated from the apical (A) or basolateral (B) side. After cell lysis p75NTR mutants were immunoprecipitated with ME20-4, analyzed by SDS-PAGE, transferred to nitrocellulose, and revealed with streptavidine coupled to peroxidase using chemiluminescence. Molecular mass markers are indicated on the left. B, quantification of apical and basolateral surface expression at steady state. Black bars represent apical expression while empty bars are for basolateral expression (n = 3). In A and B, WT, p75WT; XI, p75XI; and GPI, p75GPI-5. All constructs were preferentially expressed on the apical membrane.

main is linked to the membrane. To understand further this membrane attachment requirement we next investigated the biogenetic pathways of p75NTR in Caco-2 cells.

Biogenetic Pathways of p75WT, XI, and GPI-5 in Caco-2 Cells—The basolateral secretion of p75sec could be explained by indirect transport of membrane bound p75NTR. To test this hypothesis, cell surface delivery of p75WT, XI, and GPI-5 was monitored using a combination of [35S]cysteine pulse and chase followed by selective surface biotinylation at different times (Fig. 5A). All p75NTR constructs were detected predominantly on the basolateral surface of Caco-2 cells in the first 2 h of chase. The basolateral amount of both forms then decreased slowly, while the apical pool of p75WT, XI, and GPI-5 increased over the time of chase. After 6 h of chase all constructs were slightly enriched in the apical membrane (60%) (Fig. 5B). These kinetics of transport suggested an indirect transport and were very similar for the three constructs indicating that the cytoplasmic domain and the transmembrane domain did not play a role in this transport. When the same experiment was performed using longer times of chase (12 and 20 h) the apical proportion of p75WT, XI, and GPI-5 increased to reach 70% (not shown).

p75WT, XI, and GPI-5 Are Transcytosed from the Basolateral to the Apical Membrane—Kinetics of delivery of p75GPI-5 were
very similar to those of p75WT and XI, suggesting that the three constructs followed the same intracellular pathways on their route to the apical membrane. To ascertain that the basolateral pool of each construct was indeed transcytosed to the apical membrane we measured directly this step of transport. Confluent Caco-2 cells expressing each construct were metabolically pulsed with \[^{35}\text{S}\]cysteine for 30 min and then chased with an excess of cysteine for 2 h (maximum of basolateral expression for all constructs). Cells were then biotinylated on the basolateral membrane using a cleavable analog of biotin (S-NHS-SS-biotin) and incubated for 4 h at 37 °C in normal medium to allow transcytosis (23). The apical surface of the cells was then reduced with glutathione and p75\(^{\text{NTR}}\) was recovered by immunostreptavidin precipitations as for cell surface delivery. In these conditions about 60% of basolateral p75WT, XI, or GPI-5 was transcytosed to the apical membrane (Fig. 6). Thus it seems that the transcytosis of p75\(^{\text{NTR}}\) does not depend on its cytoplasmic or transmembrane domain but only requires a membrane anchor.

\(p75^{\text{O}}\) is Found in the Basolateral Compartment of Caco-2 and MDCK Cells—The stalk region of p75\(^{\text{NTR}}\) is very rich in putative O-glycosylation sites and is necessary for apical targeting of p75\(^{\text{NTR}}\) in MDCK cells but so far there is no evidence for a direct role of these sites in apical sorting (28). As an attempt to test the role of O-glycosylations in determining the polarity of p75\(^{\text{NTR}}\) we have mutated 5 serines and 7 threonines to alanines which are putative O-glycosylation sites in the 168–218 region (Fig. 1). The resulting receptor, \(p75^{\text{O}}\), was expressed in Caco-2 cells, and several clones expressing it were obtained and studied. We first examined its biosynthetic processing by pulse/chase and immunoprecipitations (Fig. 7A). 

![Fig. 4. Polarity of secretion of p75sec in MDCK and Caco-2 cells.](image)

**Fig. 4.** Polarity of secretion of p75sec in MDCK and Caco-2 cells. Transfected MDCK and Caco-2 cells were grown on filters and pulsed for 30 min with \[^{35}\text{S}\]cysteine and chased for 4 h in the case of MDCK and 6 h for Caco-2 cells. Apical and basolateral media were harvested and p75sec was immunoprecipitated using ME20-4 antibody. Immunoprecipitates were analyzed by SDS-PAGE, visualized by fluorography, and quantitated by scanning densitometry. A, apical medium; B, basolateral medium. (\(n = 2\)). p75sec was secreted mainly in the apical medium by MDCK cells and in the basolateral medium by Caco-2 cells.

![A](image)

**A**

WT

XI

GPI-5

Chase 0 30 60 120 240 360

0 30 60 120 240 360

![B](image)

**B**

WT

XI

GPI

![Fig. 5. Cell surface appearance of p75\(^{\text{NTR}}\) mutants in transfected Caco-2 cells.](image)

**Fig. 5.** Cell surface appearance of p75\(^{\text{NTR}}\) mutants in transfected Caco-2 cells. A, cells grown on filters were pulsed for 30 min with \[^{35}\text{S}\]cysteine and chased for the times indicated, in minutes. Newly synthesized p75\(^{\text{NTR}}\) mutants were detected at the cell surface using a combination of cell surface biotinylation, immuno and streptavidin precipitation as already described (23) and analyzed by SDS-PAGE and fluorography. AP, apical side, BL, basolateral side. B, quantification of at least to independent cell surface delivery experiments. Results were expressed as a percentage of the amount at the time of maximal expression at the cell surface. Apical (○) and basolateral (●). In A and B, WT = p75WT; XI = p75XI; and GPI = p75GPI-5.
precursor form of 65 kDa was first observed (half-time of about 2 h) and then cleaved into a faster migrating species of 50 kDa. This 50-kDa species was immunoprecipitated by the ME20-4 antibody directed against the ectodomain but not by a polyclonal antibody raised against the cytoplasmic tail of human p75\textsuperscript{NTR} (not shown). Digestion of these two forms by endoglycosidases H or F showed that the 65-kDa form was still sensitive to endoglycosidase H, while the 50-kDa form was resistant to the same enzyme, indicating that it had reached the Golgi complex (Fig. 7C). To examine the possibility that cleavage of p75\textsuperscript{O} led to the loss of the cytoplasmic and transmembrane domains, a Triton X114 phase separation assay (30) was performed on cells labeled with \textsuperscript{[35S]}cysteine and chased for 1 h. Immunoprecipitation of p75\textsuperscript{O} from the detergent and the aqueous phases showed that the 65-kDa form was enriched in the hydrophobic phase (about 60%), while the 50-kDa form was mostly detected in the aqueous phase (95%), suggesting that the latter species was not anchored to the membrane after cleavage (Fig. 7B). The half-life of the 50-kDa form was also short (2 h), suggesting either further degradation or secretion into the culture medium of the cells. Apical and basolateral culture media were thus immunoprecipitated at different times of chase after a 30 min pulse (Fig. 8A). A 50-kDa p75\textsuperscript{O} was observed in the basolateral medium after 60 min of chase indicating that secretion had occurred. A 45-kDa form that may derive from further processing of the 50-kDa was also observed in the apical medium (Fig. 8A). Secreted p75\textsuperscript{O} was enriched in the basolateral medium (75% after 6 h of chase) (Fig. 8B). The O-glycosylation status of the p75\textsuperscript{O} forms was investigated by digestion with neuraminidase and O-glycosidase as in Yeaman \textit{et al.} (28). After O-glycan digestion p75WT migrated slightly faster as described for the same protein in MDCK cells (28) while no shift in mobility could be observed for any form of p75\textsuperscript{O}, indicating that this mutant was indeed less O-glycosylated than the wild type (Fig. 9). This lack of shift in mobility after O-glycan digestion, however, does not preclude the addition of some O-glycans in p75\textsuperscript{O} since this assay is not very sensitive.

The basolateral secretion of p75\textsuperscript{O} in Caco-2 cells may not be a direct consequence of the knock out of putative O-glycosylation sites as the lack of a membrane anchor prevents apical localization by itself in this cell line (see p75sec, Fig. 4). To test this, we expressed p75\textsuperscript{O} in MDCK cells and measured its secretion in the apical and basolateral media (Fig. 10A). The
same pattern of maturation with a 65-kDa precursor form and then a 50-kDa species was observed. As in Caco-2 cells the 65-kDa form was found mainly in the detergent phase (59%), while the 50-kDa form was mainly detected in the aqueous phase (96%) (not shown). Secretion of the latter form occurred mainly in the basolateral compartment (79%) (Fig. 10, B and C) as in Caco-2 cells suggesting that the putative O-glycosylation sites that were mutated were necessary for apical targeting. On the other hand, p75sec bearing intact potential O-glycosylation sites was secreted mainly in the apical medium (this work) (28). As in Caco-2 cells the apparent molecular weight of the apically secreted p75O⁻ was 45 kDa.

**DISCUSSION**

**Transport of p75WT to the Apical Membrane Does Not Require a Cytoplasmic Domain but Requires a Membrane Anchor**—We have expressed p75WT in Caco-2 cells and have found that in all clones tested it was apically enriched (>80%) as in MDCK cells (6). A tail-minus form (p75XI) of p75WT with only 5 amino acids left on the cytoplasmic side was also found predominantly on the apical membrane of transfected Caco-2 cells. Thus the lack of a cytoplasmic domain does not change the polarity of p75WT both in Caco-2 (this work) and MDCK cells (6). When the transmembrane domain, however, was deleted from p75WT, the resulting secreted ectodomain was found predominantly in the basolateral medium of Caco-2 cells, suggesting that membrane anchoring was necessary for apical localization in contrast to MDCK cells (28). The apical to basolateral ratio of secretion in Caco-2 cells did not vary with the level of expression of p75sec (not shown) and thus was not the result of a saturation of the apical exocytic pathway.

Basolateral secretion of p75sec was likely a consequence of the intracellular transport of p75WT in Caco-2 cells. In cell surface delivery experiments, p75WT was first delivered to the basolateral surface and in greater amounts than to the apical surface. Since the technique we used is not cumulative, it is likely that more than 50% is actually going through the basolateral membrane. The amount of p75sec found in the basolateral medium (between 70 and 80%) could reflect the maximal amount of p75WT being transported to the basolateral membrane, the rest (20–30%) reaching directly the apical membrane. Thus, as opposed to MDCK cells, the apical signal borne by the ectodomain of p75WT is recognized by Caco-2 cells only after the receptor has reached the basolateral membrane. This confirms that sorting sites are different between the two cell lines (31).

**Basolateral Delivery and Transcytosis of p75WT Does Not Require a Cytoplasmic Tail**—We wished to evaluate the requirements for indirect transport and transcytosis of p75WT in Caco-2 cells using a tail-less p75WT or replacing the transmembrane domain by a GPI anchor. The two p75GPI constructs (5 and 6) were expressed on the apical membrane of Caco-2 cells, with an apical to basolateral ratio similar to what we found for p75WT and XI. Accordingly GPI-anchored proteins have been shown to be enriched in the apical membrane of Caco-2 cells and of epithelial cells in general (13). Two endogenous GPI-anchored proteins however, are present on both apical and basolateral membranes in Caco-2 cells. One is p137, which is capable to transcytose both ways (32), while the other is a glypican (33). p75GPI was first delivered to the basolateral membrane before accumulating in the apical membrane as it was also shown for 5'-nucleotidase in hepatocytes (34), suggesting that the GPI anchor does not provide a dominant sorting signal for the direct apical pathway in Caco-2 and liver cells.

Although p75WT, XI, and GPI proteins have very different cytoplasmic domains or membrane anchors, their cell surface kinetics of delivery and transcytotic capacity were very similar, suggesting that indirect transport is neither facilitated nor hampered by the presence of a cytoplasmic domain (155 amino acids in the case of p75WT). In contrast Golgi to basolateral membrane transport depends on cytoplasmic sequences in MDCK cells (35). Porcine APN expressed in Caco-2 cells was also transported to the basolateral membrane first and this transport did not rely on a cytoplasmic sequence either (31). Thus the mechanisms by which p75WT is sorted and transported to the apical side via the basolateral membrane must be different from the one proposed for the pIgR in which cytoplasmic sequences are involved in regulating both basolateral delivery and transcytosis (36). In the case of p75WT and its apical forms, the peak of appearance on the basolateral membrane was quite spread over the time of chase with some p75WT still present after 20 h, suggesting that removal of p75WT from the basolateral membrane was a passive event. Clearance of human APN, another apical protein of Caco-2 cells, from the same membrane was much faster (23) indicating that there must be some protein-specific determinants regulating entry in the endocytic/transcytotic pathway in Caco-2 cells (37). We cannot
rule out that, in Caco-2 cells, the transmembrane domain of p75NTR contains an apical sorting information that can be substituted by a GPI anchor. In MDCK cells, however, if this apical sorting information putatively present in the transmembrane domain exist it is redundant with the signal present in the ectodomain (28).

Role of O-Glycosylations in the Apical Sorting of p75NTR—In a recent study we have shown that the O-glycan-rich stalk region (168–218) of p75NTR contained an apical sorting information (28). We produced a new mutant, p75O, in which most of the serines and threonines of the stalk were changed to alanines to prevent or greatly reduce the addition of O-glycans. The new construct was expressed in both Caco-2 and MDCK cells and its apparent molecular mass of 65 kDa, instead of 75 kDa for the WT receptor, was well in agreement with the 10-kDa estimated size of the cluster of O-glycans (28), indicating that indeed most of the O-glycosylations were blocked in p75O. This was confirmed by enzymatic digestion of O-glycan chains. p75O precursor was retained in the endoplasmic reticulum since its single N-glycan chain was sensitive to endoglycosidase H, suggesting that potential O-glycan sites were necessary for proper folding of the stalk domain and exit of the endoplasmic reticulum. A faster migrating form (50 kDa) of p75O lacking the cytoplasmic and transmembrane domain was exported, however, from the endoplasmic reticulum and acquired a complex N-glycan chain as shown by its resistance to the same enzyme. This intracellular processing led to secretion of the ectodomain in the culture medium confirming that decay-accelerating factor, aminopeptidase N (38), and decay-accelerating factor (39) possess sequences sensitive to intracellular proteases and one possible role of a region rich in O-glycans close to the N-glycan sites in the sorting of apical proteins.

Impairment of O-glycan addition upon exit of the endoplasmic reticulum may have uncovered sequences sensitive to intracellular proteases and one possible role of a region rich in O-glycans close to the N-glycan sites in the sorting of apical proteins. Many plasma membrane proteins such as sucrase-isomaltase (35), and one possible role of a region rich in O-glycans is to protect proteins from degradation. Many mammalian lectins found in the exocytic pathway have been described (43) that may play a role in the recognition and transport of plasma membrane proteins but so far there is no direct evidence for a lectin-based mechanism involved in apical sorting of glycosylated proteins. N-Glycans played no role in the sorting of p75NTR as we showed in a recent study (28), and further studies will be necessary to clarify the respective roles of N- and O-glycans in the sorting of apical proteins and how many different apical sorting informations may be recognized by epithelial cells.

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REFERENCES
1. Matter, K., and Mellman, I. (1994) Curr. Opin. Cell Biol. 6, 545–554
2. Rodriguez-Boulan, E., and Powell, S. K. (1992) Annu. Rev. Cell Biol. 8, 385–427
3. Simons, K., and Wandinger-Ness, A. (1990) Cell 62, 207–210
4. Casanova, J. E., Apodaca, G., and Mostov, K. E. (1991) Cell 66, 65–75
5. Hunziker, W., Harter, C., Matter, K., and Mellman, I. (1991) Cell 66, 907–920
6. Le Bivic, A., Sabmby, J., Putat, A., Pili, N., Chao, M., and Rodriguez-Boulan, E. (1991) J. Cell Biol. 115, 607–618
7. Matter, K., Hunziker, H., and Mellman, I. (1992) Cell 71, 741–753
8. Monlauzeur, L., Rajasekaran, A., Chao, M., Rodriguez-Boulan, E., and Le Bivic, A. (1995) J. Biol. Chem. 270, 12219–12224
9. Hunziker, W., and Fumey, W. (1984) EMBO J. 13, 2963–2969
10. Arndt, K., Rosen, P. A., Kunz, I. D., Cohen, F. E., and Mostov, K. E. (1993) J. Cell Biol. 123, 1149–1160
11. Brown, D. A., Crise, B., and Rose, J. K. (1989) Science 245, 1499–1501
12. Lisanti, M. P., Caras, I. P., Davitz, M. A., and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2145–2156
13. Lisanti, M. P., Le Bivic, A., Salitel, A., and Rodriguez-Boulan, E. (1990) J. Membr. Biol. 113, 155–167
14. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
15. Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) J. Cell Biol. 122, 789–807
16. van Meer, G., and Simons, K. (1988) J. Cell Biol. 106, 51–58
17. Scheiffele, P., Perian, J., and Simons, K. (1995) Nature 378, 96–98
18. Kitagawa, Y., Sano, Y., Ueda, M., Higashio, K., Narita, H., Okano, M., Matsumoto, S., and Sasaki, R. (1994) Exp. Cell Res. 213, 449–457
19. Zurrollo, C., Lisanti, M. P., Caras, I. W., Nitsch, L., and Rodriguez-Boulan, E. (1993) J. Cell Biol. 121, 1031–1039
20. Zurrollo, C., Polistina, C., Saini, M., Gentile, K., Aloj, L., Migliacico, G., Bonatti, S., and Nitsch, L. (1992) J. Cell Biol. 117, 551–564
21. Börsig, M., and Plocher, M. G. (1988) J. Cell Biol. 107, 471–479
22. Matter, K., Brachar, M., Bucher, K., and Haupi, H. P. Cell 1990 69, 429–437
23. Le Bivic, A., Quaroni, A., Nichols, B., and Rodriguez-Boulan, E. (1990) J. Cell Biol. 111, 1351–1361
24. Barthes, J. R., Ferracci, H. M., Steiger, B., and Hubbard, A. L. (1987) J. Cell Biol. 105, 1241–1251
25. Johnson, D., Lanahan, A., Burk, C. R., Seghal, A., Morgan, C., Mercer, E., Rothwell, M., and Chao, I. (1987) Cell 47, 645–654
26. Garcia, M., Mirre, C., Quaroni, A., Reggio, H., and Le Bivic, A. (1993) J. Cell Sci. 104, 1281–1290
27. Gilbert, T., Le Bivic, A., Quaroni, A., and Rodriguez-Boulan, E. (1991) J. Cell Biol. 113, 275–288
28. Yeaman, C., Le Gall, A. H., Baldwin, A. N., Monlauzeur, L., Le Bivic, A., and Rodriguez-Boulan, E. (1997) J. Cell Biol. 139, 929–940
29. Berger, J., Howard, A. D., Brink, L., Gerber, L., Hauber, J., Cullen, B. R., Udenfriend, S. (1988) J. Biol. Chem. 263, 10016–10021
30. Bordier, C. (1981) J. Biol. Chem. 256, 1499–1501
31. Vogel, L. K., Noren, G., and Sjostrom, H. (1995) J. Biol. Chem. 270, 22933–22938
32. Ellis, J. A., and Luzio, J. P. (1995) J. Cell Biol. 127, 2071–2073
33. Mertens, G., van der Scharen, B., van der Berge, H., and David, G. (1996) J. Cell Biol. 132, 487–497
34. Schell, M. J., Maurice, M., Steiger, B., and Hubbard, A. L. (1992) J. Cell Biol. 119, 1173–1182
35. Hünziker, W., Spiess, M., Senanay, G., and Lodish, H. F. (1986) Cell 46, 227–234
36. Casanova, J. E., Breitfeld, P. P., Ross, S. A., and Mostov, K. E. (1990) Science 248, 742–745

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37. Odorizzi, G., Pearse, A., Domingo, D., Trowbridge, I. S., and Hopkins, C. R. (1996) *J. Cell Biol.* **135**, 139–152
38. Olsen, J., Cowell, G. M., Konigshofer, E., Danielsen, E. M., Moller, J., Lausten, L., Hansen, O. C., Weilinter, K. G., Engberg, J., Hunziker, W. (1988) *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **238**, 307–314
39. Medof, M. E., Lublin, D. M., Holers, V. M., Ayers, D. J., Getty, R. R., Leykam, J. P., Atkinson, J. P., and Tykocinski, M. L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2007–2011
40. Reddy, P., Caras, I., and Krieger, M. (1989) *J. Biol. Chem.* **264**, 17329–17336
41. Vogel, L. K., Nore, O., and Sjostrom, H. (1992) *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **308**, 14–17
42. Urban, J., Parczyk, K., Leutz, A., Kayne, M., and Kondor-Koch, (1987) *J. Cell Biol.* **105**, 2735–2743
43. Fiedler, K., and Simons, K. (1995) *Cell* **81**, 1–20