Sorting Competition with Membrane-permeable Peptides in Intact Epithelial Cells Revealed Discrimination of Transmembrane Proteins Not Only at the trans-Golgi Network but Also at Pre-Golgi Stages*

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Transmembrane proteins destined to the basolateral cell surface of epithelial cells contain in their cytosolic domain at least two classes of sorting signals: one class promotes exit from the endoplasmic reticulum (ER) and transport to the Golgi complex, and the other class operates at the trans-Golgi network (TGN) specifying segregation into basolateral exocytic pathways. Both kinds of addressing motifs are quite diverse among different proteins. It is unclear to what extent this feature reflects alternative decoding mechanisms or variations in motifs recognized by the same sorting factor. Here we applied a novel strategy based on permeable peptide technology and temperature-sensitive model proteins to study competition between cytosolic sorting motifs in the context of mammalian living cells. We used the transduction domain of HIV-1 Tat protein to make a membrane-permeable peptide of the cytosolic tail of GtsO45, which contains a well characterized ER exit di-acidic (DIE) motif and a tyrosine-based basolateral sorting signal (YTDI). This peptide added to the media inhibited transport of GtsO45 from both ER-to-Golgi and TGN-to-basolateral cell surface in transfected Madin-Darby canine kidney cells. Instead, it did not affect the exocytic trafficking of a GtsO45-derived chimeric protein bearing 30 juxtamembrane residues from the cytosolic domain of the epidermal growth factor receptor that contains a variant ER exit motif (ERE) and an unconventional proline-based basolateral sorting signal. These results not only proved the feasibility of competing for sorting events in intact cells but also showed that distinct plasma membrane proteins can be discriminated at pre-TGN stages, and that basolateral sorting involves different recognition elements for tyrosine-based motifs and an unconventional basolateral motif.

Transport of newly synthesized plasma membrane proteins from their site of origin at the ER to the cell surface involves vesicular or tubular carriers and stepwise sorting events operating at different stages of the exocytic route (1–4). Sorting signals located in the cytosolic domain of transmembrane proteins and their recognition by only partially defined cytosolic adaptors promote ER export and efficient transport to the Golgi complex (5–8), and then segregation into specific exocytic pathways emerging from the trans-Golgi network (TGN) to the plasma membrane (1, 2, 9–12). Intriguing characteristics of such cytosolic sorting signals include their diversity and apparent redundancy (5, 6, 13–15). This could mean that different cytosolic recognition decoders exist for each species of sorting information or that the same decoders interact with different sorting signals with variable affinity. Both alternatives could increase the possibilities of regulatory events on subsets of proteins that hold similar addressing information. How many categories of sorting factors or mechanisms exist for a similar destination or how many classes of sorting signals use one or another decoding factor are still unanswered questions. Indeed, systematic competition experiments are required. Here we used temperature-sensitive reporters and membrane-permeable peptides as feasible and convenient approaches to interfere selectively and differentially with plasma membrane protein trafficking from the ER to the Golgi and from the TGN to the basolateral cell surface in intact polarized epithelial cells, revealing unexpected aspects of the exocytic sorting machinery in mammalian cells.

Although the mechanism of ER to Golgi complex transport has been controversial regarding requirements of specific sorting signals, there is now compelling evidence supporting the notion of cargo selection mediated by the COP-II proteinaceous coat (3, 4). Distinct elements of the COP-II complex seem to be responsible for the recognition of sorting information in the cytosolic domain of cargo transmembrane proteins and the manufacture of ER exporting vesicles (16–18), in a process coordinated by the small GTPase Sar1 (19, 20). Soluble cargo also seems to leave the ER selectively by interacting with specific transmembrane proteins, which presumably act as COP-II linking receptors (21). The most characterized ER exit motifs are diphenylalanines (6–8) or a single valine (6) at the carboxyl-terminal and di-acidic motifs (DXXE, EXD, or EXE) (5, 22–24), which in certain proteins can be part of a longer moiety vesicular stomatitis virus G protein; GtsO45, temperature-sensitive VSVG protein; TGN, trans-Golgi network; EGFR, epidermal growth factor receptor; HIV-1 human immunodeficiency virus; AP, adaptor proteins; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; endo-H, endoglycosidase-H.

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‡ The abbreviations used are: ER, endoplasmic reticulum; VSVG, vesicular stomatitis virus G protein; GtsO45, temperature-sensitive VSVG protein; TGN, trans-Golgi network; EGFR, epidermal growth factor receptor; HIV-1, human immunodeficiency virus; AP, adaptor proteins; MDCK, Madin-Darby canine kidney; PBS, phosphate-buffered saline; endo-H, endoglycosidase-H.
A third class of ER export motifs based on RKXRK has been reported recently (26) in the cytosolic tails of Golgi resident glycosyltransferases, type II membrane proteins, whose recognition involved direct interaction with Sar1. How all this diversity is related to the transport process of subsets of proteins remains incompletely defined. Recent evidence in yeast suggested that cargo discrimination could occur upon exit from the ER (16, 17), for instance, leading to package glycosylphosphatidylinositol-anchored proteins in distinct transport vesicles (27). However, the still missing evidence in mammalian cells has contributed to maintain the current notion that discrimination among different plasma membrane proteins occurs only upon reaching the TGN (1).

Alternative exocytic pathways emerge from the TGN carrying distinct plasma membrane proteins according to specific sorting information (1, 9, 10, 28, 29). This is more evident in polarized epithelial cells, in which apical and basolateral exocytic pathways nurture the corresponding plasma membrane domain with specific proteins giving rise to distinct compositions (11, 12). Apical plasma membrane proteins have sorting information in their extracellular membrane, anchoring or cytosolic domains, suggesting a variety of decoding mechanisms (29). Instead, basolateral sorting signals have been found exclusively in cytosolic tails (2, 14, 30) and therefore should be recognized by cytosolic sorting factors, presumably including elements of the AP adaptor family (31–34). Although most basolateral sorting signals are based on tyrosine residues in the context of YXXΦ (where Φ is a bulky hydrophobic acid), or NPXY or involve di-leucine residues, they are highly degenerate, frequently indistinguishable from endocytic motifs (14), and can be even formed by unconventional motifs entailing none of these residues (13–15, 35). Such diversity suggests a corresponding diversity of decoder elements. Recent evidence involved the adaptor protein complexes AP-1B (31, 36, 37) and AP-4 (33) as sorting mediators of at least certain basolateral proteins, seemingly recognizing tyrosine sorting signals as well as a distinct range of non-tyrosine motifs. AP-3 could potentially expand the range (34), and there still may be additional basolateral sorting mediators to be discovered. The functional and mechanistic implications of diversity in both the basolateral sorting sequences and these AP decoders remain unknown.

The following observations prompted us to attempt a previously unexploited strategy for competing with sorting signals in intact cells. First, in vitro reconstitution systems have shown that synthetic peptides bearing sorting information for ER exit (5) or basolateral delivery (9, 38) are able to inhibit the corresponding transport step of model proteins, suggesting that it is possible to compete for cytosolic sorting events with exogenous signals. Second, membrane-permeable peptides containing transducing sequences that drive through the plasma membrane have been used recently to interfere with a variety of cytosolic protein-protein interactions (39, 40). This opens the convenient possibility of testing competition between cytosolic sorting sequences in the context of living cells. Third, VSVG protein has been widely used as reporter protein for sorting events mediated by a well characterized ER exit di-acidic (DIE) motif (5, 24, 41), recently expanded to YTDIEM (25), and for exocytic sorting from the TGN mediated by an overlapping YXXΦ basolateral sorting signal (YTD) (42, 43). Its temperature-sensitive variant GtsO45 is retained in the ER at the non-permissive temperature (44, 45) and at the TGN by the established 20 °C block (46), thus allowing the ER-to-Golgi and TGN-to-cell surface transport (47) to be analyzed separately. GtsO45 is also suitable to make hybrid proteins with segments of other proteins while conserving its temperature sensitivity (48).

We found that a peptide made up of the protein transduction domain of HIV-1 Tat followed by the cytosolic domain of GtsO45 inhibited transport of GtsO45 from the ER to the Golgi and from the TGN to the basolateral plasma membrane in intact Madin-Darby canine kidney (MDCK) cells. On the contrary, it did not affect a temperature-sensitive chimeric model protein differing only in the cytosolic domain was replaced with sequences from the EGFR receptor, which included a putative EXE (ERE) di-acidic ER exit motif (24) and an alanine-dependent basolateral sorting signal (15). These results demonstrated for the first time that mammalian cells discriminate among different plasma membrane proteins at pre-Golgi steps, during ER to Golgi complex transport, and suggest that subtly different di-acidic ER exit motifs might not be equivalent. They also showed that basolateral sorting involves different recognition elements for proteins bearing either tyrosine-based or unconventional sorting signals.

MATERIALS AND METHODS

Expression Plasmids and Antibodies—The GtsO45 cDNA (44) was subcloned into pcDNA3 (Invitrogen). A cDNA fragment encompassing residues 1–482 of GtsO45 was amplified by PCR and cloned into Nhel-Xba I-modified vector of pcDNA 3.1(−) vector (Invitrogen). Into the Xhol-HindIII site of this vector, we ligated in-frame a DNA segment encoding the cytosolic residues 645–674 of the human EGFR. This chimeric cDNA encodes the extracellular and transmembrane domains of GtsO45 followed by the 30 cytosolic juxtamembrane residues of the EGFR (G-EGFRc). This construction was corroborated by sequencing. The expression of both GtsO45 and G-EGFRc was assayed with a monoclonal antibody directed against the NH2 terminus of VSVG provided by Dr. E. Rodriguez-Boulan (Cornell University).

Permanently Transfected MDCK Cells—Strain-II MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Bioproducts for Science, Inc.), 200 units/ml penicillin, and 0.1 mg/ml streptomycin (Sigma) and fungizone (Bristol-Myers Squibb Co.). Transfections with GtsO45 and G-EGFRc DNAs were made using the LipofectAMINE Plus (Invitrogen) method according to the manufacturer’s protocol, and permanent transformants were obtained after 2 weeks of selection with G418 (0.8 mg/ml) (Invitrogen) (49, 50). Protein expression was induced by 10 mM sodium butyrate (49, 50) at the restrictive temperature of 40 °C for 20 h to accumulate the model proteins in the ER.

Domain-specific Biotinylation and Immunoprecipitation—Domain-specific biotinylation was made in MDCK cells cultured in Transwell chambers (49). The cells were washed twice in ice-cold PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-CM), and then sulfated with 1 mM CMB (25) for 30 min. After three washes in ice-cold PBS-CM, the cells were incubated with 50 mM NH4Cl for 10 min and then lysed in 20 mM Tris-HCl buffer, pH 8, 0.150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumin, and anti-protease mixture (2 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM leupeptin; Sigma). Immunoprecipitations were performed by incubating with a monoclonal anti-VSVG antibody for 1 h at 4 °C and then adding protein A-Sepharose previously incubated with 10 μg of a rabbit anti-mouse antibody. The beads were washed six times in ice-cold lysis buffer containing 500 mM NaCl, 0.1% (v/v) Triton X-100, resuspended in sample buffer, and boiled for 10 min. Proteins were resolved by SDS-PAGE, electrotransferred to nitrocellulose membranes, and the biotinylated proteins detected with horseradish peroxidase-conjugated streptavidin and ECL detection kit (Amersham Biosciences).

Immunofluorescence Microscopy and Image Quantiﬁcation—Indirect immunofluorescence was done as described (50). Permanently transfected MDCK cells grown to confluence on glass coverslips were subjected to the different experimental conditions, washed with PBS, and fixed for 30 min at room temperature with 4% paraformaldehyde in PBS supplemented with 0.1 mM CaCl2 and 1 mM MgCl2 (PBS-CM). All washes were made in PBS plus 0.2% gelatin (300 Bloom, Sigma). The cells were permeabilized with 0.2% Triton X-100 in PBS-CM for 10 min at room temperature. Incubation with anti-VSVG monoclonal antibody (1:250) and secondary antibody, fluorescein isothiocyanate-labeled antimouse IgG (1:100) was made for 30 min at 37 °C. Digital images of fluorescence were acquired on a Zeiss Axiopt microscope with a ×63 immersion objective and the 14-bit AxioCam camera and then transferred to a computer workstation running Axiovision imaging software.
For quantification, all images from a single experiment were acquired under identical settings (8 bits; 1030 × 1300 pixels; and the same exposure times). Digital images were then analyzed with MetaMorph imaging software (Universal Imaging, West Chester, PA). In each region of interest, total fluorescence intensities were measured for all samples using black and white 8-bit unsaturated and background-less images, thus minimizing nonspecific fluorescence. The entire lateral plasma membrane was selected by drawing a strip fit with a maximum width of 15 pixels. The integrated fluorescence intensities of lateral plasma membranes between two cells were divided to estimate the contribution of each cell. The perinuclear Golgi staining and vesicular structures were enclosed within lined areas, and fluorescence measures were made adjusting similar threshold intensities in each cell.

Cell-permeable Peptides—Cell-permeable peptides were manufactured at the Institute of Biochemistry, Charité, Humboldt University of Berlin. High pressure liquid chromatography-purified (95% purity) and lyophilized peptides were dissolved in Me2SO immediately before use to prevent aggregation by oxidation or denaturation. The peptides were added directly to the cell culture medium at a concentration of 40 μM.

Uptake of [35S]-Labeled Tat-GpY487Y501—The Tat-GpY487Y501 peptide (10 μg) was labeled with [35S]Met/Cys (100 μCi/mL) for 4 h at 40 °C, washed three times in PBS containing 100-fold excess of Met/Cys, and then incubated for 30 min at 40 °C with cycloheximide (10 μM) and cell-permeable peptides in in Dulbecco’s modified Eagle’s medium supplemented with 20 mM HEPES. After shifting the temperature to 32 °C for different periods of time, cell extracts were prepared for immunoprecipitation, and the immunocomplexes were digested for 17 h at 37 °C with 50 μg of Endoglycosidase-H (Calbiochem) in 100 mM acetate buffer, pH 5.6, containing 0.2% Triton X-100 and 0.02% phenylmethylsulfonyl fluoride. The digested samples were digested for 17 h at 37 °C with Endoglycosidase-H (Calbiochem) in 100 mM acetate buffer, pH 5.6, containing 0.2% Triton X-100 and 0.02% phenylmethylsulfonyl fluoride.

RESULTS

The GtsO45 and G-EGFRc Chimera as Thermo-sensitive Reporter Proteins Containing Different Sorting Information for Exiting the ER and for Basolateral Delivery—As a model system, we used permanently transfected MDCK cells expressing either the GtsO45 or the chimeric protein G-EGFRc made of the exoplasmic and transmembrane domains of GtsO45 followed by 30 residues of the juxtamembrane region of the EGFR. The cytosolic domains of both model proteins are depicted in Fig. 1A highlighting the relevant sorting sequences. GtsO45 contains the sequence YTDIEM, which acts as sorting information for efficient exit from the ER (25), and includes an overlapping YTDI, which in the wild-type VSVG protein has been identified as a tyrosine-based basolateral sorting signal (42). However, GtsO45 contains another tyrosine (Tyr587) in the context of YLCI (44) that also fits the sequence YXXΦ of basolateral sorting signals. On the other hand, the 30 juxtamembrane residues of the EGFR contain an ERE motif conforming to a di-lysine (EXE) ER exit motif (22, 24) and two basolateral sorting signals, one conforming to a di-leucine motif in position 659-LL and the second based on Pro667 and Pro670, with a possible contribution of Arg662 (15). The proline-based signal seems to be dominant over the di-leucine motif.

Domain-selective biotinylation of permanently transfected MDCK cells grown to confluence in Transwell chambers showed that at steady-state both model proteins achieved a predominantly basolateral distribution (Fig. 1B) of ~70% for G-EGFRc and 80% for GtsO45. At the non-permissive temperature (40 °C), indirect immunofluorescence showed both proteins with a reticular ER-like pattern (Fig. 2, a and b). By shifting the temperature from 40 to 20 °C for 2 h, both GtsO45 and G-EGFRc showed mainly a perinuclear staining that reflects accumulation at the TGN (Fig. 2, c and d). When transport out of the Golgi was subsequently induced by raising the temperature to 32 °C, both proteins achieved the characteristic basolateral pattern within 30 min (Fig. 2, e and f). Thus, G-EGFRc maintained the temperature sensitivity, the 20 °C block at the TGN, and the basolateral segregation of GtsO45.

A Tat-based Membrane-permeable Peptide Containing the Cytosolic Tail of GtsO45 Selectively Blocked the ER-to-Golgi and TGN-to-Basolateral Cell Surface Transport of GtsO45 but Not of G-EGFRc—Three Tat peptides were synthesized to study their effect on the ER-to-Golgi and TGN-to-basolateral plasma membrane transport of the model proteins (Fig. 3). The Tat peptide (Tat-p) contains the transduction domain of the HIV-Tat protein (residues 47–57), which confers membrane permeability properties (39, 40). This peptide served as a con-
control in all the experiments. In the Tat-GpY487-Y501 peptide, the transduction domain of the HIV-Tat precedes the entire cytosolic domain of GtsO45. Instead, the peptide Tat-GpA487-A501 is a mutant peptide of the cytosolic domain of GtsO45, in which the tyrosines Tyr487 and Tyr501 were replaced by alanines to abrogate both the known YTDI and the putative YLCI basolateral motifs.}

The effect of these Tat-carried peptides on transport of the temperature-sensitive model proteins to the Golgi complex was quantitatively analyzed by pulse-chase and endo-H resistance assays. When the cells were labeled for 4 h at 40°C, most of the GtsO45 and G-EGFRc remained sensitive to endo-H digestion (Fig. 4, A and B, compare lanes 1 and 2) indicating that they were efficiently retained in the ER at the non-permissive temperature, as shown previously by immunofluorescence. Still at the non-permissive temperature, the cells were incubated for 30 min in the presence of 10 μM cycloheximide and 40 μM of either the control Tat-p or the wild-type Tat-Gp peptide, which were maintained for the rest of the experiment. After shifting the temperature to 32°C, both endo-H-sensitive GtsO45 and G-EGFRc (lane 3) acquired endo-H resistance, ~30% at 15 min and 50% at 30 min (Fig. 4, A and B; lanes 5 and 7; bands labeled with asterisks). The Tat-GpY487-Y501 peptide having a di-acidic ER exit motif inhibited ER-to-Golgi transport of GtsO45 (Fig. 4A, lanes 4 and 6) but not of G-EGFRc, which instead acquired endo-H resistance as well as in the presence of the control Tat-p peptide (Fig. 4B, lanes 4 and 6). This indicates that transport of G-EGFRc from ER to Golgi is mediated by an element relating...
and 40°C/H9262 Tat-GpY487-Y501 selectively inhibits transport of GtsO45 from the TGN (A) were preincubated with 10 μM cycloheximide and 40 μM Tat-GpY487Y501 (b and d) or Tat-GpA487A501 (c and e) and then shifted to 32°C for 30 or 60 min, as indicated. Indirect immunofluorescence in permeabilized cells shows that Tat-GpY487Y501, but not the mutated peptide Tat-GpA487A501, lacking basolateral motifs, inhibits transport of GtsO45 from the TGN to the basolateral cell surface. B, the percentage of GtsO45 in the cell surface relative to intracellular perinuclear structures (Golgi and vesicles) was estimated by analyzing digital images with Metamorph software.

FIG. 5. Indirect immunofluorescence showing that peptide Tat-GpY487-Y501 selectively inhibits transport of GtsO45 from the TGN to the basolateral cell surface. A, transfected MDCK cells maintained at 40°C for 20 h and then at 20°C for 2 h to accumulate proteins in the TGN (a) were preincubated with 10 μM cycloheximide and 40 μM Tat-GpY487Y501 (b and d) or Tat-GpA487A501 (c and e) and then shifted to 32°C for 30 or 60 min, as indicated. Indirect immunofluorescence in permeabilized cells shows that Tat-GpY487Y501, but not the mutated peptide Tat-GpA487A501, lacking basolateral motifs, inhibits transport of GtsO45 from the TGN to the basolateral cell surface. B, the percentage of GtsO45 in the cell surface relative to intracellular perinuclear structures (Golgi and vesicles) was estimated by analyzing digital images with Metamorph software.

Interference with Exocytic Sorting Signals in Living Cells

Fig. 6. The peptide Tat-GpY487-Y501 does not inhibit transport of G-EGFRc from the TGN to the basolateral cell surface as shown by indirect immunofluorescence. A, transfected MDCK cells maintained at 40°C for 20 h and then at 20°C for 2 h to accumulate proteins in the TGN (a) were preincubated with 10 μM cycloheximide and 40 μM Tat-GpY487Y501 (b) and Tat-p (c) and then shifted to 32°C for 30 min. A, indirect immunofluorescence shows that in both conditions the protein achieved a basolateral distribution. B, percentage of the protein in the cell surface estimated as in Fig. 5. In contrast, similar amounts of G-EGFRc reached the cell surface of either Tat-GpY487-Y501 (21.9%) or the control Tat-p peptides (16.9%) (Fig. 6). None of the peptides affected the morphology or the area of the Golgi complex (data not shown).

Similar results were obtained by domain-specific biotinylation assays in cells grown to confluence on Transwell chambers. In these experiments, Tat-GpY487Y501 inhibited almost 90% of the transport of GtsO45 to the basolateral cell surface (Fig. 7A), whereas neither the mutated peptide Tat-GpA487A501 nor the peptide solvent Me₂SO (Fig. 7A) caused a significant effect. Again, transport of G-EGFRc did not change in the presence of any of these peptides (Fig. 7B). Thus, transport from the TGN to the basolateral plasma membrane of these two model proteins very likely involves distinct recognition elements.

DISCUSSION

The prevalent notion has been that plasma membrane proteins in mammalian cells are treated as similar substrates by the exocytic machinery until they reach the TGN, whereby they become segregated into alternative exocytic pathways according to specific apical or basolateral sorting information (1). We used Tat-based membrane-permeable peptide technology to interfere selectively and specifically with sorting signals in the
Interference with Exocytic Sorting Signals in Living Cells

context of living MDCK epithelial cells. A cell-permeable peptide consisting of the cytosolic tail of GtsO45 preceded by the Tat transduction domain of HIV, added to the media inhibited transport of GtsO45 from ER to Golgi, as measured by acquisition of endo-H resistance, and from the TGN to the basolateral cell surface, as shown both by quantitative immunofluorescence and domain-specific biotinylation, without affecting a chimeric protein that only differed in the cytosolic domain. These results demonstrated for the first time in mammalian cells that distinct plasma membrane proteins are discriminated not only at the TGN but also during transport from the ER to the Golgi complex. Furthermore, the differential sensitivity to the peptide bearing a tyrosine-dependent basolateral sorting signal indicates that polarized epithelial cells have alternative TGN-dependent sorting systems to segregate distinct plasma membrane proteins to the same basolateral domain.

Previous studies using permeabilized cells and in vitro reconstitution systems have shown that a peptide corresponding to the cytosolic tail of VSVG interfered with budding of VSVG carrying vesicles both at the ER (5) and the TGN (9, 38). Our results validate these in vitro observations (5, 9), but in the context of living cells, i.e. precluding unwanted dilution or unaware loss of any sorting or regulatory element. Even when we detected inhibitory effects by using similar micromolar concentrations to those used in in vitro systems (5, 9), we estimated that only 3% of the peptides enter the cell, and therefore, their competitive effectiveness probably increased in the low water concentration of the cytosol. We also expanded previous observations showing that a model plasma membrane protein, in which the cytosolic domain of GtsO45 was replaced by 30 residues from the EGFR juxtamembrane region, lost sensitivity to the blocking peptide both in its ER to Golgi and TGN to plasma membrane trafficking. At both levels, the exocytic machinery is then able to discriminate sorting information for similar destinations contained in the cytosolic tail of plasma membrane proteins, thus implying the operation of distinct sorting-adaptor elements.

There is now compelling evidence supporting the notion that exit from the ER does not occur by default but instead involves selection and concentration of cargo at ER exit sites (3, 4, 24). The ER exit motif of the cytosolic tail of GtsO45 encompasses six amino acids YTDIEM (25), including a DXXE di-acidic motif that seemingly mediates interaction with the Sec23/24 complex of the COP-II coat involved in transport from the ER to the Golgi complex (5, 20, 24, 41). Thus, the Tat-GpY487-Y501 permeable peptide most probably blocked ER to Golgi transport of GtsO45 by competing for interaction with Sec23/24p. Strikingly, the G-EGFRc chimeric protein also contains an EXE-diacidic motif (24) but showed no inhibition by the blocking peptide Tat-GpY487-Y501. Therefore, EGFRc seems to contain a di-acidic ER exit motif similar (EXE) but not equivalent to that described in GtsO45, although we cannot discard the possibility that it contains another unrelated type of ER exit signal.

The Sec23/24 complex, particularly the p24 component, most responsible for cargo selection into COP-II manufactured vesicles (16), provides a variety of binding sites able to accommodate different ER exiting signals in the COP-II complex (3, 4, 6, 7, 17, 19, 51). Furthermore, several homologues of Sec23/24 have been described in both yeast and mammalian cells and several lines of evidence suggest that they can exert selection upon different types of cargo (27, 52–55). In yeast, genetic and structural analysis complemented with in vitro competition experiments have recently disclosed functional diversity displayed by different sorting signals and the presence of at least three different binding sites for distinct ER exiting sorting signals in

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**Fig. 7.** Biotinylation assays showing that Tat-GpY487-Y501 diminishes transport of GtsO45 but not of G-EGFRc from the TGN to the basolateral cell surface in MDCK cells grown on Transwell chambers. A and B, transfected MDCK cells grown to confluence in Transwell chambers were maintained at 40 °C for 20 h and then incubated 20 °C for 2 h to accumulate the GtsO45 and G-EGFRc proteins at the TGN. Transport to the basolateral cell surface was assessed by basolateral biotinylation assays after shifting the temperature to 32 °C for 60 min in the presence of 10 μM cycloheximide and 40 μM of the indicated Tat peptides or 0.5% Me2SO, the solvent for the peptides. Proteins were immunoprecipitated, resolved by SDS-PAGE, and visualized in blot with streptavidin-peroxidase and chemiluminescence. The Tat peptide bearing a functional basolateral sorting signal inhibited the transport of GtsO45 in the ER were metabolically labeled with [35S]Met/Cys for 4 h and then shifted to 32 °C in the absence or presence of 40 μM of mutant peptide for 30 min. GtsO45 was immunoprecipitated and subjected to endo-H treatment. The fluorogram shows that in the presence of Tat-GpY487-Y501 GtsO45 did not acquire endo-H resistance and became degraded. Thus, this mutant peptide lacking the basolateral sorting signal but still maintaining the di-acidic ER exit motif (DIE) inhibited transport of the GtsO45 from ER to the Golgi, indicating that it is transduced into the cells as well as the wild-type peptide.
p24 (17, 18). Furthermore, even similar di-acidic motifs seemed not to be equivalent for the COP-II sorting machinery in yeast (17). Here we provide evidence that such distinction can also occur in mammalian cells. In our competition experiments with Tat peptides, two plasma membrane proteins carrying a di-acidic class of ER exit signal were distinguished as different substrates. This might have a molecular explanation in the structural and functional different binding sites found in Sec24 or might involve additional but yet unknown sorting elements.

On the other hand, recent evidence in yeast showed that glycosylphosphatidylinositol-anchored proteins are transported out of the ER in different vesicles than secretory proteins, indicating the existence of more than a single transporting pathway to the Golgi complex, very likely mediated by subtypes of COP-II (27). In mammalian cells, the secretory protein procollagen, which has a rigid rod-like structure too large in size to fit into conventional vesicular carriers, uses transport complexes distinct from those carrying GtsO45 or ERGIC-53 (56). It remains unknown whether discrimination of plasma membrane proteins by the ER exiting machinery determines incorporation into the same or distinct transporting vesicles.

Tat-GpY487-Y501 added to the media inhibited transport of GtsO45 from the TGN to the basolateral cell surface most probably competing for cytosolic sorting factors involved in tyrosine-mediated segregation. A mutated peptide in which the GtsO45 from the TGN to the basolateral cell surface most likely GtsO45 might have entered not only into an active cargo promotes formation of its own transporting vehicles, otherwise GtsO45 might have been eliminated by mutagenesis (14). In contrast, under the effect of the blocking peptide, GtsO45 did not appear in the apical domain but was retained in the Golgi. These observations are similar to that reported in semi-intact cells (9) and suggest that cargo promotes formation of its own transporting vehicles, otherwise GtsO45 might have entered not only into an apical path but into also the Tat-GpY487-Y501-insensitive basolateral pathway followed by the G-EGFRc chimera. Previous experiments on semi-intact MDCK cells have shown that a VSVG tail peptide blocked exit from the TGN of the VSVG protein (9, 38) and also of a chimeric protein bearing another tyrosine-based basolateral sorting signal (9). In addition, peptides containing tyrosine-based signals from other proteins also inhibited VSVG exit from the TGN, suggesting that diverse tyrosine-based sorting signals use the same factors for basolateral sorting (9). Thus, the Tat peptide we used here presumably interfered with most if not all the tyrosine-mediated sorting machinery. This peptide did not block the TGN-to-plasma membrane transport of the chimeric protein G-EGFRc containing in its cytosolic domain the basolateral sorting signal of the EGFR, which is crucially determined by two prolines (15). Therefore, basolateral targeting of the chimera G-EGFRc, and therefore of EGFR, driven by a non-conventional proline-based sorting signal very likely involves elements of the sorting machinery distinct from those engage in decoding tyrosine-based information.

The only cytosolic factors so far identified as mediators of basolateral sorting in epithelial cells, AP-1B (31, 36, 37) and AP-4 (33), both recognize tyrosine-based sorting signals but also seem to cover a broader range of sorting signals. AP-4 could mediate basolateral sorting based on di-hydrophobic motifs, and both AP-1B and AP-4 are required for basolateral segregation of proteins bearing distinct unconventional basolateral motifs. Tyrosine-dependent basolateral sorting signals are recognized by μ subunits in both AP1B and AP4 (31, 33). An additional sorting factor might be the AP-3 complex, which in non-polarized cells has been described to mediate TGN export of VSVG, seemingly by interacting through the δ subunit with the YTDIE di-acidic motif that includes a crucial tyrosine for basolateral sorting (34). Thus, the broad range of sorting signals covered by heterotetrameric AP complexes is probably due to interactions with different subunits (57) and even different molecular regions within the same subunit (37). It is unknown whether blocking the tyrosine recognition subunit leaves other AP subunits competent for additional interactions. On the other hand, recent evidence (36) showing that AP-1B is required for basolateral recycling sheds doubt on whether this and other AP adaptors, which might participate in basolateral segregation, actually exert their sorting function at the TGN or in endocytic recycling compartments. Our results showed inhibition of tyrosine-dependent trafficking of GtsO45 at the TGN and suggested that basolateral sorting of the EGFR involves a sorting machinery that can still operate when the recognition factors for tyrosine-based sorting signals are almost completely blocked by the competing Tat peptide. At the TGN, recognition of the proline-dependent basolateral information in the EGFR might involve not μ but other AP subunits or yet unidentified cytosolic sorting factors.

The in vivo competition assay with permeable peptides should be useful for identifying unknown sorting signals and establishing categories of these signals based on their susceptibility to different sets of blocking peptides. This should foster advances in dissecting and defining still unknown aspects of the sorting machinery, including assessment of common features shared by the segregation process of secretory proteins.

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REFERENCES

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