Introduction

In each compartment of the secretory pathway, proteins can either be recruited into anterograde or retrograde transport vesicles, or retained, by exclusion from such vesicles (for a review, see Mellman and Warren, 2000). The information for the choice between these alternatives generally resides within specific amino acid sequences that mediate the protein-protein or protein-lipid interactions required to determine the polypeptide’s fate. In the case of transmembrane proteins, such sorting sequences or determinants have been identified both in the cytosolic and the lumenal regions, as well as in the transmembrane domains (TMD). The TMDs in particular play an important role in determining the residence of Golgi (for a review, see Munro, 1998), and some ER, proteins (Pedrazzini et al., 1996; Yang et al., 1997; Rayner and Pelham, 1997; Watson and Pessin, 2001) in the ER retention of unassembled oligomer subunits (Bonifacino et al., 1991; Lankford et al., 1993), in the targeting of a number of apical proteins in polarised epithelial cells (Kundu et al., 1996; Huang et al., 1997; Lin et al., 1998; Dunbar et al., 2000), and in trafficking within the endosomal-vacuolar branch of the endo-exocytic pathway in yeast (Rayner and Pelham, 1997; Lewis et al., 2000; Reggiori et al., 2000). Although specific sequence motifs within TMDs may in some cases be required for their role in sorting (Dunbar et al., 2000), more frequently it appears that this function relies on simple physicochemical properties, such as length, degree of hydrophobicity or charge (Bonifacino et al., 1991; Lankford et al., 1993; Munro, 1998; Pedrazzini et al., 1996; Letourneur and Cosson, 1998; Reggiori et al., 2000). Such properties constitute, in some cases, the basis for specific interactions with sorting receptors (Letourneur and Cosson, 1998); however it has also been suggested that they mediate a lipid-based sorting mechanism by driving transmembrane proteins into domains of the bilayer that are either recruited into or excluded from budding transport vesicles (Bretscher and Munro, 1993; Pedrazzini et al., 1996).

Because multiple, hierarchically acting sorting determinants are often present on the same protein (for a review, see Mostov et al., 2000), their identification in multidomain polypeptides or...
oligomers has proved to be difficult. As simple models to study the role of the TMD in trafficking, we have been using tail-anchored (TA) proteins. Proteins of this class, which are defined by a cytosolic N-terminal domain anchored to the bilayer by a TMD close to the C-terminus (Borgese et al., 1993; Kutay et al., 1993), provide convenient models for investigating trafficking of membrane proteins in the absence of a luminal domain. Using cytochrome b(5) (b(5)) as a representative TA protein, we were able to uncover for the first time a role for the TMD in determining the localisation of an ER-resident protein. Like other ER-restricted TA proteins, b(5) has a relatively short TMD, a feature that is absolutely required for its ER residency. This was demonstrated by a b(5) mutant with a lengthened TMD, which escapes from the ER and reaches the plasma membrane (Pedrazzini et al., 1996). The TMD-dependent sorting mechanism first described for b(5) is shared also by other TA proteins (Rayner and Pelham, 1997; Yang et al., 1997).

Here, we have pursued our studies of the role of the TMD in sorting of TA proteins. To exclude any effect of the catalytic domain of b(5), we have replaced it with GFP and then systematically investigated the effect of TMDs of different length, hydrophobicity and sequence on the intracellular distribution of the corresponding fusion proteins. Moreover, we have extended our observations to polarised epithelial cells and asked which plasma membrane domain is reached by TA proteins with extended TMDs. We find that GFP fused to the extended tail of b(5) is localised to the basolateral surface of MDCK cells; however it can be partially relocated to the apical domain by addition of a short luminal sequence containing an N-glycosylation consensus. A similar effect is obtained by replacing the extended tail of b(5) with the TMD of the apically targeted TA protein, syntaxin 3.

Materials and Methods

Plasmid constructions

The plasmids used in this study were all derived from a basic GFP reporter construct (GFP-17), which is illustrated in Fig. 1A. It consists of an extended version of GFP fused at its C-terminus, via a linker sequence, to the tail region of the ER isoform of rat b(5). The tail region contains the entire sequence (Pro94-Asp134) that remains membrane associated after trypsin cleavage of native b(5), including the 17-residue TMD (Fig. 1C), flanked upstream and downstream by polar sequences (UPS and DPS respectively) (Fig. 1A-C). The linker consists of the myc epitope followed by [(Gly)4Ser]3 (Fig. 1B). Unique restriction sites are present at the border between the linker and the UPS and at both borders of the TMD to permit the introduction of altered sequences by simple cassette mutagenesis. The entire cDNA was inserted in the Hind3-XbaI sites of the mammalian expression vector pCDNA1 (Invitrogen). The details of the construction of this plasmid are described in a recent publication (Borgese et al., 2001) in which it is referred to as GFP-ER.

Constructs with altered TMD length and hydrophobicity (GFP-14, GFP-19, GFP-22, GFP-25 or GFP-17-HH) (Fig. 1C) were obtained by inserting paired synthetic oligonucleotides, coding for the desired sequences and designed to have Xho1 and Age1 compatible extremities, into the basic GFP-17 plasmid cut with Xho1 and Age1. Similarly, GFP-22-Nglyc and GFP-22-MutNglyc (Fig. 1C), which bear a C-terminal tag corresponding respectively to the C-terminal region of bovine opsin or to the same region mutated in its N-glycosylation consensus sequences, were constructed by substituting the appropriate oligonucleotide pairs within the Age1-XbaI sites of GFP-22. The GFP-ST, GFP-Syn3 and GFP-Syn4 (Fig. 1C) constructs were obtained by substituting the entire Xho1-XbaI region of GFP-17 with oligonucleotide pairs coding respectively: (i) for the TMD of rat α-2,6-sialyl transferase flanked at the N-terminus by its three Lys residues, and at the C-terminal end by the downstream polar region of b(5); (ii) for the 29 C-terminal residues of rat syntaxin 3; (iii) for the 29 C-terminal residues of rat syntaxin 4. The GFP-17-ΔUPS construct (not listed in Fig. 1), in which b(5)’s UPS was deleted, was obtained by substituting the BamH1-Xho1 fragment of GFP-17 with an oligonucleotide cassette to obtain the modified linker sequence (AUPS linker; Fig. 1B) directly attached to the TMD region.

We also produced two constructs, GFP-22-Δmyc and GFP-22-MutNglyc-Δmyc (not listed in Fig. 1), in which the corresponding constructs (GFP-22 and GFP-22-MutNglyc) were modified to contain a linker lacking the myc epitope (Δmyc linker – Fig. 1B). To this purpose, the sequence coding for enhanced GFP in pEGFP-N1 (Clontech) was amplified with an upper primer (5' ACCCAAGCTTGGACATGTTGAGACGAAGG-3') covering nucleotides 673-692 of the plasmid and preceded by an extra sequence containing a Hind3 site, and a lower primer (5'-GGCGATCCGGCACCCTCAATCCCTCGGAAACCTCCTGCCTGTTACAGCTCGTCCA TGC-3') comprising nucleotides 1376-1395 of the plasmid followed by a stretch coding for [GGGS]3 and containing a BamH1 site. The amplified fragment, cut with Hind3 and BamH1, was used to replace the Hind3-BamH1 fragment of similarly digested GFP-22 or GFP-22-MutNglyc, to create the corresponding Δmyc plasmids.

Cell culture, transient transfections and selection of stable transformatants

CV-1 cells were grown and transiently transfected by the Ca2PO4 method as previously described (De Silvestris et al., 1995). MDCK strain II cells were cultured as previously described (Borgese et al., 1996). To obtain clones stably expressing our constructs, cells were cotransfected by the Ca2PO4 method with GFP-reporter constructs in pCDNAI and with a plasmid conferring resistance to G-418 (pCB6) in a 20:1 ratio. After 10 days of selection with G-418 (GIBCO BRL, Gaithersburg, MD) at 0.9 mg/ml, resistant colonies were visually inspected with an inverted microscope equipped for epifluorescence (Zeiss IM35), and GFP-expressing clones were picked and expanded in complete medium containing 0.4 mg/ml G-418. For each construct, all experiments were performed on at least two separate clones, with identical results.

To obtain confluent monolayers of polarised stably transfected cells with easily detectable levels of our constructs, cells were seeded onto 24 or 6.5 mm diameter polycarbonate Transwell filters with 0.4 μm pores (Corning Costar Corp, Cambridge, MA) at a density of 250,000 cells/cm2 and cultured for 4 days, with daily changes of the medium. At the end of the fourth day, the monolayers were exposed to medium containing 10 mM Na+ butyrate for 8-12 hours. This medium was then replaced with butyrate-free medium, and the cells were returned to the incubator for a further 4 hours before fluorescence or biotinylation analysis (see below).

In some experiments, we used electroporation to transiently transfect MDCK cells, as described by Rowe et al. (Rowe et al., 2001). After elimination of dead cells by centrifugation over a cushion of Ficoll (Ficoll Plus from Pharmacia Biotech Italia, Cologno Monzese, Milan, Italy), live cells were plated at confluent density onto Transwell filters and analysed after 4 days of culture.

Antibodies

A monoclonal antibody (mAb R2 – 15) against the N-terminal peptide of bovine opsin was a gift of Paul Hargrave (Univeirsity of Florida, Gainesville, FL). A mAb (6.23.3) against a 58 kDa basolateral protein of MDCK cells was donated by Kai Simons (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Anti-giantin antiserum was provided by M. Renz (Institute of Immunology
and Molecular Genetics, Karlsruhe, Germany). Other antibodies were from the indicated commercial sources: polyclonal anti-GFP, Medical and Biological Laboratories, Naka-ku Nagoya, Japan; monoclonal anti-Protein Disulfide Isomerase (PDI), StressGen Biotechnologies, Victoria BC, Canada; rat monoclonal anti-mouse E-cadherin (clone no. DECMA-1), Sigma Italia, Milano, Italy; a polyclonal antibody against the N-terminal peptide of human caveolin I (N-20 antibody), Santa Cruz Biotechnology.

Secondary fluorescent antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Biotinylated or peroxidase-conjugated secondary antibodies and fluorochrome-conjugated streptavidin were from Amersham Pharmacia Biotech Italia.

**Quantitative analysis of confocal Z series**

To estimate the percentage of GFP constructs localising to the Golgi complex, transfected CV-1 cells were fixed and stained with anti-giantin antibodies. Z series of confocal sections at 1 μm intervals were acquired separately with the FITC and Texas Red filters from 25 randomly chosen cells. The laser power and gain were adjusted to maintain the signal below saturation levels. To avoid possible errors owing to bleaching, 50% of the Z series were acquired from the apical to the basal side and 50% were acquired in the opposite direction. The Z series were imported as stacks into the Metamorph program. The mean pixel intensity and area of each cell was measured throughout the stack. After background subtraction, the distribution of label, as a percentage of the total, was calculated. For the anti-opsin antibodies, background for each section was obtained from areas devoid of expressing cells. For E-cadherin, areas inside of the cells, with borders removed by 1 μm from the lateral membrane, were taken as background. Data from eight separate determinations were averaged and standard errors were calculated.

**Treatment with Peptide N-glycanase F (PNGase F) and Endoglycosidase H (Endo H)**

MDCK transfectants expressing GFP-22-Nglyc were grown to subconfluence on 10 cm Petri dishes and treated with Na⁺ butyrate as described above. The cells from one dish were lysed with 0.5 ml TNE + 1% Triton X-100. After clarification by centrifugation, the Triton soluble proteins were precipitated with –20°C acetone, resuspended in 40 μl of a 1:2 slurry of streptavidin agarose beads (ImmunoPure Immobilised Streptavidin Gel from Pierce). Attached proteins were solubilised with Laemmli denaturation buffer and analysed by SDS-PAGE using the reagents supplied by the manufacturer. For 3 hours of incubation, the samples were supplemented with Laemmli denaturation buffer and analysed by SDS-PAGE western blotting.

**Sucrose flotation gradients of detergent lysed cells**

Subconfluent MDCK transfectants, grown on 10 cm Petri dishes and treated with 10 mM Na⁺ butyrate as described above, were scraped with 1 ml of ice-cold TNE buffer containing 1% Triton X-100 and homogenised with 10 strokes of a Dounce homogeniser. The lysate obtained from two Petri dishes was immediately diluted with an equal volume of 80% w/v sucrose in TNE and loaded under a step gradient, consisting of two 4 ml layers of 30% and 5% sucrose in TNE. After centrifugation at 39,000 g for 19 hours at 4°C in the SW 41 rotor (Beckman Instruments, Palo Alto, CA), 12 ml fractions were collected from the top. These were supplemented with 2 ml of PBS containing 50 μg/ml bovine Haemoglobin (Sigma) as carrier, and 3 ml 20% trichloracetic acid. Precipitates were washed with ~20°C acetone and then solubilised with Laemmli denaturing buffer. Equal aliquots of the fractions were analysed by SDS-PAGE western blotting followed by enhanced chemiluminescence.

**Results**

**TMD hydrophobicity as a determinant in the sorting of GFP TA constructs in CV-1 cells**

Our previous work demonstrated that extension of the TMD of b(5) by five residues results in a protein that leaves the ER and...
Fig. 1. Representation of the constructs used in this study. (A) Schematic representation of the basic TA reporter construct, GFP-17. An enhanced version of GFP (oval) is attached at its C-terminus to a linker (filled thin rectangle, L), consisting of the myc epitope followed by a repeated Gly-Ser sequence. This is attached to the entire tail region of rat b(5), which contains the TMD, flanked upstream and downstream by polar sequences (UPS and DPS, respectively). The regions between the indicated unique restriction sites in the cDNA can be easily substituted with paired synthetic oligonucleotides. The expected topology of the construct after insertion into membranes is also indicated. (B) Amino acid sequence (one-letter code) of the Linker and the UPS region present in all the constructs listed in panel (C); within the linker the residues of the myc epitope are in italics. Also shown are the sequences of the Δmyc linker present in the Δmyc constructs, and the ΔUPS linker present in a construct in which b(5)’s UPS was deleted (these constructs are not listed in panel C – see Text). (C) Sequences of the TMD and DPS region of GFP TA constructs. The first six constructs have TMDs derived from b(5) in which amino acids have been deleted, inserted or substituted. The TMD of all these constructs is followed by the DPS of b(5). The numbers 14 through 25 in the construct names indicate the length of the TMD as predicted by hydrophilicity analysis with the scale of Engelman et al. (Engelman et al., 1986) over a window of seven residues. The residues that are predicted to be in a hydrophobic environment are shown in boldface. The construct GFP-17 contains the wild-type tail region of b(5). In the construct GFP-17-HH, the number of residues was not changed, but four substitutions (underlined) increase the TMD hydrophobicity (this results in the inclusion of one more residue in the hydrophobic region, as predicted by hydrophilicity analysis). In GFP-ST, the TMD of sialyl transferase replaces the one of b(5). In GFP-22-Nglyc, the DPS of b(5) is connected, via the couple SR, to the N-terminal sequence of bovine opsin, which contains two N-glycosylation consensus sites. The site that is predicted to be sufficiently distant from the bilayer to be used is boxed. In GFP-22-MutNglyc, both N-glycosylation sites of the preceding construct have been eliminated by Asn→Gln substitution (boldface). In GFP-Syn3 and Syn4, b(5)’s UPS connects to the last 29 residues of rat syntaxin 3 and 4, respectively. For all constructs, the numbers in italics enclosed by parentheses in the left column indicate the hydrophobicity of the TMD region, calculated by using the value of the scale of Engelman et al. (Engelman et al., 1986) for each residue in the sequence between the last upstream charged amino acid and the Arg residue in the DPS or for GFP-Syn 3 and 4, between the last upstream charged amino acid and the C-terminal residue.

reaches the cell surface (Pedrazzini et al., 1996). To systematically investigate the effect of the physicochemical characteristics of the TMD of TA proteins without the possible influence of the cytosolic heme-binding domain of b(5), we produced a variety of constructs, in which GFP replaced b(5)’s cytosolic domain and in which we varied the length or hydrophobicity of the TMD (Fig. 1). As shown in Fig. 2, GFP linked to the C-terminal tail region of b(5) (construct GFP-17) had a typical ER pattern, indicating that this part of b(5) has the necessary targeting information. Within this region, the UPS (Fig. 1) is not required, as demonstrated by the ER localisation of the GFP-17-ΔUPS construct, in which b(5)’s UPS was deleted (Fig. 2). In contrast, as reported elsewhere (Borgese et al., 2001), the C-terminal DPS is of fundamental importance for...
differential targeting of TA proteins between the ER and the mitochondrial outer membrane. A GFP construct with a deletion of three residues in the TMD (GFP-14) remained in the ER, whereas addition of 2 amino acids to obtain GFP-19 resulted in a shift to an heterogeneous distribution, with intracellular as well as some surface staining (arrows in relevant panel of Fig. 2). The constructs with longer TMDs, GFP-22 and GFP-25, showed a prominent surface localisation, in addition to intracellular staining especially in the Golgi region. Since elongation of the TMD results in an increase in total hydrophobicity, we considered the possibility that hydrophobicity rather than length was the important sorting determinant. The hydrophobicity of the TMD region of the constructs, calculated by adding the value of the scale of Engelman et al. (Engelman et al., 1986) for each residue in the sequence between the last upstream charged residue and the Arg residue in the DPS, is indicated in italics next to the name of each construct in Fig. 1. To test whether the effect on sorting was caused by the increased length or hydrophobicity, we produced a construct, GFP-17-HH, in which no amino acids were added to the b(5) tail, but four residues were substituted for more hydrophobic ones, as shown in Fig. 1. This construct, like the ones with lengthened TMD, exited the ER and was localised to the Golgi region as well as at the cell surface (Fig. 2).

Rayner and Pelham (Rayner and Pelham, 1997) reported that in the TMD of the yeast ER-resident TA protein Ufe 1p, the order of the residues, in addition to length, is important for whether the TMD of a bona fide Golgi-resident protein, α-2,6-sialyl transferase, could cause retention in the context of a TA protein. To this end, the TMD of b(5) was replaced with the one of sialyl transferase, including a short basic region on the cytosolic side of the membrane, to obtain GFP-ST (Fig. 1C). As shown in Fig. 2, this construct, although concentrated in the Golgi region, was also capable of reaching the cell surface. Plasma membrane staining was visible also in cells with low levels of expression of the construct (arrows). The double Golgi/surface localisation of GFP-ST is better seen in the confocal images of Fig. 3A, in which colocalisation with the Golgi marker giantin (panels d-f) and with surface glycoproteins labelled with Con A (panels a-c) is apparent. To investigate whether the failure of GFP-ST to be completely retained in the Golgi was because of its overexpression, we determined the percentage of GFP-ST colocalising with giantin in randomly selected cells showing different levels of expression of the transfected cDNA. As shown in Fig. 3B (grey bars), over a wide range (~30 fold) of average GFP fluorescence, cells showed a similar proportion of Golgi staining, indicating that overexpression was not the cause of transport of the constructs to the cell surface. Similar results were obtained with the GFP-17-HH and GFP-22 constructs, although the proportion of Golgi staining with these constructs was lower, especially for GFP-22 (A.B. and N.B., unpublished). To see whether Golgi-localised GFP-ST could be subsequently transported to the cell surface we analysed cells treated with cycloheximide for 4 hours (Fig. 3B; pink bars). Although Golgi
staining remained clearly detectable, the treatment caused a statistically highly significant decrease in its relative intensity (for the 25 cells examined 4.74%±0.66 (s.e.m.) versus 8.09%±0.63 for untreated cells, P<0.001 by Student’s t test), suggesting a slow transport of GFP-ST to the cell surface.

The results of Figs 2 and 3 indicate that low TMD hydrophobicity is the critical factor determining ER residence of TA proteins. Moreover, the TA constructs that escaped from the ER were all capable of travelling to the cell surface, regardless of the length of their TMD, although constructs with short and hydrophobic TMDs (GFP-17-HH and GFP-ST) were also partially retained in the Golgi.

A plasma membrane delivered GFP TA protein (GFP-22) is preferentially localised to the basolateral surface in MDCK cells

At least two routes for transport from the Golgi complex to the cell surface exist and are thought to correspond, respectively, to the apical and basolateral pathways in polarised epithelial cells (Muesch et al., 1996; Yoshimori et al., 1996). To investigate whether the TMD length of our GFP constructs influenced their post-Golgi sorting, we turned to polarised MDCK cells and produced clones stably expressing the plasma-membrane-directed constructs GFP-22 or GFP-25 as well as the ER-retained construct GFP-17.

A comparison of the localisation of GFP-17 and GFP-22 in MDCK cells is shown in Fig. 4. As in CV-1 cells, GFP-17 (panel A) appeared confined to intracellular compartments and showed a distribution similar to that of PDI (Fig. 4B,C) as expected for an ER protein. In contrast, GFP-22, although partly intracellular, was also present at the cell surface (Fig. 4D), together with a 58 kDa basolateral protein (Keller and Simons, 1998) (Fig. 4E,F). Confocal x-z sections indicated that GFP-22 was preferentially localised to the basolateral membrane of the transfected cells (Fig. 4G) and was not detectable at the apical surface, revealed by bound lectin administered in the upper compartment of a Transwell filter.
chamber (Fig. 4H,I). Identical behaviour was observed for the GFP-25 construct (not shown).

N-glycosylation of a C-terminal lumenal tag causes partial relocation of GFP-22 to the apical surface

Basolateral sorting of membrane proteins is generally mediated by specific signals that are located in the cytosolic tails (Matter and Mellman, 1994). These signals usually dominate over apical determinants, with which they often coexist within the same polypeptide (for a review, see Mostov et al., 2000). On the other hand, there is also evidence that some transmembrane proteins can reach the basolateral surface without a signal, simply by exclusion from apical carriers (Lin et al., 1998). We initially considered the possibility that the myc epitope in our constructs, because of the di-hydrophobic couple Leu-Ile that it contains, could be responsible for basolateral localisation and produced constructs without this epitope (Dmyc constructs) (Fig. 1), but this deletion did not have any obvious consequence on sorting (Fig. 6C). We then investigated whether GFP-22 carries a dominant basolateral signal in any part of its sequence by providing it with an apical determinant. This was achieved by tagging its C-terminus with a peptide corresponding to the N-terminus of bovine opsin and containing an N-glycosylation site (GFP-22-Nglyc) (Fig. 1C). N-linked oligosaccharides are known to favour apical targeting of some secretory (Scheiffele et al., 1995) and membrane (Gut et al., 1998) proteins.

First, we investigated whether GFP-22-Nglyc was glycosylated as expected. Lysates obtained from stably expressing transfectants were digested either with PNGase F, with Endo H or mock digested and then analysed by western blotting with anti-opsin mAbs. As shown in Fig. 5, two bands were present in the undigested samples, which were both converted to a lower Mr (~40 kDa) species by PNGase F (lanes 1 and 2). However, the higher Mr band was resistant to Endo H, whereas the more rapidly migrating one (arrow in lanes 1 and 4) was sensitive. Thus, GFP-22-Nglyc is efficiently glycosylated, and most of the N-linked oligosaccharide undergoes Golgi processing. The Endo-H-sensitive polypeptide probably represents the fraction of molecules that are in the ER at steady state. It should be mentioned that a small amount of unglycosylated GFP-22-Nglyc was also present; this species is detected more efficiently by the anti-GFP antibodies used in the blots of Figs 7 and 8.

We then analysed the distribution of GFP-22-Nglyc in polarised cells. As shown in Fig. 6A, this construct produced a strong apical signal, although its presence on the basolateral membrane was also detectable. The N-linked glycan, and not the lumenal amino acid sequence, was responsible for the altered targeting, because a construct containing a modified opsin tag, in which the glycosylation consensus site was eliminated by conversion of its Asn residue to Gln (GFP-22-
MutNglyc) (Fig. 1C) reverted to a basolateral localisation (Fig. 6B). Fig. 6 also shows a vertical section of a clone expressing a Δmyc construct (GFP-22-MutNglyc-Δmyc) (Fig. 6C) whose distribution was similar to that of the corresponding myc-containing protein (Fig. 6B).

Because GFP-22Nglyc and GFP-22-MutNglyc have an extracellular epitope, it became possible to analyse their surface distribution quantitatively. We used a method based on immunolabeling of the extracellular opsin sequence in non-fixed, non-permeabilised cells, as well as a biochemical biotinylation assay (Fig. 7). Fig. 7A shows confocal sections taken from an apical or lateral plane of a field of cells expressing GFP-22-Nglyc and stained for extracellular opsin, as well as for the adhesion protein E-cadherin. Total GFP fluorescence is shown in panels a and e, and merged images of all three fluorochromes are shown in d and h. The opsin epitope in non-fixed cells was accessible to antibodies both from the apical and from the basolateral surface. The intracellular GFP construct was not labelled by the anti-opsin mAbs (compare e with f and with the merge in h). Fluorescence intensity of the extracellular opsin tag was quantified throughout Z series of confocal sections of monolayers of GFP-22-Nglyc- or GFP-22-MutNglyc-expressing cells, and the distribution was compared with that of E-cadherin. As shown in Fig. 7B, surface GFP-22-Nglyc displayed a prominent peak in the apical region (left panel), which was not apparent in the case of GFP-22-MutNglyc (right panel). With both constructs a relatively low degree of labelling was obtained in the junctional region, probably because of the difficulty of penetration of the antibody in these non-fixed preparations.

The results of the confocal analysis were fully substantiated by the biotinylation assay (Fig. 7C). GFP-22-MutNglyc (lanes 5 and 6), like E-cadherin, was biotinylated only from the basolateral chamber, whereas the glycosylated construct (lanes 3 and 4) was biotinylated also at the apical surface (45% apical and 55% basolateral by densitometry of the blots). Interestingly, within the GFP-22-Nglyc-expressing cells, only the mature glycosylated form was accessible to apical biotin (square bracket), whereas the non-glycosylated form (arrowhead) was biotinylated exclusively from the basolateral side.

Sucrose gradient analysis of TA construct solubility in cold detergent solution

In order to gain insight into the mechanism underlying the targeting of GFP-22-Nglyc to the apical surface, we analysed whether it partitions into cold detergent-insoluble sphingolipid and cholesterol-enriched complexes (low-density detergent-insoluble glycosphingolipid-enriched membrane domains – DIGs – or rafts), which are thought to be involved in apical sorting (Simons and Ikonen, 1997). Sucrose gradient flotation was used to analyse a possible association of GFP-22-Nglyc and GFP-22-MutNglyc with DIGs. The distribution of the GFP constructs was compared with that of caveolin I, which partitions into DIGs (Lisanti et al., 1994), and with that of a non-raft protein, E-cadherin (Fig. 8A).

As expected, cadherin remained in the load zone and was not detectable in the upper fractions of the gradient even after prolonged exposures of the blots; in contrast, a sizeable proportion of caveolin floated into the low-density fractions (21 and 38% of total caveolin in fractions 1-7, as determined by densitometry of the GFP-22-Nglyc and GFP-22-MutNglyc blots, respectively). An extremely minor portion of both GFP constructs floated into the raft-containing fractions. However, close inspection revealed that the mature glycosylated form of GFP-22-Nglyc was recruited preferentially with respect to the non-glycosylated form of the same protein and to GFP-22-MutNglyc. This can be seen more clearly in Fig. 8B, where the western blot pattern of an aliquot of fraction four, corresponding to the 30%/5% sucrose interface, is compared with the one obtained with 1/10th the amount of a fraction from the load zone (fraction 11). The mature glycosylated form of GFP-22Nglyc (square bracket) was enriched in fraction four relative to the Endo-H-sensitive and non-glycosylated polypeptide (arrow and arrowhead, respectively), and a smaller proportion of GFP-22-MutNglyc than of mature glycosylated GFP-22-Nglyc floated to the 30%/5% sucrose interface. After reducing the amounts of load zone fractions (8-12) to 1/10th the amount loaded for the low density fractions (1-7) (as exemplified in Fig. 8B) we carried out densitometric analyses of the blots, by which the percentage of floating mature glycosylated GFP-22-Nglyc was estimated at ~2.8%, whereas the corresponding value for the Endo-H-sensitive and for the unglycosylated form, as well as for GFP-22-MutNglyc, was ~1%.

Reporter constructs bearing the tails of syntaxin 3 and 4 are sorted differently in MDCK cells

From the results described above, no differences in post-Golgi sorting between the 22- and 25-residue TMD TA constructs were detected, and apical delivery was induced only by addition of an artificial glycosylated lumenal tag. However, some TA proteins, without lumenal domains, do have a polarised surface distribution in epithelial cells (Low et al., 1996; Delgrossi et al.,
1997). To investigate whether the TMDs of these endogenous TA proteins are involved in apical/basolateral sorting, we produced fusion constructs in which GFP was attached to the tail region of syntaxin 3 or 4, two TA target-Soluble N-ethylmaleimide sensitive factor Attachment protein Receptors (t-SNAREs), which are sorted respectively to the apical and basolateral domain in MDCK cells (Low et al., 1996). The distribution of the two constructs, GFP-Syn3 and GFP-Syn4 (Fig. 1C) was analysed in polarised monolayers by confocal microscopy. As shown in Fig. 9, neither of the constructs had a completely polarised surface distribution; however, GFP-Syn3 produced a pronounced apical signal (Fig. 9A-C), whereas GFP-Syn4 appeared to be more concentrated in the basolateral domain (Fig. 9D) where it colocalised with E-cadherin (Fig. 9E,F). These results suggest that the TMDs of syntaxins play a role in sorting in polarised epithelial cells.

Discussion

A number of TA proteins that reside in the ER require a short TMD to avoid escaping to downstream compartments of the secretory pathway (Pedrazzini et al., 1996; Rayner and Pelham, 1997; Yang et al., 1997; Honsho et al., 1998). As TMDs with completely artificial sequences show this length-dependent effect (Honsho et al., 1998), their role in sorting must be caused by their general physicochemical properties rather than sequence-specific protein-protein interactions. In the present study, we have further analysed the effect of different TMDs on the sorting of model TA proteins, consisting of an N-terminal GFP linked to different C-terminal hydrophobic regions. As very little was known about the mechanisms of sorting of TA proteins to different surface domains in polarised cells, we have also analysed sorting of some of our model proteins in MDCK cells. We discuss in turn these two aspects of our work.

TMD-dependent sorting of GFP TA constructs

For our investigation, we chose GFP as a neutral and easily detectable reporter for the TMD-dependent sorting of TA proteins. As found for other proteins with this topology, the tendency to escape from the ER of our GFP constructs correlated with TMD length. Two novel observations in this study are, however, worth underlining: first, the degree of hydrophilicity rather than length alone appears to determine the capacity of a TMD to prevent ER exit; second, all of the constructs that could exit the ER, with TMDs characterised by different combinations of length and hydrophobicity, were able to reach the plasma membrane at least to some extent. Even the construct bearing the TMD of sialyl transferase, previously shown to be the key determinant for Golgi localisation of type II proteins, in addition to being concentrated in the Golgi complex as expected, showed a clear surface localisation. Watson and Pessin (Watson and Pessin, 2001) reported a Golgi localisation for GFP linked to the TMD of the Golgi TA protein syntaxin 5. Yang et al. (Yang et al., 1997) also reported that the ER TA enzyme UBC6 was relocated to the Golgi after moderate lengthening of its TMD, whereas further elongation resulted in transport to the cell surface. However, in line with our results, a recent paper reports that a group of Golgi-resident coiled-coil TA proteins requires a sequence of the cytosolic domain adjacent to the TMD to be retained in the Golgi (Misumi et al., 2001).

It has been suggested that short TMDs cause retention of Golgi-resident proteins because their length in the α-helical conformation more closely matches the width of the Golgi bilayer than that of the plasma membrane (Breitschwerdt and Munro, 1993). The latter, presumably because of its high cholesterol content, is characterised by increased thickness compared with intracellular membranes. Although the fundamental role of the length of the TMD in Golgi protein localisation (Munro, 1998) is not questioned, and although we do not know at present why sialyl transferase’s TMD failed to be completely retained in the Golgi in the context of our TA construct, it is intriguing that the short TMD of a Golgi protein can reside on the cell surface, notwithstanding the mismatch between its expected length and the thickness of the plasma membrane. One might speculate that at the plasma membrane it is confined to microdomains of reduced thickness, depleted in cholesterol and sphingolipids.

In summary, it appears that for TA proteins containing a neutral reporter as the cytosolic domain, the crucial sorting decision is whether or not to exit the ER. Sorting at subsequent stations of the secretory pathway appears to be looser, as all constructs that exit the ER attain at least some surface localisation. The choice whether or not to remain in the ER depends on the hydrophobicity of the TMD: short, moderately hydrophilic ones determine ER residence, whereas short or long ones with increased hydrophobicity favour ER exit.

In the present study, we have not addressed the mechanism by which polar TMDs effect ER residence, whether by real retention (exclusion from anterograde transport vesicles) or retrieval from a downstream compartment by retrograde traffic. However, in a previous investigation (Pedrazzini et al., 2000) we showed that both mechanisms operate to keep b(5) in the ER. Thus, the moderately hydrophobic TMD of ER-resident TA proteins could be involved in both retention and retrieval. Moderate hydrophobicity is indeed a requirement for interaction of TMDs with rer1p (Letourneur and Cosson, 1998), a recycling receptor operating at the ER-Golgi interface (Sato et al., 2001). On the other hand, the membrane of the ER is enriched in short chain, unsaturated fatty acyl containing phospholipids and is poor in cholesterol and sphingolipids (for a review, see Sprong et al., 2001). This lipid composition, resulting in a particularly fluid bilayer, might provide a favourable environment for moderately polar TMDs and contribute to their retention in the ER.

Sorting of GFP TA proteins with extended TMD in polarised epithelial cells. Effect of an N-linked glycan

Transport from the Golgi complex to the cell surface occurs by at least two different routes, which are thought to correspond to the apical and basolateral pathways in polarised epithelial cells (Muensch et al., 1996; Yoshimori et al., 1996). It is difficult to study these different pathways in non-polarised cells, which lack clearly demarcated surface domains. To investigate the post-Golgi sorting of our plasma-membrane-directed TA constructs, we therefore turned to MDCK cells and found that both GFP-22 and GFP-25 had a polarised distribution on the basolateral surface.

Basolateral transport of membrane proteins is usually
mediated by signals in the cytosolic tails of these proteins. These signals are generally dominant over apical ones, which are of more variable nature, and can be in any region of a transmembrane protein (for reviews, see Matter and Mellman, 1994; Mostov et al., 2000). We investigated the possible presence of a basolateral signal in our constructs by adding an apical determinant – an N-linked glycan (Scheiffele et al., 1995) – to the C-terminus of GFP-22. Because of the recessive nature of apical sorting determinants, we expected this sorting determinant to be without effect in the case of a signal-mediated transport of GFP-22 to the basolateral membrane.

Instead, we found that the N-linked glycan fulfilled its targeting potential quite efficiently, relocating roughly 50% of the construct to the apical domain. This result is consistent with the idea that GFP-22 and GFP-25 are targeted to the basolateral domain without a signal, simply by exclusion from apically directed transport carriers. Support for the existence of this type of exclusion mechanism has been provided from studies on Influenza virus Hemagglutinin mutants (Lin et al., 1998). We cannot, of course, rule out the possibility that our constructs have a weak basolateral targeting signal, recessive to the N-linked glycan. This would not be present in the myc epitope,
as its deletion had little effect on basolateral sorting, but could conceivably be present in the UPS of b(5)'s tail or in GFP itself. The effect of adding the opsin sequence to GFP-22 was quite striking and deserves some further discussion. First, the N-glycosylation consensus was used efficiently, and the N-linked glycan underwent Golgi processing, confirming that TA proteins reach the surface after translocation of their C-terminus across the ER membrane and transport through the secretory pathway (Jäntti et al., 1994; Kutay et al., 1995; Pedrazzini et al., 1996). Second, the apical targeting it caused was dependent on glycosylation, as point mutation of the consensus resulted in a protein that was again targeted to the basolateral surface. Third, the addition of this extracellular opsin epitope made it possible to quantitatively evaluate the surface distribution of GFP-22-Nglyc and GFP-22-MutNglyc by morphology and biochemistry. By both techniques, the glycosylated version was distributed approximately equally between the two surface domains. Interestingly, in the same cells, the fraction of GFP-22-Nglyc that escaped glycosylation was detected only on the basolateral membrane by biotinylation, indicating that the glycosylated and non-glycosylated forms of the construct are sorted from each other within the same cells and ruling out the possibility that apical targeting was an artefact caused by different expression levels of GFP-22-Nglyc and GFP-22-MutNglyc.

The role of N-linked glycans in apical targeting has been the subject of debate (for a review, see Rodriguez-Boulan and Gonzales, 1999). On the one hand, it has been suggested that oligosaccharides may be recognised by a lectin receptor involved in apical transport from the trans Golgi network (Fiedler and Simons, 1995; Scheiffele et al., 1996). Second, the apical targeting it caused was dependent on glycosylation, as point mutation of the consensus resulted in a protein that was again targeted to the basolateral surface. Third, the addition of this extracellular opsin epitope made it possible to quantitatively evaluate the surface distribution of GFP-22-Nglyc and GFP-22-MutNglyc by morphology and biochemistry. By both techniques, the glycosylated version was distributed approximately equally between the two surface domains. Interestingly, in the same cells, the fraction of GFP-22-Nglyc that escaped glycosylation was detected only on the basolateral membrane by biotinylation, indicating that the glycosylated and non-glycosylated forms of the construct are sorted from each other within the same cells and ruling out the possibility that apical targeting was an artefact caused by different expression levels of GFP-22-Nglyc and GFP-22-MutNglyc.

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other hand, an alternative proposal (Rodriguez-Boulan and Gonzales, 1999) suggests that oligosaccharides play an indirect role in apical sorting by stabilising proteinaceous apical sorting signals. The role of N-linked glycans in apical sorting was first unequivocally demonstrated for a secretory protein (Scheiffele et al., 1995) and subsequently reported for three membrane protein chimeras, which in the absence of glycosylation are trapped in the Golgi complex (Gut et al., 1998), and for a glycosyl-phosphatidylinositol-anchored growth hormone chimera (Benting et al., 1999). The GFP-22-Nglyc construct used in the present study differs from the above models because the bulk of the polypeptide mass is cytosolic and the lumenal domain is small (29 residues), barely long enough to allow placement of the glycosylation consensus at the minimal distance from the bilayer required for it to be functional (Nilsson and von Heijne, 1993). Thus, it seems unlikely that the apical relocation we observed was an indirect effect of glycosylation caused by either altered folding of the cytosolic domain or unmasking or stabilisation of a cryptic apical targeting determinant contained in the opsin tag. Therefore, we believe that our observations indirectly support the hypothesis of a direct role of N-linked glycans in apical sorting (Fiedler and Simons, 1995) and that the GFP-22-Nglyc construct could be a useful tool in the hunt for putative lectins involved in apical sorting.

Like the function of glycans in polarised sorting, the role of sphingolipid-cholesterol rafts within the exoplasmic leaflet of the Golgi membrane as platforms for apical targeting (Simons and Ikonen, 1997) is also controversial. Partitioning of proteins within these lipid domains is generally evaluated indirectly by assessing their association with cold detergent-insoluble low-density material. Such an association has been shown for glyco-phosphatidylinositol-linked proteins (Brown and Rose, 1992), which are targeted apically in MDCK cells (Lisanti et al., 1989), and also for apical transmembrane proteins (for a review, see Harder and Simons, 1997; Mostov et al., 2000). Recently, however, exceptions to this relationship between apical targeting and detergent insolubility have been reported (Lin et al., 1998; Zheng et al., 1999; Benting et al., 1999; Lipardi et al., 2000). In our system, neither GFP-22-Nglyc nor its non-glycosylated counterpart were significantly associated with DAG-containing fractions after flotation on sucrose gradients, although more of the mature glycosylated polypeptide than the other forms was Triton insoluble. As the proportion of glycosylated protein recovered in raft-containing fractions was small (<3%), the relevance of this observation to the apical sorting of GFP-22-Nglyc remains to be investigated.

Sorting of GFP-syntaxin tail fusion proteins in polarised epithelial cells

Syntaxins are t-SNAREs that play a central role in recognition and fusion of vesicles and target membrane (for a review, see Chen and Scheller, 2001). In polarised epithelial cells, syntaxins 3 and 4 are enriched at the apical and basolateral membrane, respectively (Low et al., 1996; Fujita et al., 1998; Delgrossi et al., 1997); this different localisation is thought to contribute to the specificity of vesicle fusion with these surface domains (Galli et al., 1998). However, the mechanism of specific targeting of the two syntaxin isoforms themselves is at present poorly understood.

Although the extended TMDs deriving from b(5) in GFP-22 and 25 were incapable of determining apical localisation, we considered the possibility that the syntaxin TMDs might contain information for polarised sorting. Therefore, we expressed two fusion proteins, which resulted from the replacement in our constructs of b(5)’s TMD and DPS with the C-terminal tails of syntaxin 3 or 4, and we observed a clear difference in their localisation in MDCK cells, with the tail of syntaxin 3 determining a more pronounced apical distribution of GFP.

It is now known that the TMDs of SNAREs play an important role in determining specificity in vesicle-SNARE/t-SNARE interactions (McNew et al., 2000), as well as in SNARE localisation along the secretory pathway (Banfield et al., 1994; Rayner and Pelham, 1997; Lewis et al., 2000; Watson and Pessin, 2001). The result reported here demonstrates yet another role for syntaxin TMDs. Although it is likely that the cytosolic domains of syntaxin 3 and 4 also contain targeting information, as reported for other SNAREs (Banfield et al., 1994; Grote et al., 1995; Watson and Pessin, 2000), our results indicate that the TMDs contribute to the final polarised distribution of the two syntaxins.

At present, we do not know what feature of the two syntaxin TMDs determine their differential targeting. The two sequences are of similar length and differ only slightly in hydrophobicity; both are considerably more hydrophobic than the TMD of GFP-25 (total hydrophobicity 62.6 and 57.7 for syntaxin 3 and 4, respectively, calculated as explained in the legend to Fig. 1). It is possible that the slightly higher hydrophobicity of syntaxins 3’s TMD or the abundant hydroxylated residues in the one of syntaxin 4 (Fig. 1C) are important elements in sorting. The GFP TA constructs we have described here should offer a good starting point to investigate the features that define the sorting role of the TMDs of these two syntaxin isoforms.

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