An investigation of the role of transmembrane domains in Golgi protein retention

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The single transmembrane domains (TMDs) of the resident glycosylation enzymes of the Golgi apparatus are involved in preventing these proteins moving beyond the Golgi. It has been proposed that either the TMDs associate, resulting in the formation of large oligomers of Golgi enzymes, or that they mediate the lateral segregation of the enzymes between lipid microdomains. Evidence for either type of interaction has been sought by examining the retention of sialyltransferase (ST), an enzyme of the mammalian trans Golgi. No evidence could be obtained for specific interactions or ‘kin recognition’ between ST and other proteins of the trans Golgi. Moreover, it is shown that the previously described kin recognition between enzymes of the medial Golgi involves the luminal portions of these proteins rather than their TMDs. To investigate further the role of the ST TMD, the effects on Golgi retention of various alterations in the TMD were examined. The addition or removal of residues showed that the efficiency of retention of ST is related to TMD length. Moreover, when a type I plasma membrane protein was expressed with a synthetic TMD of 23 leucines it appeared on the cell surface, but when the TMD was shortened to 17 leucines accumulation in the Golgi was observed. These observations are more consistent with lipid-based sorting of ST TMD, but they also allow for reconciliation with the kin recognition model which appears to act on sequences outside of the TMD.

Keywords: Golgi/kin recognition/retention/sialyltransferase/transmembrane domain

Introduction

The secretory pathway of eukaryotic cells comprises a series of discrete membrane-bound organelles between which transport occurs by a process of vesicle budding and targeted fusion. On the exocytic route, proteins and phospholipids are initially inserted into the endoplasmic reticulum (ER) where folding, assembly and basic covalent modification occurs. From the ER, transport is to the Golgi apparatus via an intermediate compartment. The Golgi itself consists of several compartments arranged cis to trans, and it is here that proteins acquire extensive covalent additions such as complex N- and O-linked oligosaccharides, sulfation and palmitylation, as well as being the site of synthesis of sphingolipids and glycolipids (Roth, 1987). The Golgi also has a sorting function. The cis compartments are the sites at which escaped resident proteins of the ER are retrieved, and the last compartment, the trans-Golgi network (TGN), is an exocytic junction point with specific pathways for the plasma membrane and its subdomains, for the lysosomal system and for secretory granules. Each compartment within the exocytic pathway thus contains a unique set of resident enzymes and sorting components. This requires that mechanisms exist to maintain compartmental identity despite the continuous exocytic flow of membrane and protein through the pathway (Luzio and Baning, 1993; Pelham and Munro, 1993). How this occurs for the residents of the Golgi is particularly intriguing as they must be capable of leaving the ER after synthesis, but then accumulate in the Golgi and not at a later point in the pathway.

All Golgi residents so far examined are membrane proteins, with most of these being the glycosylation enzymes which share a common structure: a small cytoplasmic tail, a single type II transmembrane domain (TMD) and a luminal catalytic domain (Kleene and Berger, 1993; Natsuka and Lowe, 1994). Studies on two such enzymes, α-2,6-sialyltransferase (ST) and β-1,4-galactosyltransferase, led to the conclusion that the TMD has a major effect on their localization, a result confirmed for several other Golgi residents (Munro, 1991; Nilsson et al., 1991; Colley et al., 1992; Russo et al., 1992; Wong et al., 1992; Machamer, 1993; Hobman et al., 1995). In some cases, contributions from other parts of the protein were also observed, although in every case the TMD clearly had retaining activity of its own (Munro, 1991; Dahdal and Colley, 1993; Burke et al., 1994; Teasdale et al., 1994). Moreover this TMD-mediated retention of Golgi enzymes is apparently conserved between mammals and yeast (Chapman and Munro, 1994b; Schwientek et al., 1994). Distinct from this, it has been found for the TGN proteins TGN38 and furin and for the mannose 6-phosphate receptor, that signals in their cytoplasmic tails maintain their location by specifying retrieval from the plasma membrane, in some cases acting in addition to a retention signal in the TMD (Bos et al., 1993; Humphrey et al., 1993; Reaves et al., 1993; Wong and Hong, 1993; Conibear and Pearse, 1994; Molloy et al., 1994; Ponnambalan et al., 1994). Such recycling from the plasma membrane is not seen for the Golgi glycosylation enzymes retained by their TMDs (Chapman and Munro, 1994a; Teasdale et al., 1994).

Although the mechanism underlying this TMD-mediated retention has not yet been resolved, two distinct models have been proposed. The first is that Golgi enzymes form oligomers too large to enter into forward-moving transport vesicles (Pfeffer and Rothman, 1987; Weisz et al., 1993). In support of this, there is evidence that some Golgi enzymes can form multimeric structures in vivo, because it has been observed that when one enzyme of the medial Golgi is held in the ER, another
medial enzyme also accumulates with it (Nilsson et al., 1994). This led to the proposal of a specific ‘kin recognition’ model in which Golgi enzymes form long linear hetero-oligomers held together by interactions through their TMDs (Nilsson et al., 1993). However, attempts to define by mutagenesis what constitutes a TMD with Golgi-retaining activity have failed to identify any key residues (Munro, 1991; Dahdal and Colley, 1993). Moreover, when Golgi enzyme TMDs are compared with those of plasma membrane proteins, the only difference so far discerned is that the Golgi TMDs are on average five residues shorter and contain more of the bulky residue phenylalanine, a difference also observed for the TMDs of the tSNARE vesicle-targeting proteins (Bretscher and Munro, 1993; Banfield et al., 1994; Munro, 1995). This led to a second model in which these two sets of TMDs would confer different behaviours in the secretory pathway by virtue of differences in their physical properties (Bretscher and Munro, 1993; Masibay et al., 1993). Within the bilayer of a Golgi cisterna, mixed lipid populations would separate into lipid microdomains with distinct compositions and hence thickness and degree of structural perturbability. The Golgi enzymes would selectively partition into one domain and so be prevented from entering transport vesicles comprising the other domain.

Here, the features of these two models, as they pertain to the trans Golgi enzyme ST, are examined. It is shown that there is no apparent kin recognition between enzymes of the trans Golgi using the assay employed to show kin recognition between medial enzymes. Furthermore, it appears that this interaction between medial enzymes occurs through the luminal portions of the proteins rather than through their TMDs. Moreover, analysis of the effects of mutations in the ST TMD on retention are more consistent with the TMD exerting its effect because of its physical properties rather than its ability to form protein–protein interactions. Thus, the two models can be reconciled by suggesting that different mechanisms are acting through different parts of the Golgi enzymes.

Results

Investigation of kin recognition involving ST

To investigate the mechanism by which ST is retained in the trans Golgi, I initially looked for evidence for the types of protein–protein interaction that have been reported for enzymes of the medial Golgi (Nilsson et al., 1994). In these experiments it was observed that when the enzyme N-acetylgalcosaminyltransferase I (NAGT I) was held in the ER by the addition of an ER-specific retention signal, then another enzyme of the medial Golgi (mannosidase II) also accumulated in the ER, but enzymes of the trans Golgi did not. To determine if ST could form similar specific interactions, the protein was expressed in COS cells with the ER retention signal KDEL attached to its C-terminus. Although this signal is usually found on soluble ER proteins, it is also found on some membrane proteins of the ER and has been shown to confer ER retention to a heterologous membrane protein (Sweet and Pelham, 1992; Tang et al., 1992). Figure 1B shows that although the ST–KDEL does indeed accumulate in the ER, when the location of another trans Golgi enzyme, β-1,4-galactosyltransferase, was examined in the trans-

![Fig. 1. Relocation of ST to the ER does not affect other trans Golgi proteins. (A) Schematic representations of the ST chimeras. The TMD is shown by a black box, and the myc and HA tags are named. (B) Double label immunofluorescent localization of endogenous galactosyltransferase and ER-retained ST in a COS cell transfected with STHK. (C) Double label immunofluorescent localization of ST with (STHK) and without (STM) an ER retention signal when coexpressed in the same cells. Cotransfected COS cells were either left untreated or treated with 1 μg/ml brefeldin A for 20 min and then fixed immediately or washed and allowed to recover for 4 h prior to fixation.](image)
to the ER and hence brings about a mixing of contents of the two organelles. As expected, the two forms of ST colocalize in the ER of brefeldin-treated cells, but upon recovery following removal of the drug, the non-retained form of ST returns to the Golgi (Figure 1C). Thus, unlike the medial Golgi enzymes, ST does not appear to form specific kin interactions in the ER.

**NAGT I interacts with mannosidase II through its lumenal portion**

The experiments described above differ from those of Nilsson et al. (1994) in that the Golgi enzymes are held in the ER using the C-terminal KDEL retention signal rather than the N-terminal double-arginine signal from invariant chain (Schutze et al., 1994). To ensure that the KDEL signal is compatible with the formation of specific interactions between Golgi enzymes, it was attached to NAGT I. Figure 2 shows that NAGT I-KDEL accumulates in the ER and causes mannosidase II to accumulate in this compartment (NN), whereas the accumulation of ST-KDEL in the ER does not affect the location of mannosidase II (SS). Thus the specific interactions seen previously can also be observed using the KDEL localization signal. Since ST-KDEL does not appear to interact with mannosidase II in this assay, it was next asked which part of ST had to be replaced with a part of NAGT I to result in an interaction with mannosidase II. When the signal anchor region of ST-KDEL was replaced with the signal anchor region of NAGT I, the chimera accumulated in the ER but had no discernible effect on the Golgi location of mannosidase II (NS, Figure 2). In contrast, when the lumenal domain of ST-KDEL was replaced with the equivalent region of NAGT I, the clear accumulation of mannosidase II in the ER could be seen, even though the signal anchor region of this construct comes from ST (SN, Figure 2). This result raises the strong possibility that the observed interaction between medial Golgi enzymes does not involve the TMDs of the proteins but rather their lumenal portions.

**Varying the length of the ST TMD**

If the TMDs of Golgi enzymes are not involved in specific protein–protein interactions to form oligomers, then what other function might they have? To test further the proposal that their physical properties are important in their ability to serve as retention signals, the effects of making insertion and deletion mutations in the TMD of ST were examined. Figure 3 shows a series of mutants in which hydrophobic residues were either inserted into or deleted from the middle of the TMD of ST. These mutant signal anchor regions were expressed in transfected COS cells as fusions to the monomeric reporter protein lysozyme. The degree of Golgi retention was assayed by determining the amount of each mutant expressed on the cell surface by antibody binding, and normalizing this with respect to the total amount of expressed protein as determined by protein blotting. Figure 3C shows that the removal of a single residue from the middle of the TMD does not increase the cell surface expression of the ST–lysozyme chimera, with the protein still accumulating in the Golgi apparatus as judged by immunofluorescence (data not shown). Lengthening the TMD by one or more residues gradually increases the cell surface expression, but the level does not approach that seen with the control cell surface TMD (DPPIV) until five or more residues are added. The slight increase in retaining activity seen when the insertion is increased from three to four residues was seen reproducibly, but it should be noted that the four residue chimera was the only one to accumulate to substantially lower levels than the rest (~30% reduced), suggesting that it may be less stable on the cell surface. We have seen similar effects with some other fusion proteins with unnatural TMDs, suggesting that there may be a mechanism to clear aberrant or maladapted proteins from the plasma membrane, a phenomenon reported for temperature
sensitive viral glycoproteins (Balch and Keller, 1986). Nonetheless, it is clear that the addition or subtraction of a single residue does not have a substantial effect on the Golgi retention activity of the ST TMD, although such changes would be expected to have a large effect on the spatial organization of residues along any interacting surface a helical TMD might form. Longer insertions do have a substantial effect on retention, and these results are more consistent with the proposal that it is the short hydrophobic length of the ST TMD that is the key to its ability to serve as a retention signal.

Shortening a TMD can reduce cell surface expression

To test the generality of the above observations, the effect of shortening the TMD of a plasma membrane protein was determined. DPPIV is a type II protein of the plasma membrane. When its signal anchor region is fused to lysozyme, the resulting chimera is also expressed on the plasma membrane (GDDD). The TMD of DPPIV is 23 residues long, in contrast to the 17 residues of ST. Therefore the behaviour was examined of an altered version of GDDD in which six residues had been removed from the DPPIV TMD. Figure 4A shows that the relative cell surface expression of this protein is substantially reduced, albeit not to the level seen with the ST signal anchor region. Immunofluorescent localization of the intracellularly retained GΔ6DDD shows that instead of appearing on the plasma membrane, it accumulates in the

Golgi apparatus (Figure 4B). Consistent with this, GDDD and GΔ6DDD show similar proportions of endoglycosidase H-resistant material at the steady state, suggesting that both constructs leave the ER at similar rates (data not shown). Thus it appears that shortening the length of the TMD of a cell surface protein is sufficient to alter its intracellular distribution.

Role of phenylalanines in retention

The residue phenylalanine is more abundant in Golgi than plasma membrane TMDs, and indeed occurs four times in the ST TMD, suggesting that this residue may make some contribution to retention. Expression plasmids were made encoding ST–lysozyme chimeras with the TMD altered such that all four phenylalanines were either replaced with isoleucine, or one or other of the two pairs of phenylalanines changed in the same way (Figure 5A). These latter alterations were made in the context of +2 (the form of GSSS which has two hydrophobic residues inserted in its TMD and which is only partially retained in the Golgi; Figure 3) on the grounds that this might allow for the detection of more subtle changes in retention. Figure 5B shows that changing all the phenylalanines produced only a small, albeit reproducible, increase in the cell surface expression of the GSSS chimera. This effect did not seem to depend on the phenylalanines at a particular end of the TMD, as the two pairwise changes to +2 construct had equal small effects (~2-fold) which were additive when combined. Thus it appears that
Almost all Golgi proteins are type II proteins and so the TMD has to act not only as an anchor to hold the protein in the bilayer but also as a signal to direct its insertion during synthesis. To test further the retaining characteristics of the ST TMD, its ability to function in a type I protein was examined. Expression plasmids were constructed encoding the extracellular domain of the lymphocyte surface antigen CD8 attached to either the DPPIV or the ST TMD, followed by a short cytoplasmic tail (Figure 6A). CD8 is a substrate for the addition of O-linked sugars, the sialylation of which in the Golgi causes a marked alteration in gel mobility (Jackson et al., 1993). Pulse–chase analysis shows that when expressed in cells, the two chimeras acquire Golgi modifications at similar rates and have similar stabilities (Figure 6B). Immunofluorescent localization shows that while the DPPIV chimera (CD8-D) accumulates on the plasma membrane as expected, the ST chimera shows a very different distribution with accumulation in the Golgi apparatus (Figure 7A, CD8-S). The quantitation of anti-CD8 binding to the cell surface shows an 8-fold reduction (data not shown). Thus the ST TMD is able to alter the intracellular localization of a protein even when reversed into a type I orientation, which might have been expected to interrupt any specific protein–protein interactions with other Golgi residents.

If TMD length is a critical feature of the Golgi protein retention signal, then it should be possible to alter the intracellular location of a type I protein chimera by varying the length of a TMD comprising a simple hydrophobic homopolymer. Two such expression plasmids were made encoding the CD8 extracellular domain attached to a TMD of either 17 or 23 leucines. A pulse–chase analysis of transfected cells showed that these chimeras display the same stability as each other and as CD8-S and CD8-D (Figure 6B). CD8-17L and CD8-S leave the ER at essentially the same rate, although CD8-23L leaves slightly more slowly. The reasons for this are unclear and are currently being investigated further. Nonetheless, when the intracellular localization of the CD8-23L and CD8-17L chimeras is compared, it can be seen that once the CD8-23L leaves the ER it accumulates on the plasma
membrane, in contrast to CD8-17L, which shows Golgi accumulation that persists even after a prolonged chase in cycloheximide (Figure 7B). Thus, the hydrophobic length of a TMD can apparently affect the rate at which a protein exits the Golgi apparatus.

Discussion

Here, the retention of ST in the Golgi has been examined with the aim of determining whether the role of the TMD in retention is to participate in protein–protein interactions or to provide a signal which allows the protein to be sorted by physical means. Direct evidence for specific interactions between Golgi proteins has previously come from the observation that when the medial enzyme NAGT I was held in the ER, a second medial enzyme mannosidase II also accumulated in the ER. This ‘kin recognition’ led Nilsson et al. (1994) to propose that Golgi enzymes exist in linear hetero-oligomers held together by specific interactions between their TMDs. ST is an enzyme of the trans Golgi and it is reported here that specific interactions between ST and either itself or another trans enzyme galactosyltransferase cannot be detected using a similar assay system. Of course the possibility remains that only the interactions between the medial enzymes are capable of forming in the ER, although the overlapping distribution of Golgi enzymes is such that as much as one half of NAGT I and mannosidase II are in the same trans compartment that contains galactosyltransferase and ST. Hence any specific interactions should be capable of forming under the same conditions in vivo. However, more importantly, the results presented here show that the interaction between NAGT I and mannosidase II is mediated, at least in part, by the lumenal section of the proteins, with no detectable interaction occurring through the signal anchor region of NAGT I. This observation is consistent with a previous report that the lumenal portion of NAGT I contributes to its Golgi localization, presumably by mediating interactions with mannosidase II (Burke et al., 1994). Thus, while it appears that Golgi enzymes can multimerize, this need not be mediated through their TMDs. This leaves the question of how this latter region of the protein contributes to retention.

The results of mutagenesis presented here are more consistent with the TMD of ST affecting the intracellular distribution of the protein by virtue of its physical properties, rather than by it being involved in a specific protein–protein interaction. First, the addition or subtraction of a single residue in the middle of the TMD only has a small effect on retention, while lengthening the TMD by five or more residues greatly reduces retention. Secondly, reducing the length of the TMD of a cell surface protein
by six residues alters its location, with the protein now accumulating in the Golgi. Finally, if a protein is anchored in the bilayer by a featureless polymer of leucine, then varying the length of this stretch affects the distribution of the protein, with a shorter length slowing exit from the Golgi. These results are consistent with previous studies in which key residues for retention in Golgi TMDs could not be found. I have shown previously that ST is still retained in the Golgi if its 17 residue TMD is replaced by 17 leucines but not if it is replaced by 23 (Munro, 1991). The studies reported here strengthen the notion that TMD length is a general retaining feature for the Golgi, although TMD shape may also have a role because the bulky phenylalanine residues in ST make a small contribution to retention and this residue is particularly abundant in Golgi TMDs (Bretscher and Munro, 1993; Munro, 1995).

How could the length and shape of a TMD act as a sorting signal? It has been proposed previously that the observed changes in lipid composition along the secretory pathway might be involved (Bretscher and Munro, 1993). The ER is the start of the secretory pathway and yet it has a very different lipid composition from the plasma membrane. This difference is probably of considerable importance to the cell. The plasma membrane has to serve as an impermeant but flexible barrier to protect the cell and to constrain small metabolites. Its high content of cholesterol and sphingolipids will confer impermeance by thickening the bilayer and reducing acyl chain flexibility. Conversely, the ER has low levels of these lipids, allowing it to provide a pliable biosynthetic environment suitable for the insertion, modification and assembly of proteins, lipids and other hydrophobic molecules. Somewhere along the secretory pathway the lipid composition of the bilayer must change, and the Golgi is the place where both glycolipids and the majority of sphingomyelin are made, and where cholesterol levels appear to rise (Orci et al., 1981; Van Meer, 1989; Coxey et al., 1993). Indeed, one function of the Golgi may be to prevent vesicular communication between the ER and plasma membrane from averaging lipid compositions. Thus, the very least that the observed difference in TMD length implies is that the proteins have evolved to fit into the changing bilayer. Biophysical studies have shown that deformation of the bilayer by mismatch between TMD length and bilayer thickness is energetically unfavourable (Mouritsen and Bloom, 1993). Thus it seems reasonable that as proteins moving through the pathway encounter the point (or points) where bilayer composition changes from ER-like to plasma membrane-like, then proteins with longer TMDs will move forward more easily than those with shorter ones. Indeed, it has been observed for many years by electron microscopic studies that the plasma membrane appears thicker than internal membranes (Sandellius et al., 1986). TMD shape could also play a role if the thicker, more impermeant, plasma membrane bilayer cannot readily accommodate bulky residues such as phenylalanine.

How might a bilayer transition within the pathway occur in practice? At present, our understanding of the lipid composition and vesicle dynamics of the Golgi apparatus is still vague enough to tolerate a wide variety of models. However, it is useful to emphasize two distinct possibilities. In the first, the formation of lipid microdomains, triggered by either sphingolipid synthesis or cholesterol accumulation, would be the site of formation of forward-moving vesicles which would exclude Golgi enzymes. Indeed, it has been proposed that even the observed partitioning of cholesterol-rich and cholesterol-poor domains in pure phospholipid bilayers is driven by the mismatch in hydrophobic thickness (Sankaram and Thompson, 1990). This model would be analogous to the sorting of glycosyl phosphorylatedinositol-anchored proteins into glycolipid rafts proposed for sorting in polarized cells (Simons and Van Meer, 1988). Indeed, Van Meer (1989) suggested some years ago that the selective synthesis of sphingolipids in the Golgi, rather than in the ER like other lipids, implied that they may have a specific sorting function in this organelle. Alternatively, there is increasing evidence that, in at least some transport steps, proteins can be selectively collected into forward-moving vesicles (Balch and Farquhar, 1995; Fiedler and Simons, 1995; Schimmöller et al., 1995). If sufficient plasma membrane proteins, or even specialist vesicle ‘resident’ proteins, were brought together, then their long TMDs would be expected to attract a thicker, plasma membrane-like bilayer which could then exclude Golgi enzymes from the forming vesicle. Of course, many other views are possible, for instance there must be at least some degree of retrograde transport within the Golgi and the above models could be reversed with retrograde vesicles selectively excluding forward-moving proteins. Nonetheless, there must be a bilayer transition within the exocytic pathway, and the TMD length-dependent behaviour reported here suggests that this transition occurs in the Golgi and that it could be an agent in the localization of the Golgi residents themselves.

Oligomerization

The above proposals do not exclude a role for oligomerization in the localization of Golgi enzymes, and indeed they cannot. The formation of large oligomeric structures can apparently prevent forward movement through the secretory pathway of certain proteins of the ER (Delahunty et al., 1993; Field et al., 1994; Schweizer et al., 1994). It is also clear that the medial Golgi enzymes do form oligomeric structures, although the results presented here suggest that this is not mediated by their TMDs. Rather, it may be that mannosidase II and NAGT I, along with other medial enzymes, sugar transporters, etc., form a multienzyme complex such as those found for many other enzymes that catalyse successive steps in a biosynthetic or degradative pathway. This complex would be formed by interactions between the luminal domains of the enzymes, whose TMDs would then allow the complex to be sorted by a lipid-based or other such mechanism. The distribution of a protein between different lipid domains will be governed by the difference in free energy of the TMD in the two different bilayers. This difference might well be small, and so an increase in the number of TMDs in the structure being sorted would have the effect of increasing the extent to which one bilayer is preferred to another. This might result in large complexes accumulating earlier in the Golgi than smaller ones. The enzymes in the trans Golgi may also form complexes, but they are less likely to be so large or stable because they catalyse a more diverse and bifurcating array of modifications for
which substrate passing is less likely to be important or useful. For the trans Golgi enzymes ST and galactosyl-transferase, the residues flanking the TMD have been shown to contribute to retention. Some of this effect may be a result of the necessity to define the ends of the hydrophobic portion by virtue of basic charges or specific interactions with phospholipid head groups, but it is also quite possible that some degree of luminal domain oligomerization occurs which aids retention but is not strong enough to be seen in the ER retention assay used here (Munro, 1991; Dahdal and Colley, 1993; Teasdale et al., 1994).

However, it should also be noted that studies on coronavirus M proteins, which have three TMDs and are retained in the Golgi apparatus, have shown that oligomer formation is not a sole determinant of Golgi retention. Mouse hepatitis virus M protein forms detergent-resistant oligomers of heterogeneous size, but mutant forms which accumulate on the plasma membrane show only a slight reduction in the amount of the very largest complexes (Armstrong and Patel, 1991; Krijnse Locker et al., 1995). Moreover, the related M protein from infectious bronchitis virus does not appear to form such oligomers and yet is retained in the Golgi (Weisz et al., 1993; Krijnse Locker et al., 1995).

Lipid-based sorting and aggregation are thus not mutually exclusive models, but rather they probably act through different parts of the Golgi enzymes. Indeed, one could imagine that further mechanisms, such as a recycling or selective degradation, could also play a role. Such a complex situation for Golgi protein localization is almost inevitable given the facts that not only are Golgi enzymes distributed differentially between the cisternae of the Golgi complex, but these distributions can vary between different cell types (Roth et al., 1986; Velasco et al., 1993). The relative contributions of the different mechanisms for particular enzymes will have to be examined in detail. Even so, the analysis of the TMDs of Golgi enzymes presented here suggests that the Golgi is the site of a fundamental transition in the internal membranes of the cell which is probably undertaken to allow eukaryotic cells to maintain two very different types of bilayer. This transition point may have been exploited as one means of maintaining the complex internal composition of the Golgi apparatus.

Materials and methods

Plasmid construction

The plasmid STM is the rat α-2,6-ST gene with a myc tag-encoding sequence attached to the C-terminus, expressed from the adenovirus major late promoter in a SV40 origin-containing vector, and has been described previously (Munro, 1991). STHK is similar, except that the C-terminus of the encoded ST is "IRCAMYAPYPDVYPASEKDEL, IRC being the C-terminus of native ST (Weinstein et al., 1987). SSMK encodes ST followed by YM3KLISELINN, and has been described previously (Munro et al., 1991). NSMK encodes human NAGT I followed by the same sequence but without the first Y. In the SSMK, NSMK, and SSMK are chimeras of these two proteins. In NSMK, the sequence at the fusion point is DPAGNSPKS, where A is residue 48 of NAGT I and P is residue 45 of ST, with GSN being encoded by the linker. For SSMK, the fusion point is Kovevi, with the first V being residue 51 of ST, the E being residue 53 of NAGT I and G from the linker. Plasmids SSMK, NSMK, and SSMK are based on expression vector CDM8 (Seed, 1987). We have observed that the NAGT I CDNA contains a cryptic splice donor which is spliced to the acceptor of the SV40 intron in the 3′ untranslated region (UTR) of the CDMA expression cassette. Thus, CDMA was cut with XbaI and DraI, the 5′ overhang filled in and the vector recircularized. This deletes 505 bp of the 3′ UTR including the intron, and eliminates the cryptic splicing of the NAGT I cDNA (unpublished observations). This vector was used for NNNK and SNMK.

The expression plasmids encoding lysozyme chimeras were all derived from the previously described GSSS and GDDD COs cell expression plasmid containing the adenovirus major late promoter (Munro, 1991). For insertions and deletions in the ST TMD, the TMD-encoding sequence of GSSS was altered to create SpeI and Ndel sites (encoding ILV and VIC), leaving the encoded protein unchanged. Appropriate oligonucleotides were then inserted between these sites. For insertions, the extra residues were encoded by GTAGTCTGTCGTCCGTTGCTCTCTC for the longest (VLALVLAL) and the shorter insertions by progressive N-terminal deletions of the sequence following the first G. Phenylation to isoleucine changes (T → A in first position of codons) and the GA6D mutation were made by assembling the DPPV or ST signal anchor region from oligonucleotides as described previously (Munro, 1991).

The CD8 plasmids are based on CD8-B, an adenovirus major late promoter-containing COS cell expression vector described previously (Chapman and Munro, 1994a). In each case the entire extracellular portion of CD8 ending —ACD is followed by QK, the appropriate TMD and a cytoplasmic tail of KRLK.

Antibodies and immunofluorescence

COS or CHOP cells, 24 h after transfection using DEAE-dextran as described previously (Munro and Pelham, 1987; Hefferman and Dennis, 1991), were split onto eight-well slides (C.A. Hendley (Essex) Ltd). On the day following day they were incubated with drugs and fixed. Fixation, permeabilization with Triton X-100, immunofluorescence and mounting were performed as described previously (Munro and Pelham, 1987). For epitope-tagged proteins and lysozyme chimeras, fixation was with 2% paraformaldehyde/0.1% glutaraldehyde, followed by 1 mg/ml sodium borohydride. For native proteins, 4% paraformaldehyde was used. For galactosyltransferase, methanol (5 min) was employed, followed by acetone (1 min) at -20°C.

Mouse monoclonal antibodies were used to detect the myc epitope (9E10, ATCC CRL 1729; Munro and Pelham, 1987), the HA epitope (12CA5; Field et al., 1988), lysozyme (F10; R. Polijak, personal communication) and the Golgi (22-II-D8B; Celis et al., 1988). Rat monoclonal Campath 5c (YTC141.1) was used to detect CD8 (Bindon et al., 1989). Rabbit polyclonal antisera against mouse mannosidase II (Moremen et al., 1991), bovine galactosyltransferase (Russo et al., 1992), hen lysozyme and the myc epitope were generously provided by Kelley Moremen, Joel Shaper and Mike Lewis. Fluorescein isothiocyanate- and Texas Red-conjugated secondary antibodies were obtained from Amersham International (mouse and rabbit) or Southern Biotech (rat). Immunofluorescent images were collected on an MRC 600 confocal microscope.

Quantification of cell surface expression of lysozyme fusion proteins

COS cells were transfected in 10 cm dishes. After ~24 h, they were split into six-well plates. At 40 h post-transfection, cells were washed once in ice-cold serum-containing medium and then incubated for 30 min at 4°C in 1 ml of Optitens (Gibco) containing 10% fetal calf serum (FCS) and 0.1 µg/ml monoclonal F10 labelled with 125I to 30–100 µCi/µg using chloramine T. After washing four times in DMEM/1% FCS at 4°C, the cells were harvested with 1 ml of 1% SDS/0.1 M NaOH and the bound 125I counted. The inclusion of protein in the binding and washing steps was found to prevent cell loss. Total chimera expression was determined by solubilizing cells with wells with 400 µl SDS sample buffer. After sonication, proteins were separated by SDS-PAGE, transferred to nitrocellulose, blocked with PBS/10% FCS, probed with rabbit anti-lysozyme sera, followed by 125I-labelled Protein A (Amersham), and the lysozyme chimera content quantified using a Molecular Dynamics PhosphorImager.

Pulse-chase analysis

CD8-expressing plasmids were transfected into COS cells as above. After ~24 h the cells were split into six-well plates. At 40 h post-transfection the cells were rinsed once in PBS, once in methionine-free DMEM and pulse with 100 µCi/ml [35S]methionine for 15 min in methionine-free DMEM/1% dialysed FCS, and then either lysed immediately or chased in a complete medium before lysis in 50 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% NP40 and 1 mM phenylmethylsulfonyl fluoride. CD8 fusion proteins
were precipitated from the post-nuclear supernatant by adding Campath 8c followed by 2 h incubation with protein G-Sepharose (Sigma) at 4°C. The beads were then washed five times in lysis buffer, the bound proteins eluted with SDS sample buffer and separated by SDS–PAGE, the gel dried and the proteins detected and quantified using a Molecular Dynamics PhosphorImager.

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References

Armstrong,J. and Patel.S. (1991) The Golgi sorting domain of coronavirus E1 protein. J. Cell Sci., 98, 567–575.

Balch,W.E. and Farquhar,M.G. (1995) Beyond bulk flow. Trends Cell Biol., 5, 16–19.

Balch,W.E. and Keller,D.S. (1986) ATP-coupled transport of vesicular stomatitis virus G protein: functional boundaries of secretory compartments. J. Biol. Chem., 261, 14690–14696.

Banfield,D.K., Lewis,M.J., Rabouille,C., Warner,G. and Pelham,H.R.B. (1994) Localization of Sec5, a putative vesicle targeting molecule, to the cis-Golgi network involves both its transmembrane and cytoplasmic domains. J. Cell Biol., 127, 357–371.

Bretscher,A. and Munro,S. (1993) Cholesterol and the Golgi apparatus. Science, 261, 1280–1281.

Bretschger,M.S. and Munro,S. (1993a) Retrieval of TGN proteins from the cell surface requires endosomal acidification. EMBO J., 12, 2219–2228.

Bretschger,M.S. and Munro,S. (1993b) Functioning of the yeast Golgi apparatus requires an ER protein encoded by ANP1, a member of a new family of genes affecting the secretory pathway. EMBO J., 12, 4896–4907.

Colley,K.J., Lee,E.U. and Paulson,J.C. (1992) The signal anchor and stem regions of the β-galactosidase α-2,6-sialyltransferase may each act to localize the enzyme to the Golgi apparatus. J. Biol. Chem., 267, 7784–7793.

Conboy,E. and Pearse,B.M.F. (1994) A chimera of the cytoplasmic tail of the mannose 6-phosphate IGFl receptor and lysosome localizes to the TGN rather than prelysosomes where the bulk of the endogenous receptor is found. J. Cell Sci., 107, 923–932.

Coorey,R.A., Penthange,P.G., Campbell,G. and Blanchette-Mackie,E.J. (1993) Differential accumulation of cholesterol in Golgi compartments of normal and Niemann–Pick type C fibroblasts incubated with LDL: a cytotoxic freeze–fracture study. J. Lipid Res., 34, 1165–1176.

Dahdal,R.Y. and Colley,K.J. (1993) Specific sequences in the signal anchor of the β-galactosidase α-2,6-sialyltransferase are not essential for Golgi localization. J. Biol. Chem., 268, 26310–26319.

Delahunt,M.D., Stafford,F.J., Yuan,L.C., Shaz,D. and Bonifacino,J.S. (1993) Uncleaved signals for glycosylphosphatidylinositol anchoring cause retention of precursor proteins in the endoplasmic reticulum. J. Biol. Chem., 268, 12017–12027.

Fiedler,K. and Simons,K. (1995) The role of N-glycans in the secretory pathway. Cell, 81, 309–312.

Field,J., Nikawa,J., Broek,D., MacDonald,B., Rodgers,L., Wilson,J.A., Lerner,R.A. and Wigler,M. (1988) Purification of a RAS-responsive adenyl cyclase complex from Saccharomyces cerevisiae by use of an epitope addition method. Mol. Cell. Biol., 8, 2159–2165.

Field,M.C., Moran,P., Li,W.L., Keller,G.A. and Caras,I.W. (1994) Retention and degradation of proteins containing an uncleaved glycosphosphatidylinositol signal. J. Biol. Chem., 269, 10830–10837.

Heffernan,M. and Dennis,J.W. (1991) Polyoma and hamster papovavirus large T antigen-mediated replication of expression shuttle vectors in Chinese hamster ovary cells. Nucleic Acids Res., 19, 85–92.

Hobman,T.C., Woodward,L. and Farquhar,M.G. (1995) Targeting of a heparan-sulfate membrane protein complex to the Golgi: rubeusa virus E2 glycoprotein contains a transmembrane Golgi retention signal. Mol. Biol. Cell, 6, 7–20.

Humphrey,J.S., Peters,P.J., Yuan,L.C. and Bonifacino,J.S. (1993) Localization of TGN38 to the trans-Golgi network: involvement of a cytoplasmic tyrosine-containing sequence. J. Cell Biol., 120, 1123–1135.

Jackson,M.R., Nilsson,T. and Peterson,P.A. (1993) Retrieval of transmembrane proteins to the endoplasmic reticulum. J. Cell Biol., 121, 317–333.

Klee,R. and Berger,E.G. (1993) The molecular and cell biology of glycosyltransferases. Biochim. Biophys. Acta, 1154, 283–325.

Krijnse Locker,J., Opstelten,D.J.E., Ericsson,M., Horzinek,M.C. and Rottier,P.J.M. (1995) Oligomerization of a trans-Golgi/trans-Golgi network retained protein plays a critical role in the Golgi complex and may be part of its retention. J. Biol. Chem., 270, 8815–8821.

Kumar,R., Yang,J., Larsen,R.D. and Stanley,P. (1990) Cloning and expression of a N-acetylgalcosaminyltransferase I, the medial Golgi transferase that initiates complex N-linked carbohydrate formation. Proc. Natl Acad. Sci. USA, 87, 9984–9985.

Littman,D.R., Thomas,Y., Maddon,P.J., Chess,L. and Axel,R. (1985) Isolation and sequence of the gene encoding TR: a molecule defining functional classes of T lymphocytes. Cell, 40, 237–246.

Luzio,J.P. and Banting,G. (1993) Eukaryotic membrane traffic — retrieval and retention mechanisms to achieve organelle residence. Trends Biochem. Sci., 18, 395–398.

Machamer,C.E. (1993) Targeting and retention of Golgi membrane proteins. Curr. Opin. Cell Biol., 5, 606–612.

Madsen,S., Balaji,P.V., Legge,E.F., Hildebrandt,F. and Qasba,P.K.J. (1993) The Golgi retention signal of bovine β1,4-galactosyltransferase. J. Biol. Chem., 268, 9908–9916.

Molloy,S.S., Thomas,L., VanSlyke,J.K., Stenberg,P.E. and Thomas,G. (1994) Intracellular trafficking and activation of the furin proprotein convertase: localization to the TGN and recycling from the cell surface. EMBO J., 13, 18–33.

Moremen,K.W., Touster,O. and Robbins,P.W. (1991) Novel purification of the catalytic domain of Golgi α-mannosidase. J. Biol. Chem., 266, 16876–16885.

Mouritsen,O.G. and Bloom,M. (1993) Models of lipid–protein interactions in membranes. Annu. Rev. Biophys. Biomol. Struct., 22, 145–171.

Munro,S. (1991) Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention. EMBO J., 10, 3577–3588.

Munro,S. (1995) A comparison of the transmembrane domains of Golgi and plasma membrane proteins. Biochem. Soc. Trans., 23, 527–530.

Munro,S. and Pelham,H.R.B. (1987) A C-terminal signal prevents secretion of luminal ER proteins. Cell, 48, 899–907.

Natsuka,S. and Lowe,J.B. (1994) Enzymes involved in mammalian oligosaccharide biosynthesis. Curr. Opin. Struct. Biol., 4, 683–691.

Nilsson,T., Luocq,J.M., Mackay,D. and Warren,G. (1991) The membrane spanning domain of β1,4-galactosyltransferase specifies Golgi localization. EMBO J., 10, 3567–3575.

Nilsson,T., Siuslaewicz,P., Hoe,M.E. and Warren,G. (1993) Kin recognition: a model for the retention of Golgi enzymes. FEBS Lett., 330, 1–4.

Nilsson,T., Hoe,M.H., Siuslaewicz,P., Rabouille,C., Watson,R., Hunte,F., Watzele,G., Berger,E.G. and Warren,G. (1994) Kin recognition between medial Golgi enzymes in HeLa cells. EMBO J., 13, 562–574.

Orci,L., Montesano,R., Meda,P., Malaisse-Lagae,F., Brown,D., Perrelet,A. and Vassalli,P. (1981) Heterogeneous distribution of fipolin–cholesterol complexes across the cisternae of the Golgi apparatus. Proc. Natl Acad. Sci. USA, 78, 293–297.

Pelham,H.R.B. and Munro,S. (1993) Sorting of membrane proteins in the secretory pathway. Cell, 75, 603–605.

Pfeffer,S. and Rothman,J.E. (1987) Biosynthetic protein transport and sorting by the endoplasmic reticulum and Golgi. Annu. Rev. Biochem., 56, 829–852.
Ponnambalam, S., Rabouille, C., Luzio, J.P., Nilsson, T. and Warren, G. (1994) The TGN38 glycoprotein contains two non-overlapping signals that mediate localization to the trans-Golgi network. J. Cell Biol., 125, 253-268.

Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E.G., Warren, G. and Nilsson, T. (1995) Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. J. Cell Sci., 108, 1617–1627.

Reaves, B., Horn, M. and Banting, G. (1993) TGN38/41 recycles between the cell surface and the TGN: brefeldin A affects its rate of return to the TGN. Mol. Biol. Cell, 4, 93–105.

Roth, J. (1987) Subcellular organization of glycosylation in mammalian cells. Biochim. Biophys. Acta, 906, 405–436.

Roth, J., Taatjes, D.J., Weinstein, J., Paulson, J.C., Greenwell, P. and Watkins, W.M. (1986) Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal absorptive and goblet cells. J. Biol. Chem., 261, 14307–14312.

Russo, R.N., Shaper, N.L., Taatjes, D.J. and Shaper, J.H. (1992) β-1,4-galactosyltransferase: a short NH2-terminal fragment that includes the cytoplasmic and transmembrane domain is sufficient for Golgi retention. J. Biol. Chem., 267, 9241–9247.

Sandellius, A.S., Penel, C., Auderset, G., Brightman, A., Millard, M. and Morre, D.J. (1986) Isolation of highly purified fractions of plasma membrane and tonoplast from the same homogenate of soybean hypocotyls by free-flow electrophoresis. Plant Physiol., 81, 177–185.

Sankaram, M.B. and Thompson, T.E. (1990) Modulation of phospholipid acyl chain order by cholesterol. A solid state 2H nuclear magnetic resonance study. Biochemistry, 29, 10676–10684.

Schimmöller, F., Singer-Krüger, B., Schröder, S., Krüger, U., Barlowe, C. and Riezman, H. (1995) The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. EMBO J., 14, 1329–1339.

Schutze, M.P., Peterson, P.A. and Jackson, M.R. (1994) An N-terminal double arginine motif maintains type II membrane proteins in the endoplasmic reticulum. EMBO J., 13, 1696–1705.

Schweizer, A., Rohrer, J., Haue, H.P. and Kornfeld, S. (1994) Retention of P63 in an ER–Golgi intermediate compartment depends on the presence of all three of its domains and on its ability to form oligomers. J. Cell Biol., 126, 25–39.

Schwentk, T., Lorenz, C. and Ernst, J.F. (1994) Golgi localization in yeast is mediated by the membrane anchor region of rat liver sialyltransferase. J. Biol. Chem., 270, 5483–5489.

Seed, B. (1987) An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. Nature, 329, 840–842.

Simons, K. and Van Meer, G. (1988) Lipid sorting in epithelial cells. Biochemistry, 27, 6197–6202.

Sweet, D.J. and Pelham, H.R.B. (1992) The Saccharomyces cerevisiae SEC20 gene encodes a membrane glycoprotein which is sorted by the HDEL retrieval system. EMBO J., 11, 423–432.

Tang, B.L., Wong, S.H., Low, S.H. and Hong, W. (1992) Retention of a type II surface membrane protein in the endoplasmic reticulum by the lys–ar–leu sequence. J. Biol. Chem., 267, 7072–7076.

Teasdale, R.D., Matheson, F. and Gleeson, P.A. (1994) Post-translational modifications distinguish cell surface from Golgi-retained β-1,4galactosyltransferase molecules. Golgi localization involves active retention. Glycobiology, 4, 917–928.

Van Meer, G. (1989) Lipid traffic in mammalian cells. Annu. Rev. Cell Biol., 5, 247–275.

Velasco, A., Hendricks, L., Moremen, K.W., Tulsiani, D.R.P., Touster, O. and Farquhar, M.G. (1993) Cell-type dependent variations in the subcellular distribution of α-mannosidase I and II. J. Cell Biol., 122, 39–51.

Weinstein, J., Lee, E.U., McEntee, K., Lai, P.-H. and Paulson, J.C. (1987) Primary structure of β-galactosidase α-2,6-sialyltransferase. Conversion of the membrane bound form of the enzyme to soluble forms by cleavage of the H2-terminal signal anchor. J. Biol. Chem., 262, 17735–17743.

Weisz, O.A., Swift, A.M. and Machamer, C.E. (1993) Oligomerization of a membrane protein correlates with its retention in the Golgi complex. J. Cell Biol., 122, 1185–1196.

Wong, S.H. and Hong, W. (1993) The SYXYRL sequence in the cytoplasmic domain of TGN38 plays a major role in trans-Golgi network localization. J. Biol. Chem., 268, 22853–22862.

Wong, S.H., Low, S.H. and Hong, W. (1992) The 17-residue transmembrane domain of β-galactosidase α-2,6-sialyltransferase is sufficient for Golgi retention. J. Cell Biol., 117, 245–258.

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