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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 lumenal 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 Banting, 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 lumenal 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 lumenal 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.

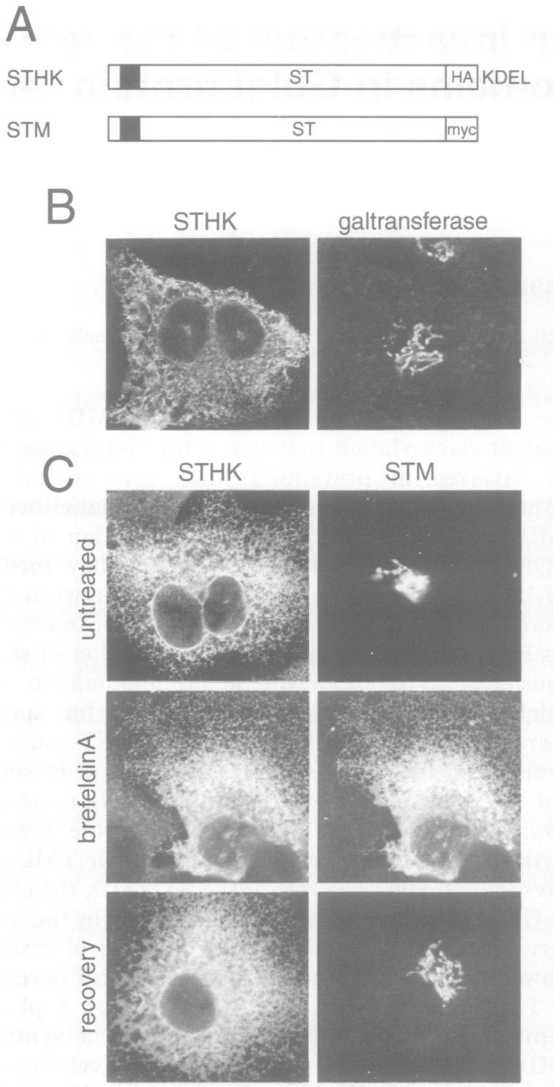

Fig. 1. Relocation of ST to the ER does not affect other *trans* Golgi proteins. (**A**) Schematic representations of the ST chimeras. The TMD is shown by a black box, and the *myc* and HA tags are named. (**B**) Double label immunofluorescent localization of endogenous galactosyltransferase and ER-retained 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.

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*-acetylglucosaminyltransferase 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 transfected cells, it had not accumulated in the ER but was still in the Golgi. One possible explanation is that ST forms specific oligomers which do not include galactosyltransferase. Because ST accumulates in the *trans* Golgi even when overexpressed 50-fold (Rabouille *et al.*, 1995), then any oligomerization-based retention might be expected to involve at least an interaction between the molecules of ST. Thus two different forms of ST were coexpressed, one with an ER retention signal and one without (Figure 1A), and their locations were compared by virtue of them containing different peptide tags. Figure 1C shows that these two proteins also accumulate in the separate locations of ER and Golgi. To increase the opportunity for the proteins to oligomerize, the cells were treated with brefeldin A which causes the Golgi to fuse

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

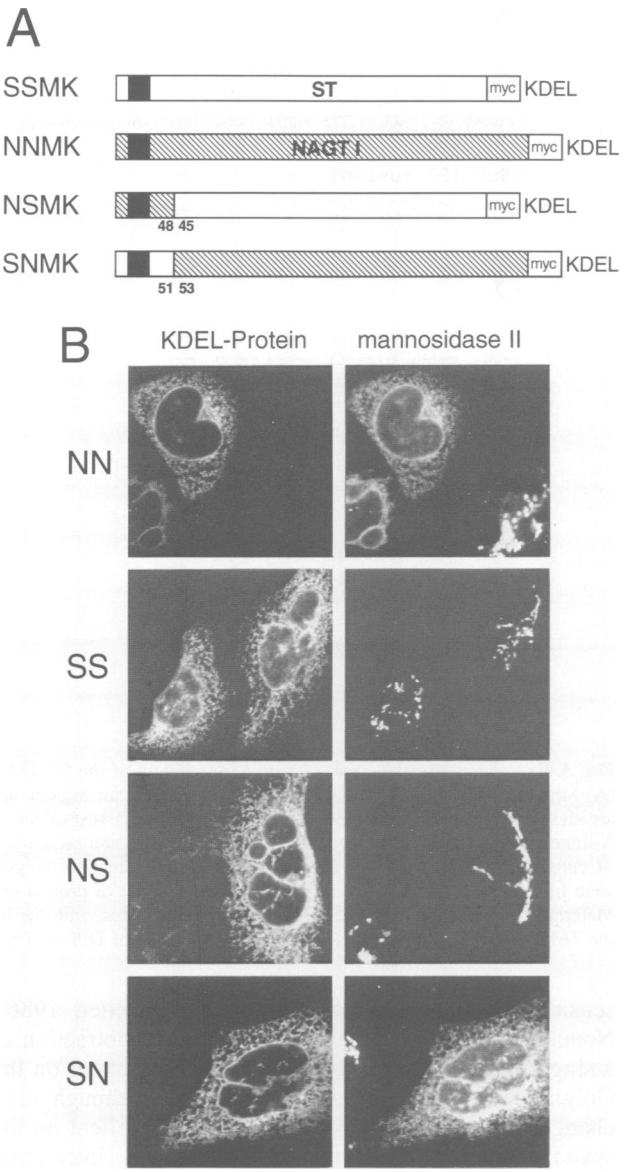

Fig. 2. The lumenal domain of NAGT I mediates kin recognition with mannosidase II. (**A**) Schematic representations of the Golgi enzyme chimeras used in the kin recognition studies. The TMD is shown by a black box, and the *myc* tags and KDEL sequences are named. For the SN and NS chimeras, the residue numbers at the break points in the chimeras are shown. (**B**) Double label immunofluorescent localization of endogenous mannosidase II and ER-retained Golgi enzymes in CHOP cells transfected with the indicated plasmids. In untransfected cells, mannosidase II is always located in the typical perinuclear Golgi distribution.

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

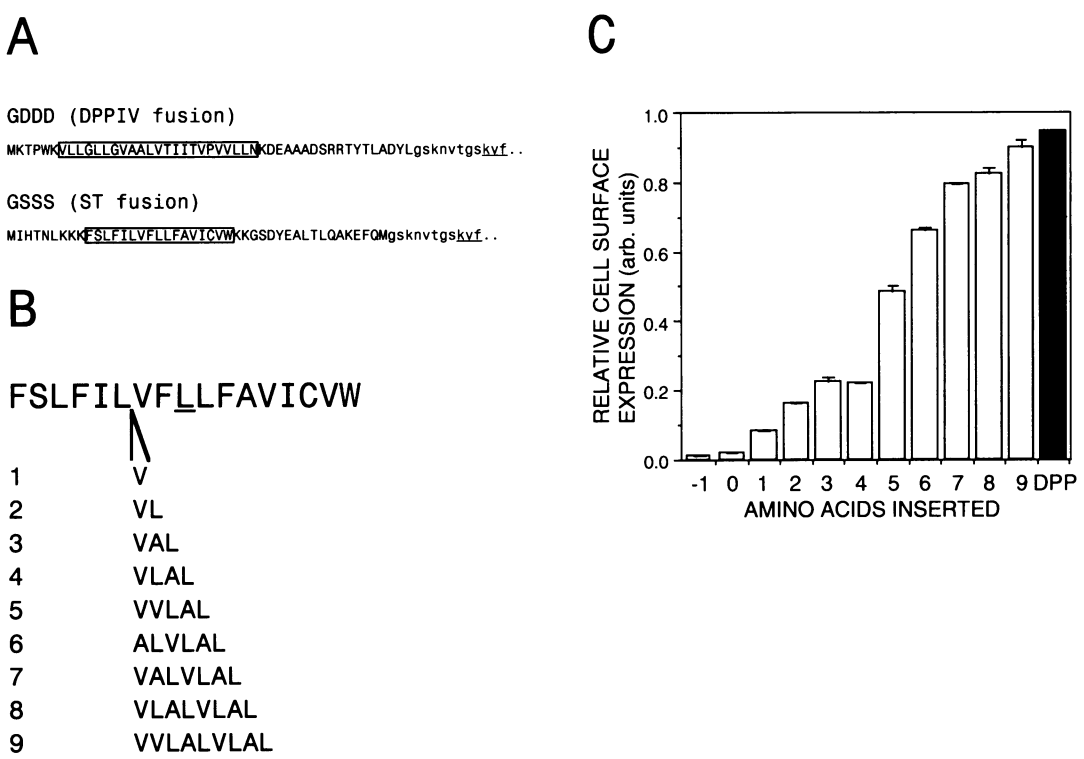

Fig. 3. Examination of the effect of varying the length of the ST TMD on its ability to confer Golgi retention to a ST–lysozyme fusion protein. (**A**) Structures of the lysozyme fusions to the signal anchor regions of DPPIV (residues 1–47) and ST (residues 1–44). The signal anchor residues are shown in upper case, the TMDs are boxed, and the first residues of mature lysozyme are underlined, with an interposed linker encoding a site for *N*-linked glycosylation (Munro, 1991). (**B**) The location and sequences of the insertions in the ST TMD of GSSS. The residue deleted in the −1 construct is underlined. (**C**) Relative cell surface expression of lysozyme in COS cells transfected with the indicated TMD length variants. Cells were transfected with the relevant plasmids. The amount of protein on the surface relative to the total in the cells was determined as described in Materials and methods. All values are means of duplicates, with the individual values indicated by the error bars. The 'DPP' construct is one where the TMD of GSSS has been replaced exactly with that of DPP ('SDS' in Munro, 1991).

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 the +2 construct had equal small effects (~2-fold) which were additive when combined. Thus it appears that

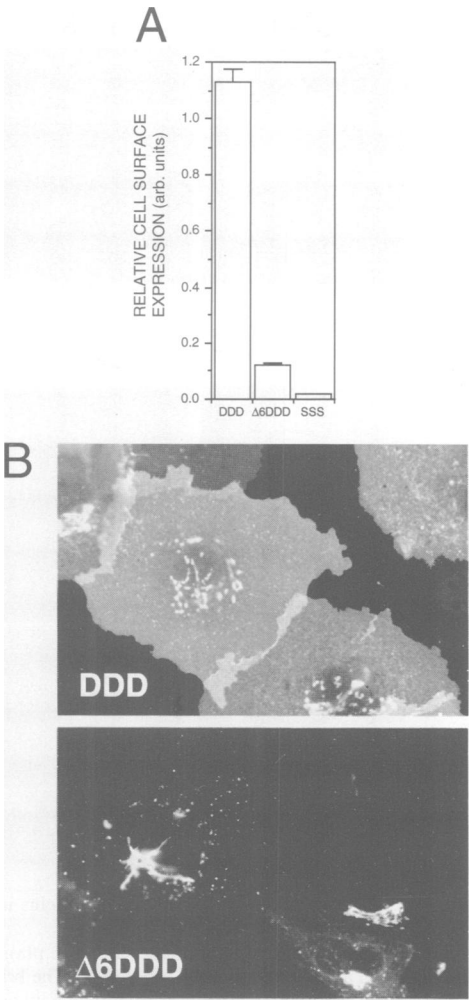

Fig. 4. Shortening the TMD of a cell surface protein reduces its cell surface accumulation. (**A**) Relative cell surface expression in transfected COS cells of GDDD and GSSS compared with GDΔ6D, a lysozyme chimera identical to GDDD except for the removal of the six residues PVVLLN at the end of the TMD (see Figure 3A). (**B**) Immunofluorescent localization of GDDD and GDΔ6D in transfected COS cells. Note that in some cells surface staining is visible with GDΔ6D.

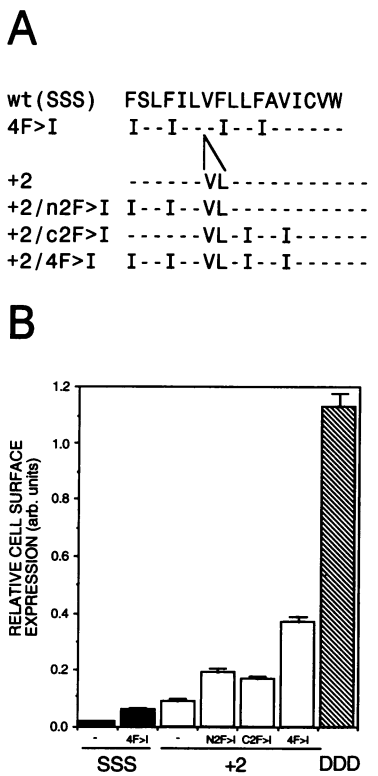

Fig. 5. The phenylalanines in the ST TMD contribute to its ability to confer Golgi retention to a ST signal anchor–lysozyme chimera. (**A**) Sequences of the TMDs in the versions of GSSS, the ST–lysozyme fusion protein, based on either the wild-type GSSS TMD or the +2 version which is extended by two amino acids. (**B**) Relative cell surface expression of lysozyme chimera in COS cells transfected with the phenylalanine variants. Values are means of triplicates, with the highest and lowest values indicated by the error bars

while the phenylalanine residues may make a small contribution to the retaining activity of the ST TMD, altering its length has more substantial effects.

TMD-mediated retention of a fusion protein with a type I orientation

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

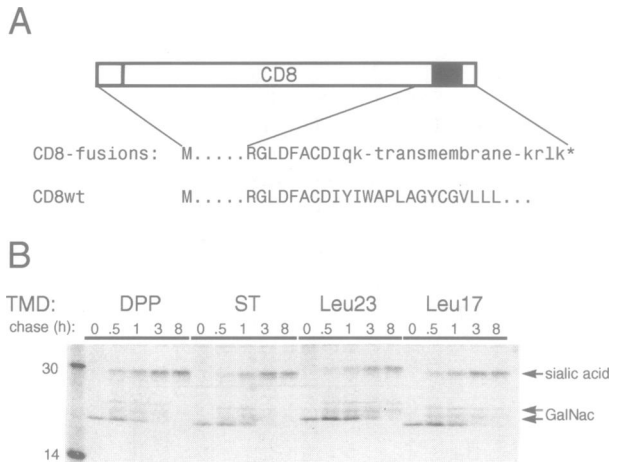

Fig. 6. CD8 fusion proteins with different TMDs. (**A**) Structures of the CD8 fusions encoded by the plasmids used in this study. The TMD and cytoplasmic tail of the dimeric T cell surface protein CD8 (CD8wt; Littman *et al.*, 1985) were replaced with four different TMDs all followed by the same short cytoplasmic tail. The transmembrane portions (black) were either those of ST or DPP (boxed in Figure 3A) or a stretch of 17 or 23 leucines. (**B**) Maturation of the CD8 fusions followed by a pulse–chase analysis of the transfected cells. COS cells transfected with the relevant plasmids were pulsed with [^{35}S]methionine for 20 min and chased for the indicated times, before the CD8 fusion proteins were harvested by immunoprecipitation and analysed by SDS–PAGE. The newly synthesized form of the protein acquires a reduced mobility as it is modified with *O*-linked *N*-acetylgalactosamine (GalNac) which is then galactosylated and sialylated in the Golgi apparatus, the latter modification causing a marked reduction in mobility (Jackson *et al.*, 1993). $T_{1/2}$ values for acquisition of this modification are 75, 100, 110 and 250 min for D, S, L17 and L23, respectively.

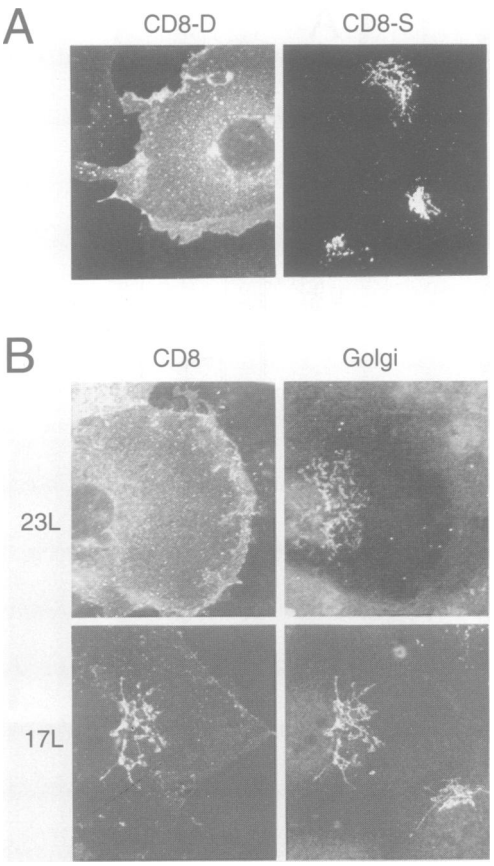

Fig. 7. The intracellular localization of CD8 fusion proteins in COS cells depends on their membrane-spanning sequence. (**A**) Immunofluorescent staining of cells transfected with plasmids expressing CD8 with either DPP (D) or ST (S) TMD. The bright perinuclear structures seen with CD8-S also stain with antibodies against endogenous Golgi proteins (data not shown). (**B**) Double label immunofluorescent localization of CD8 fusions with TMDs of either 23 or 17 leucine residues, and an endogenous Golgi marker. Cells transfected with the relevant plasmids were chased for 3 h in 0.5 mM cycloheximide prior to fixation, and then stained with an anti-CD8 rat monoclonal and an anti-Golgi mouse monoclonal (22-II-D8B) followed by species-specific fluorescent antibodies.

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 had 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 (Sandelius *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 phosphatidylinositol-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 lumenal 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 galactosyltransferase, 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 lumenal 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 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 ···IRCYMAYPYDVPDYASEKDEL, IRC being the C-terminus of native ST (Weinstein *et al.*, 1987). SSMK encodes ST followed by YMEQKLISEEDLNSEKDEL, and NNMK encodes human NAGT I followed by the same sequence but without the first Y (Kumar *et al.*, 1990). NSMK and SNMK are chimeras of these two proteins. In NSMK, the sequence at the fusion point is DPAGSNSPKS, where A is residue 48 of NAGT I and P is residue 45 of ST, with GSNS being encoded by the linker. For SNMK, the fusion point is KVGEVI, with the first V being residue 51 of ST, the E being residue 53 of NAGT I and G from the linker. Plasmids SSMK, NNMK, NSMK and SNMK 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 CDM8 expression cassette. Thus, CDM8 was cut with *Xba*I and *Dra*I, 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 NNMK and SNMK.

The expression plasmids encoding lysozyme chimeras were all derived from the previously described GSSS and GDDD COS cells expression plasmids 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 *Spe*I and *Nde*I 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 GTAGTCCTGGCCCTCGTGTTGGCTCTC for the longest (VVLALVLAL) and the shorter insertions by progressive N-terminal deletions of the sequence following the first G. Phenylalanine to isoleucine changes (T → A in first position of codons) and the GDΔ6D mutant were made by assembling the DPPIV or ST signal anchor region from oligonucleotides as described previously (Munro, 1991).

The CD8 plasmids are based on CD8-D, 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 ···ACDI 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; Heffernan and Dennis, 1991), were split onto eight-well slides [C.A.Hendley (Essex) Ltd]. On the following day they were treated 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 8c (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 Optimem (Gibco) containing 10% fetal calf serum (FCS) and 0.1 μg/ml monoclonal F10 labelled with ^{125}I 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 ^{125}I 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 from 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 ^{125}I-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 pulsed with 100 μCi/ml [^{35}S]methionine for 15 min in methionine-free DMEM/1% dialysed FCS, and then either lysed immediately or chased in 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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