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Sequences within and adjacent to the transmembrane segment of α-2,6-sialyltransferase specify Golgi retention

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The glycosyltransferase α-2,6-sialyltransferase (ST) is a Type II membrane protein localized to the Golgi apparatus. The first 44 amino acids of this protein were able to specify Golgi retention of a fused marker protein, lysozyme. This section of ST contains a transmembrane segment which serves as a non-cleaved signal anchor. When lysozyme was fused to an equivalent region of a cell surface protein it now appeared on the cell surface. Analysis of chimeras between the two proteins revealed that the transmembrane segment of ST specifies Golgi retention. Furthermore, altering this segment in full-length ST results in the protein accumulating on the cell surface. However, the retaining effect of the transmembrane domain of ST is augmented by the presence of adjacent lumenal and cytoplasmic sequences from ST. If these sequences are spaced apart by a transmembrane domain of the same length as that of ST they too can specify Golgi retention. Thus retention in the Golgi of ST appears to involve recognition of an extended region of the protein within and on both sides of the bilayer.

Key words: Golgi/retention/α-2,6-sialyltransferase/targeting/transmembrane domain

Introduction

In eukaryotic cells, proteins destined for the secretory pathway are inserted into the endoplasmic reticulum and then proceed through a series of membrane bounded compartments to their final destination in the cell. It is now a widely held belief that in non-polarized cells a soluble or membrane protein can proceed to the plasma membrane without special targeting signals (reviewed in Pfeffer and Rothman, 1987). However, proteins destined for internal structures are thought to contain specific targeting signals which are used by the cell to direct them to their destinations. Considerable progress has been made in understanding the sorting of soluble and membrane proteins of the endoplasmic reticulum (ER) as well as that of soluble proteins of the lysosome and the yeast vacuole (Pelham, 1989; Nilsson *et al.*, 1989; Rothman *et al.*, 1989; Kornfeld, 1990). However, very little is known about how the Golgi apparatus, arguably the central organelle of the secretory pathway, maintains its resident population of proteins.

After assembly and addition of core N-linked sugars in the ER, proteins proceed to the Golgi apparatus where further modifications occur including the trimming of the N-linked sugar core and subsequent addition of complex carbohydrate structures, as well as palmitoylation, tyrosine sulphation and the addition of O-linked sugars (for reviews see Kornfeld and Kornfeld, 1985; Pugsley, 1989). The Golgi is arranged in a stacked cisternal structure with enzymes generally located in separate cisternae in the order in which they act (reviewed in Dunphy and Rothman, 1985; Roth, 1987). In the *cis* and *medial* cisternae the core sugars are trimmed and common additions made and in the *trans* cisternae and the *trans*-Golgi network (TGN) a variety of different complex sugar structures are assembled in a protein and cell-type specific manner. The TGN is also the point at which proteins destined for lysosomes, storage vesicles and domains of the plasma membrane are sorted from one another (Griffiths and Simons, 1986).

In recent years the genes encoding a number of Golgi enzymes involved in carbohydrate modification have been isolated and all encode membrane proteins with a Type II orientation (i.e. their amino-termini are in the cytosol) and a single membrane spanning domain (Paulson and Colley, 1989; Kumar *et al.*, 1990; Yamamoto *et al.*, 1990; Sarkar *et al.*, 1991). In addition a protein of the TGN with a Type I orientation has been identified although its function is unknown (Luzio *et al.*, 1990). Since membrane proteins of both orientations can pass through the Golgi apparatus, there must be a mechanism for ensuring that the resident population of Golgi enzymes is maintained. Investigation of this problem has so far been restricted to a viral glycoprotein which accumulates in the Golgi prior to budding—the E1 glycoprotein of coronaviruses. Analysis of E1 from the avian infectious bronchitis virus (IBV) has suggested that one of its three transmembrane segments is involved in its localization (Machamer and Rose, 1987). However, work on the homologous protein from mouse hepatitis virus A59 has shown that small deletions in the cytoplasmic tail of the virus eliminate Golgi retention (Armstrong *et al.*, 1990; Armstrong and Patel, 1991). The reasons for these differences remain unclear.

As an alternative approach I decided to investigate an endogenous Golgi enzyme with a single membrane spanning domain to try to determine how it is prevented from accumulating on the plasma membrane. The enzyme chosen for study was rat α-2,6-sialyltransferase (ST) (EC 2.4.99.1) which is located in the *trans* Golgi and the TGN, (Roth *et al.*, 1985; Taatjes *et al.*, 1988). Like the other transferases so far investigated, ST has a short amino-terminal cytoplasmic domain, an uncleaved hydrophobic signal anchor and a large catalytic lumenal domain (Weinstein *et al.*, 1987; Paulson and Colley, 1989). Like other transferases it is also found in serum apparently having been clipped from its membrane anchor by an endogenous protease with the resulting fragment beginning at amino acid 64 of the original protein (Beyer *et al.*, 1981; Weinstein *et al.*, 1987; Lammers and Jamieson, 1988). This suggests that the 63 amino-terminal residues contain sequences that are required, at least in part, for the retention of the intact enzyme in the cell. Indeed if

the secreted domain is expressed with a cleavable leader peptide positioned at residue 57, the protein is rapidly secreted (Colley *et al.*, 1989).

To investigate the Golgi retaining role of the amino-terminal section of ST, I made a hybrid gene which expressed a fusion between the first 44 amino acids of ST and a secreted protein, chicken lysozyme. This fusion protein did not appear on the cell surface but accumulated in the Golgi whilst an analogous fusion with a cell surface Type II protein accumulated on the plasma membrane. Analysis of a series of chimeric constructs showed that the transmembrane section of the Golgi enzyme contains a retention signal and that altering this region in the full-length ST resulted in the protein appearing on the cell surface. Finally, a further series of chimeras showed that sequences in the lumenal section of ST can also cause Golgi retention.

Results

Generation and analysis of lysozyme fusion proteins

The first 63 amino acids of ST include a cytoplasmic tail, a transmembrane signal anchor and a lumenal stalk connecting the catalytic domain of the enzyme to the membrane (Figure 1). Sequences encoding the first 44 amino acids of ST were fused to the coding region for chicken lysozyme, a secreted protein of known structure to which many antibodies are available (plasmid GSSS, Figure 1). In preliminary experiments no difference could be detected in the behaviour of this fusion and a longer one containing the first 55 amino acids of ST and so only the shorter fusion was used for subsequent studies (data not shown). As a control, the lysozyme cDNA was also fused to the equivalent region from a cell surface membrane protein dipeptidylpeptidase IV (DPPIV) (GDDD, Figure 1). DPPIV is a Type II membrane protein found on a variety of epithelial, endothelial and lymphocytic cell types (Hong and Doyle, 1987, 1990; Ogata *et al.*, 1989). It was chosen because, like ST, it has a cytoplasmic domain of only a few amino acids, and a single membrane-spanning domain flanked by lysine residues (Figure 1).

In order to be able to check the membrane orientation of the lysozyme fusions encoded by GSSS and GDDD, the constructs were made with a site for N-linked glycosylation inserted between the membrane protein sequences and the lysozyme coding region (Figure 1). For comparison, versions were also made in which either the glycosylation site was absent (USSS and UDDD, Figure 1), or the non-cleaved signal anchors were replaced with a cleavable leader peptide (LPS and LPD, Figure 1). The expression plasmids encoding these proteins were transfected into COS cells and the gel mobility of the newly made proteins examined by [^{35}S]Met labelling and immunoprecipitation. Figure 2A shows that the lysozyme fusion proteins produced by GDDD and GSSS had a lower mobility than their unglycosylated counterparts encoded by UDDD and USSS. Furthermore, if the cytoplasmic and transmembrane domains of GDDD and GSSS were replaced by a leader peptide, the resulting proteins now had an increased mobility (Figure 2A, LPS and LPD), indicating that the products of GDDD and GSSS were not being cleaved after their transmembrane sections. When the various proteins were treated with endoglycosidase H to remove N-linked carbohydrate, the mobility of those fusions containing the glycosylation site was increased (Figure 2A). Thus the fusions GDDD and GSSS appear to be inserted in the membrane in the correct orientation and are not substrates for signal peptidase.

Cellular localization of fusion proteins

The fusion constructs were then transfected into COS cells and the localization of the chimeric proteins determined by immunofluorescence with antibodies against lysozyme. As can be seen in Figure 3, the DPPIV fusion, GDDD, produced high levels of cell surface staining as well as some staining of internal structures. However, the fusion with ST, GSSS, showed very little cell surface staining, but instead had bright perinuclear staining with some cells showing fainter cytoplasmic staining in a reticulum typical of the ER. Transfected COS cells typically show heterologous expression levels and in the more highly expressing cells the ER staining was more prominent. Scanning a large number of cells revealed the occasional cell with faint plasma membrane staining (<5% of the transfected cells). The result with the unglycosylated forms appeared very similar, although USSS seemed to show consistently more ER staining than GSSS (not shown), and in all subsequent experiments glycosylated lysozyme fusions were used. Next, the cells transfected with GSSS were double-labelled with antisera against a Golgi enzyme, β-1,4-galactosyltransferase and as shown in Figure 3B, the product of GSSS was clearly colocalized with galactosyltransferase.

To investigate further the intracellular fate of the products of GSSS and GDDD, cells transfected with the two plasmids were labelled with a pulse of [^{35}S]Met and chased for 3.5 h instead of harvesting immediately. SDS–PAGE of the immunoprecipitated fusion proteins revealed that both had acquired a heterogeneous and reduced mobility but could be resolved into a single sharp band by treatment with endo F (Figure 2B). Furthermore the two fusion proteins seem to have the same stability over the period of the chase. The mobility reduction during the chase suggests the addition of complex carbohydrate structures which occurs in the *trans* Golgi and TGN (Kornfeld and Kornfeld, 1985; Roth, 1987). The degree and heterogeneity of this mobility reduction suggests the addition of long carbohydrate chains, the most common of which is poly-*N*-acetyl lactosamine (Kornfeld and Kornfeld, 1985; Fukuda, 1985). This carbohydrate is recognized by the lectin *Datura stramonium* agglutinin and indeed recent experiments have shown the immunoprecipitated proteins expressed by GDDD and GSSS are recognized by this lectin (Cummings and Kornfeld, 1984; Yamashita *et al.*, 1987, data not shown). Thus whilst the products of GDDD and GSSS have a similar stability, only the former accumulates on the surface whilst the latter appears to accumulate in the Golgi having acquired complex carbohydrate additions consistent with a location on the *trans* side of this structure.

Localization of fusion proteins at late times following transfection

As mentioned above some of the cells transfected with GSSS show staining of the ER as well as of the Golgi (Figure 3A). The frequency and intensity of this staining increases at later times following transfection, i.e. after ~36 h when the expression level starts to plateau (data not shown). Thus cells fixed and stained after 33 h show fainter ER staining and in fewer cells than at 40 h, the time at which the cells were

fixed for the immunofluorescent analyses shown in Figures 3, 5 and 7. COS cell expression vectors of the type used in this study contain an SV40 replication origin and as the plasmid replicates in the transiently transfected cells the expression level of the protein it encodes increases. Expression levels in excess of 10^6 protein molecules per cell are typically seen with replicating COS cell vectors (e.g. Simmons and Seed, 1988). Thus it is possible that the extremely high level of expression begins to produce aberrant localization of the fusion protein.

Another phenomenon also increasingly apparent at later times is the release of the product of GSSS into the medium.

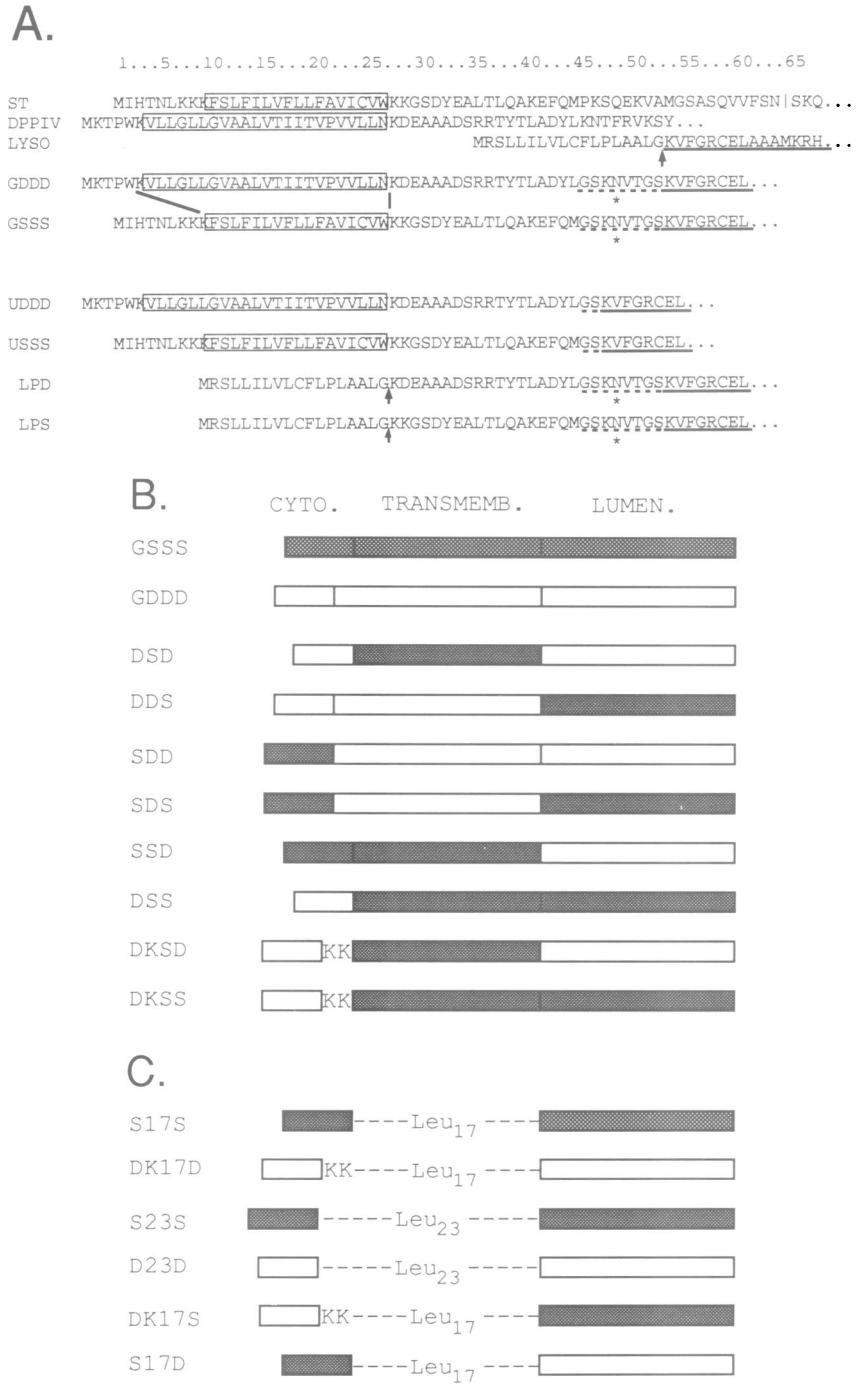

Fig. 1. Sequences of the starting proteins and fusions used in this study. (**A**) The amino-terminal amino acid sequences of rat α-2,6-sialyltransferase (ST), rat dipeptidylpeptidase IV (DPPIV) and chicken pre-lysozyme (LYSO). The hydrophobic membrane spanning domains (defined by the extent of uncharged residues) are boxed. Also marked is the amino-terminus of the serum form of ST (|) and the cleavage site following the leader peptide (arrow). Shown below are the fusion proteins encoded by the plasmids GDDD and GSSS. For GSSS the mature portion of lysozyme (underlined) is fused to the first 44 amino acids of ST with an eight amino acid spacer (dashed) containing a potential site for N-linked glycosylation (*). GDDD is the analogous construct made with the first 47 amino acids of DPPIV. The lysines which flank the transmembrane segments are linked by lines and these formed the common crossing over point for the chimeric constructs described below. (**B**) and (**C**) Diagram of the various chimeras used in this study with the region that is fused to the lysozyme plus glycosylation site being indicated. The two extra lysine residues inserted in some constructs at the start of the membrane spanning domain are indicated (KK).

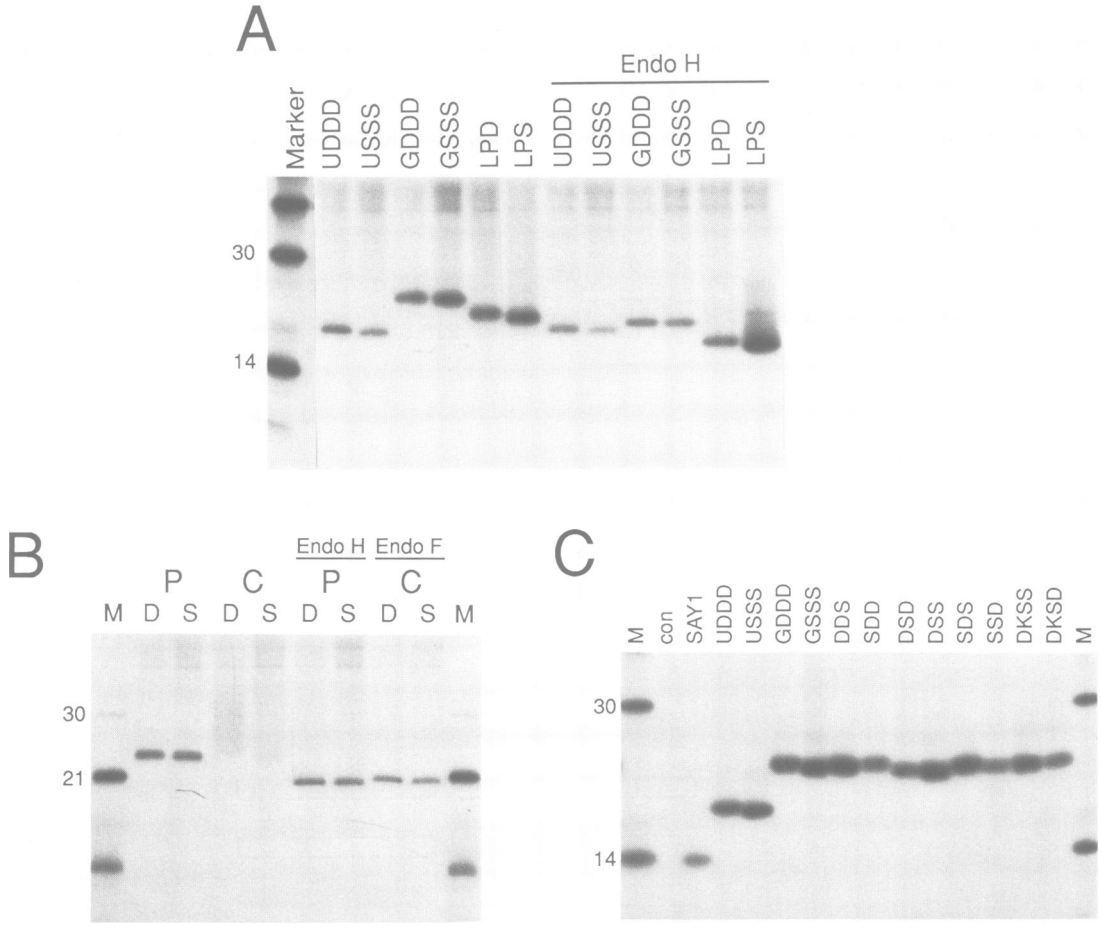

Fig. 2. Anti-lysozyme immunoprecipitations from transfected and [^{35}S]Met-labelled COS cells. Fluorographs of ^{35}S-labelled lysozyme fusion proteins isolated by immunoprecipitation with an anti-lysozyme serum and separated by SDS−PAGE. (**A**) Cells transfected with the indicated plasmids were pulse-labelled for 20 min, 33 h following transfection, lysed and immunoprecipitations performed. The precipitates were divided equally and incubated with or without endoglycosidase H prior to analysis. The molecular weights in kDa of the size markers are shown, the 14 kDa marker being lysozyme. (**B**) COS cells transfected with plasmids GDDD (D) or GSSS (S) were pulse-labelled as above and then lysed immediately (P) or following a 3.5 h chase (C). The immunoprecipitated lysozyme fusion proteins were divided equally for incubation with or without the indicated endoglycosidases prior to electrophoresis. The molecular weight in kDa of the size markers (M) is indicated. (**C**) COS cells transfected with the indicated plasmids and 48 h later, pulse-labelled for 20 min prior to lysis and immunoprecipitation. M: Size markers as in (A).

Whilst GSSS appears to be as stable in cells as GDDD at early times following addition of DNA (e.g. Figure 2B, 33 h after transfection), at later times considerable quantities of the protein are released into the medium in an apparently clipped form. This is shown by a pulse−chase analysis in Figure 4 where protein is precipitated from cells and from the medium at various times following a pulse 44 h post-transfection. Clearly protein is leaking into the medium from GSSS whilst this is not seen with GDDD. Note that the nascent product of GSSS does not chase as rapidly into higher molecular weight forms as does that of GDDD, which would be consistent with the high levels of the former in the ER.

A third effect which appears to be time dependent is the appearance of lysozyme in lysosomes. Immunofluorescence of COS cells transfected with GDDD and GSSS often revealed punctate staining throughout the cytoplasm. These structures did not stain with the antisera to galactosyl-transferase (panels C and D, Figure 3B). The number of these blobs varied considerably between cells with their frequency and brightness increasing with time following transfection. They could be seen with both GSSS and GDDD and with the chimeric constructs discussed later and were only visible if glutaraldehyde had been used in the fixation of the cells. These dots were identified as early lysosomes by double label immunofluorescence using a monoclonal antibody 1B5 (Mark Marsh, personal communication; data not shown).

Lysosomal localization seems to be a property of lysozyme itself, because when COS cells express the wild type lysozyme gene, most of the protein is rapidly secreted into the medium, but the residual protein accumulates in these lysosomal structures (data not shown). The appearance of the GSSS product in lysosomes may represent the released proteins mentioned above behaving in the same way as normal lysozyme although the GDDD product would have to be accumulating via another route. Alternatively the localization may result from mis-folding of some small fraction of the protein due to the high expression level. Whatever its cause this effect should not interfere with the conclusions drawn from the behaviour of the lysozyme fusion proteins. Firstly, when lysozyme is attached to the segment of DPPIV, most of the protein passes all the way through

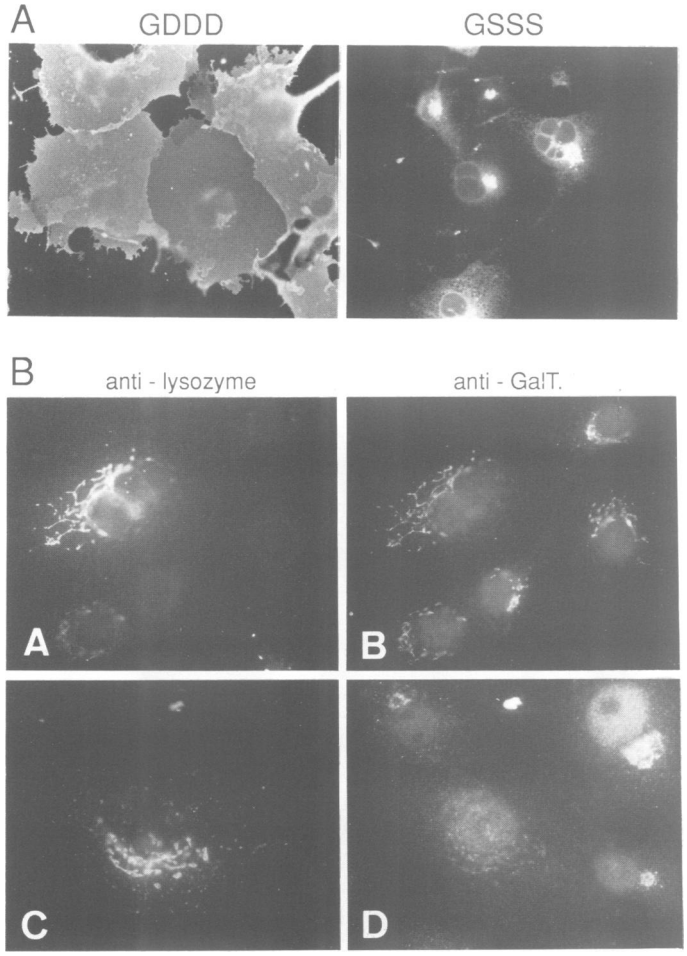

Fig. 3. Immunofluorescent localization of the lysozyme fusion proteins. (**A**) COS cells transfected with GDDD or GSSS as indicated were fixed 40 h later with paraformaldehyde/glutaraldehyde, permeabilized with Triton X-100, and stained with the anti-lysozyme monoclonal F10 followed by an FITC-labelled second antibody. (**B**) COS cells transfected with GSSS and after 40 h fixed and permeablized with either methanol/acetone (panels A and B) or as above with paraformaldehyde/glutaraldehyde. The cells were than double labelled with F10 (panels A and C) and antisera against galactosyltransferase (panels B and D) followed by species-specific fluorescent secondary antibodies. More cells are seen with the anti-galactosyltransferase antibodies because only a fraction of the COS cells are transfected with the expression plasmid. Note that the paraformaldehyde/glutaraldehyde fixation results in a reduced signal and elevated non-specific binding with the anti-galactosyltransferase sera. In the cell fixed in this way the F10 antibody stains many small punctate structures which can be seen scattered about the brighter ribbons of Golgi. The image of GSSS shown in A has been exposed to show the fainter ER staining as well as the brighter Golgi staining. To allow clear visualization of the Golgi structures for the colocalization experiments shown in (B), cells with fainter ER staining were chosen and the exposure reduced so that the ER staining is no longer clearly visible.

the secretory pathway to the cell surface. Thus any failure of a fusion protein to appear on the cell surface is unlikely to be due to the lysozyme part of the fusion. Secondly, lysozyme itself passes rapidly through the Golgi apparatus on its way to either the cell surface or lysosomes. Thus any retention of a lysozyme fusion protein in this compartment is again unlikely to be due to the lysozyme portion. Finally, when cells transfected with GSSS were treated with 0.5 mM cycloheximide for 3 h the bright Golgi staining was unchanged suggesting that this localization is not due to protein passing through on the way to lysosomes (data not shown).

As mentioned above, all of these effects are mainly features of late times following transfection when the fusion proteins accumulate to high levels. However, at both early and late times there is a striking difference in the localization of the products of GSSS and GDDD, strongly suggesting that this is a meaningful result, and so it was decided to examine the basis for it in more detail.

Construction of ST–DPPIV chimeras

The amino-terminal 44 residues of ST in GSSS, but not the equivalent region of DPPIV in GDDD, are sufficient to prevent a fusion protein from appearing on the cell surface and so presumably must contain specific residues necessary for this effect. This section of ST is exposed to three distinct compartments of the cell; the cytoplasm, the interior of the lipid bilayer, and the lumen of the Golgi. However, these residues are also responsible for the protein being inserted into, and then anchored in, the lipid bilayer. Thus it seemed unlikely that it would be possible to map the residues responsible for the localization of ST by a simple deletion analysis. To determine if sequences in one of these regions were sufficient for the effect seen with the intact ST sequence, oligonucleotides encoding each of the separate sections of ST and the equivalent sections of DPPIV were combined in all possible combinations and fused to lysozyme. The resulting chimeras are named by three letters indicating the origin of their cytoplasmic, membrane spanning and

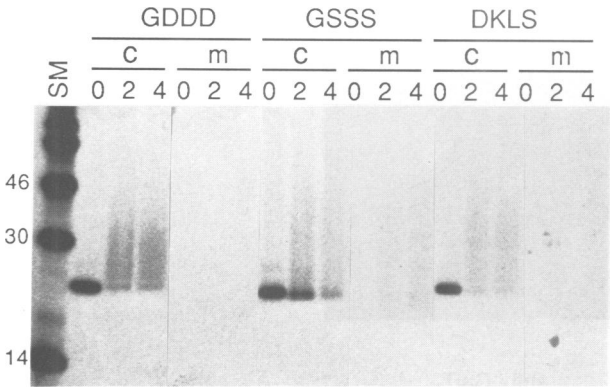

Fig. 4. Release of lysozyme fusion proteins from transfected cells. COS cells transfected with the indicated plasmids (DKLS indicates DK17S) 44 h previously were pulsed for 20 min with [^{35}S]Met and then either lysed immediately (0) or chased for 2 (2) or 4 (4) h before cells (c) and medium (m) were harvested. Equivalent amounts of anti-lysozyme immunoprecipitates were separated by SDS–PAGE and visualized by fluorography. SM: molecular weight markers as in Figure 3A.

lumenal domains, respectively, e.g. DSS, SDD, etc. (Figure 1B). Expression plasmids encoding these proteins were transfected into COS cells, and initially the ability of the chimeric sequences to serve as Type II membrane anchors was investigated as before by examining the gel mobility of pulse labelled protein. Figure 1C shows that the six chimeras migrated with a mobility equivalent to the uncleaved, glycosylated forms of GSSS and GDDD. Thus all of the chimeras appear to insert themselves into the membrane in the correct orientation and are not cleaved by signal peptidase.

Localization of the chimeric constructs

The cellular localization of the six chimeras was then examined by immunofluorescence of transfected COS cells as shown in Figure 5. The top row of panels shows the constructs where two of the three sections are from ST (DSS, SDS and SSD) and only with SDS does the fusion protein appear on the plasma membrane. Conversely, the middle panels show the constructs where only one of the three sections is from ST (DDS, DSD and SDD) and bright plasma membrane staining is seen with all but DSD. Golgi

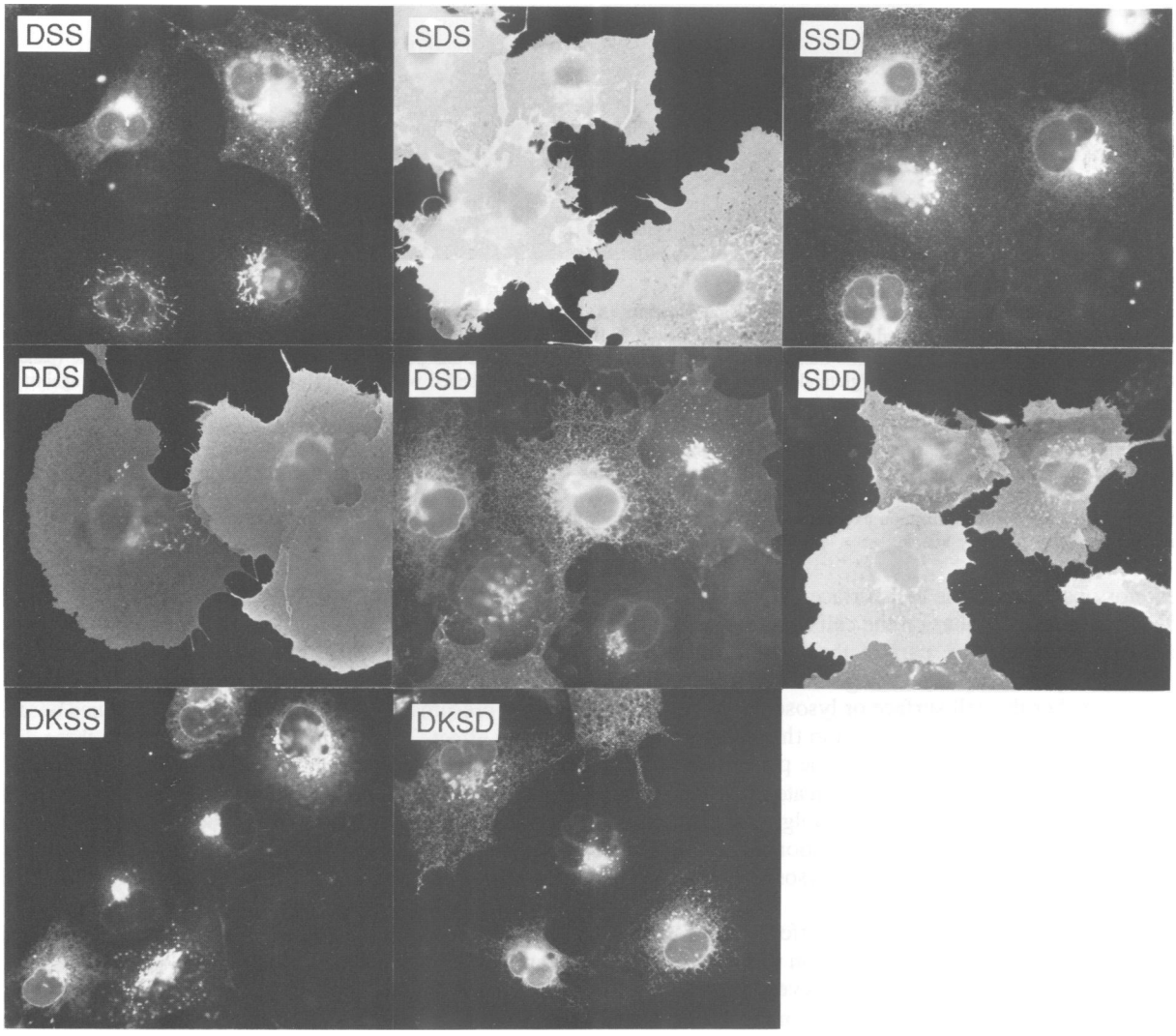

Fig. 5. Immunofluorescent localization of ST–DPPIV chimeras. COS cells were transfected with the indicated chimeras fused to lysozyme and after 40 h fixed and permeabilized and treated for immunofluorescence as in Figure 3A.

localization of SSD, DSS and DSD was confirmed by double labelling with galactosyltransferase (data not shown). Thus it appears that the central transmembrane section from ST is necessary and sufficient to prevent cell surface expression of a lysozyme fusion protein and instead give bright Golgi staining.

It can be seen in Figure 5 that whilst the DSD staining pattern is clearly more like GSSS than GDDD, there is faint plasma membrane staining visible on some of the cells—see cells at top right and bottom left of the panel. However, this considerable leakage of proteins past the Golgi was not seen with either SSD or DSS. This suggests that whilst the transmembrane domain clearly contains localization information, both the cytoplasmic and lumenal sequences flanking the transmembrane segment can contribute to its effect. One obvious difference between the cytoplasmic domains of ST and DPPIV is that there are three lysine residues adjacent to the transmembrane domain in the former protein and only one in the latter. Thus a version of DSD, and also of DSS, was made in which two additional lysines were inserted before the transmembrane domain—DKSD and DKSS respectively (Figure 1). Immunofluorescence analysis shows that distribution of both proteins is indistinguishable from that of GSSS (Figure 5). Thus whilst the three lysines in the ST cytoplasmic domain are clearly not sufficient for retention within the Golgi (see SDD, Figure 5) it appears that they contribute to the effect of the transmembrane domain in retention. Positively charged residues flanking

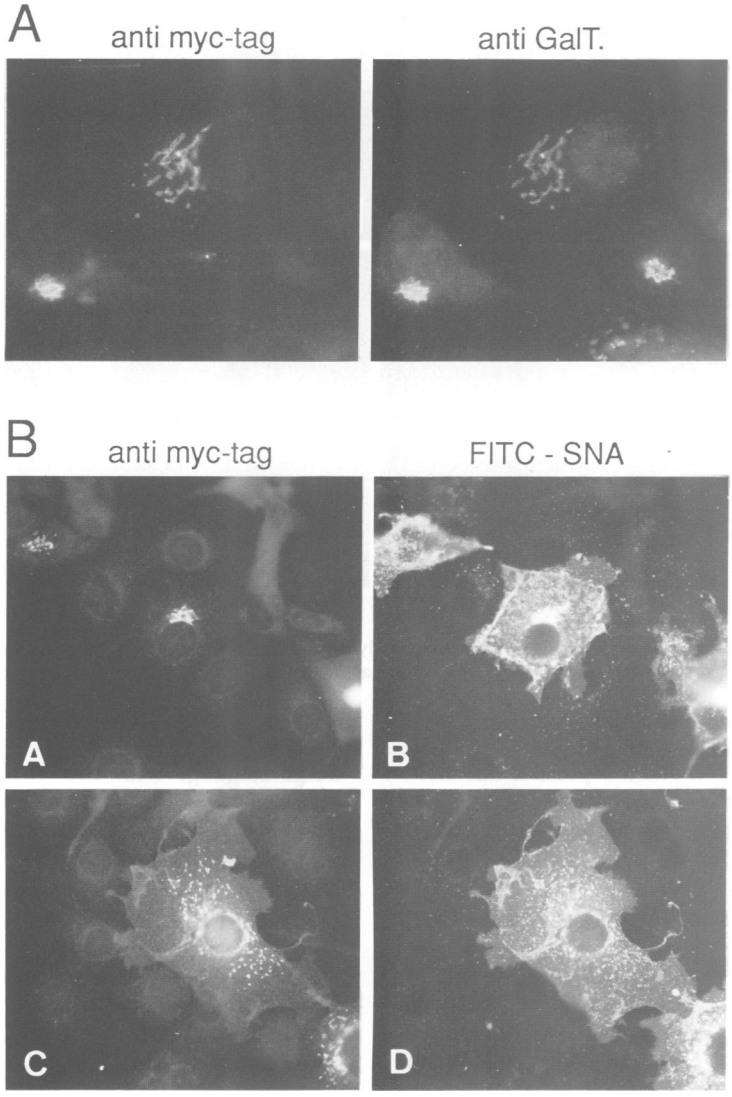

Fig. 6. Altering the transmembrane domain of full-length sialyltransferase results in cell surface accumulation. (**A**) Double label immunofluorescent localization of myc-tagged sialyltransferase and endogenous galactosyltransferase in COS cells transfected with plasmid STM which encodes a myc tagged ST. Cells were fixed with methanol/acetone 72 h after transfection and stained with the anti-myc monoclonal 9E10 and anti-GalT antisera. Although these full-length ST constructs were stable in COS cells they were expressed at much lower levels than the lysozyme fusion proteins (data not shown). The reason for this is unclear, perhaps the longer coding region is not efficiently translated, but it necessitated analysing the cells longer after transfection. (**B**) Double labelling of transfected COS cells with 9E10 (anti-myc-tag) and FITC-labelled *Sambucus nigra* agglutinin (FITC-SNA). The cells in panels A and B were transfected with STM whilst those in C and D were transfected with DTM which encodes myc-tagged ST with the membrane spanning domain altered to that of DPPIV. The cells were fixed and permeabilized as in Figure 3A, 72 h after transfection. Untransfected COS cells contain sialic acid with α-2,3- but not α-2,6-linkages (data not shown). The punctate staining seen in panels B and D in untransfected cells is presumably from endocytosed serum proteins carrying α-2,6-sialic acid residues.

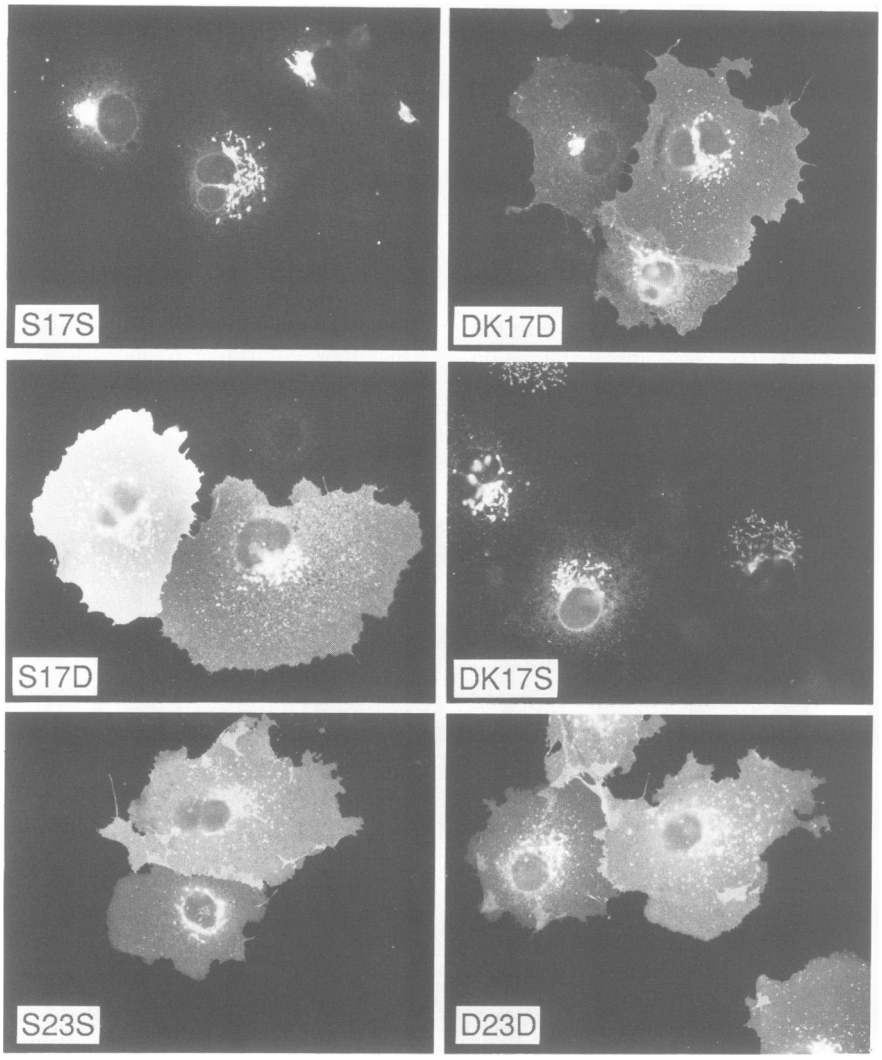

Fig. 7. Immunofluorescent localization of lysozyme fusion proteins with polyleucine transmembrane segments. Lysozyme fusion proteins in COS cells transfected with the indicated plasmids were immunofluorescently localized as Figure 3A.

transmembrane domains are thought to help hold the membrane segment in the bilayer and it is possible that these extra charges help maintain the transmembrane segment of ST at a critical height within the bilayer (reviewed in Hartman *et al.*, 1989; von Heijne and Manoil; 1990).

The transmembrane domain of ST is required for the retention of the full-length enzyme

Because all of the above experiments analysing the role of various sections of ST were performed using an artificial lysozyme fusion protein, it is important to determine if the conclusions pertain to the intact enzyme. Thus a full-length cDNA for rat α-2,6-ST was inserted in a COS cell expression vector and a peptide-tag from c-myc was attached to the C-terminus to allow detection of the protein (plasmid STM) As shown in Figure 6A the tagged ST expressed from STM accumulated in Golgi structures entirely coincident with galactosyltransferase. In a few highly expressing cells ER staining could also be observed (not shown). An altered version of ST was also made with the transmembrane section replaced with that of DPPIV (plasmid DTM) and the expression pattern of the two proteins compared by immunofluorescence. Whilst the unmodified ST showed Golgi staining, with cell surface staining never being observed, the construct with the altered transmembrane segment (DTM) often showed plasma membrane staining, especially on the higher expressing cells (Figure 6B). The Golgi staining with the altered transmembrane construct was also very bright, which may reflect binding of the enzyme to substrates in the Golgi. Indeed double labelling with *Sambucus nigra* agglutinin showed that both proteins caused the accumulation of α-2,6-linked sialic acid in the transfected cells (Shibuya *et al.*, 1987). Thus ST with the DPPIV transmembrane domain is still enzymatically active but is no longer restricted to the Golgi apparatus and instead appears on the cell surface. This confirms that the transmembrane segment of ST is an important element of the signal that retains the protein in the Golgi.

Retention by the sequences flanking the transmembrane domain

The above examination of the ST−DPPIV chimeras showed that both SSD and DSS were better retained than DSD, suggesting that the lumenal and cytoplasmic sections

contribute to the effect of the transmembrane segment, with the role of the cytoplasmic domain being possibly the provision of adjacent positive charges. However, the cytoplasmic and lumenal sections of ST do not exert a retaining effect in the context of SDS which appears on the plasma membrane like GDDD (Figure 5). The transmembrane segment of DPPIV is not only different in sequence from that of ST, but it is also different in length. Thus if both the flanking sections of ST contribute to retention, then it seems quite possible that they will not act in concert unless spaced apart the same distance as in ST which has a transmembrane section of 17 amino acids compared with the 23 of DPPIV. To test this possibility, the effects of replacing the transmembrane regions of GSSS and GDDD with 'neutral' sequences of either 17 or 23 leucines were examined. These constructs, S17S, S23S, DK17D and D23D, are shown in Figure 1C. The DPPIV cytoplasmic tail attached to the 17 leucine sequence has the extra lysines adjacent to the transmembrane domain in case these are necessary to position the shorter hydrophobic stretch in the bilayer as discussed above. By pulse labelling all the encoded proteins were found to be made glycosylated and intact in transfected COS cells (not shown).

Immunofluorescent staining of transfected COS cells expressing S23S and D23D shows bright cell surface staining with both constructs (Figure 7). However, the cells expressing S17S showed Golgi and occasional faint ER staining whilst those expressing DK17D show clear plasma membrane staining (Figure 7). Thus the poly-leucine sequence of either length is not sufficient for Golgi localization, but addition of the ST cytoplasmic and lumenal sections on either side of the 17 leucine sequence results in Golgi retention, whilst the protein with same sequences 23 amino acids apart appears on the cell surface. To investigate this further, the domains were swapped to make the chimeric constructs S17D and DK17S (Figure 1C). Figure 7 shows that, when expressed in cells, DK17S shows Golgi retention and S17D plasma membrane expression. The brightly stained perinuclear structures seen with S17S and DK17S exactly colocalize with galactosyltransferase (not shown). Taken together, these data suggest that the lumenal domain of ST contains Golgi targeting information which can cause retention if the sequence is present at the same distance from the cytoplasmic domain as it is in wild type ST.

The Golgi retained protein expressed from DK17S does not appear to be released into the medium at late times, unlike the product of GSSS (Figure 4, DK17S is marked DKLS). Interestingly, DK17S also shows less ER staining than GSSS or DKSD even at times well after transfection, and has a correspondingly more rapid accumulation of complex carbohydrate in a pulse–chase (Figure 4). However, it should be noted that the expression levels of the constructs containing the Leu_{17} stretch are typically less than those of the other fusions—e.g. compare the pulse labelled lane in Figure 4 for DK17S with that for GSSS and GDDD. The reasons for this are unclear but it may allow the lumenal domain to show better retention than would be seen at a higher expression level. Even so the difference between the products of DK17S and DK17D clearly demonstrates that the lumenal section of ST can contribute to the Golgi localization of the protein although this effect is only seen if the cytoplasmic and lumenal domains are spaced as in the wild type protein.

Discussion

The amino-terminal 44 residues of ST prevent an attached protein reaching the cell surface

This paper reports that the amino-terminal signal–anchor region of a Golgi enzyme, α-2,6-sialyltransferase, is sufficient to cause the Golgi localization of a fusion protein. This is in contrast with a fusion to an analogous portion of a plasma membrane protein, which instead accumulates on the cell surface. Attempts to map the localization sequence in greater detail showed that the transmembrane domain contains targeting information, although its action is augmented by the sequences flanking it. Furthermore, altering the transmembrane region of the full-length transferase results in it appearing on the cell surface. In parallel to these studies, a similar series of experiments has been carried out by another group on a different Golgi transferase, β-1,4-galactosyltransferase (Nilsson *et al.*, 1991). They find that the transmembrane region of the enzyme is capable of conferring accumulation of a fusion protein in the *trans*-Golgi but complete retention is dependent on the sequences adjacent to the transmembrane domain—in this case the cytoplasmic tail. Initial experiments on coronavirus E1 glycoprotein have suggested that the first of three transmembrane segments is involved in the retention of the protein from at least one strain of virus (Machamer and Rose, 1987). Recent experiments have shown that this transmembrane domain is capable of retaining a fused protein in the Golgi although the different results obtained with E1 glycoproteins from different coronaviruses have yet to be resolved (Swift and Machamer 1991; Armstrong *et al.*, 1991). There are no obvious homologies between any region, transmembrane or otherwise, of the several Golgi transferases cloned. Perhaps the most striking feature of the transmembrane region of ST is that it has four phenylalanine residues arranged such that they would line up along one side of an α-helix. Interestingly, the first TM segment of the IBV Coronavirus E1 contains one tyrosine and three phenylalanines arranged in a similar manner although this membrane spanning helix is arranged in the opposite direction across the bilayer to that of ST.

If retention of a membrane protein involves recognition of sequences within its transmembrane domain, it is perhaps not surprising that this interaction can also involve the contiguous flanking sequences. If a transmembrane segment such as that of ST were to interact with that of another transmembrane protein, also arranged perpendicularly to the bilayer, then the sequences adjacent to the transmembrane segments would probably be in contact as well. In the case described here these flanking interactions appear to be sufficiently significant that if the lumenal and cytoplasmic domains of ST are separated by a stretch of leucines of the same length as that of the normal ST transmembrane domain, then the protein is still retained in the Golgi.

Location of the retained protein within the cell

The ST-derived fusion proteins which do not reach the cell surface clearly accumulate in the Golgi apparatus as judged by immunofluorescent colocalization with galactosyltransferase. Immuno-electron microscopic localization of α-2,6 ST in rat hepatocytes and intestinal goblet cells has revealed the enzyme and its product to be located to the *trans*-cisternae and the TGN (Roth *et al.*,1985; Taatjes *et al.*, 1988). This is consistent with cell fractionation studies and with the

requirement for the enzyme in the latter part of the pathway of carbohydrate modification (reviewed in Kornfeld and Kornfeld, 1985; Roth, 1987). However, in intestinal absorptive cells the enzyme is also found in more medial cisternae (Roth *et al.*, 1986; Taatjes *et al.*, 1988) and in α-2,6-ST expressing CHO cells, α-2,6 linked sialic acid is found throughout the Golgi stacks suggesting either a broader distribution of the enzyme or mobility within the Golgi of one or more of its substrates (Lee *et al.*, 1989).

In this study both the Golgi-retained and cell surface lysozyme fusions acquire complex carbohydrate side chains whose considerable size suggests that they are likely to be poly-*N*-acetyl lactosamine and recent studies have shown that a lectin specific for this structure, *D.stramonium* agglutinin, can bind to the chains (data not shown). It has previously been suggested that poly-*N*-acetyl lactosamine is added to glycosylation sites held close to the lipid bilayer as is the case with these constructs (Fukuda *et al.*, 1988). However, it has recently been reported that when a glycosylation site is inserted into the human lysozyme gene the resulting protein also acquires poly-*N*-acetyl lactosamine, suggesting that this addition may be due to some property of the lysozyme part of the fusions (Horst *et al.*, 1991). Addition of poly-*N*-acetyl lactosamine is thought to occur late in the Golgi after the action of GlcNAc-transferase V (van den Eijnden *et al.*, 1988). The elongation of the chains requires two enzymes, GlcNac-transferase (i) and a β-1,4-galactosyltransferase (van den Eijnden *et al.*, 1983; Basu and Basu, 1984). The former enzyme has not been localized but the latter enzyme is in the *trans* cisternae and the TGN (reviewed in Strous, 1986). Furthermore, the polylactosamine product of these two enzymes has been localized to the *trans* cisterae and TGN by lectin staining (Egea *et al.*, 1989; Roth and Goldstein, 1989). This would be consistent with the fusions accumulating at the *trans* side of the Golgi. In order to determine the precise location of the fusion proteins in COS cells, immuno-electron microscopic localization is currently being attempted.

Accumulation of the Golgi-retained fusions in the ER and release into the medium

The fusion proteins containing the ST transmembrane segment showed considerable staining of the endoplasmic reticulum, especially at late times following transfection. This was also seen with cells expressing high levels of the full-length ST and with HeLa cells expressing the β-1,4-galactosyltransferase fusions analysed by Nilsson *et al.* (1991). Similarly, at late times in coronavirus infection, the E1 glycoprotein accumlates in the ER as well as the Golgi, and virus budding can be seen into both structures (Tooze *et al.*, 1984). Replicating COS cell vectors of the sort used in this paper produce extremely high levels of the encoded protein, often in excess of 10^6 molecules per cell (Simmons and Seed, 1988). However, the plasma membrane DPPIV fusions do not show high levels of ER staining and pulse–chase analysis suggests that they rapidly acquire complex sugars (Figure 6B). This suggests that Golgi retained proteins may have the general property of backing up into the ER, rather than progressing beyond the Golgi, when overexpressed. It is possible that this is caused by the retention mechanism that normally holds them in the Golgi now operating in the ER.

Another unexpected observation was that the fusions containing the ST transmembrane segment were slowly released into the medium—again more obviously at late times following transfection. The ST transmembrane segment appears to contain residues which are involved in Golgi retention and it is possible that when free they are recognized by a system of retention and degradation in either the Golgi or the ER, analogous to that proposed to recognize the unassembled subunits of the T-cell receptor in the ER (Bonifacino *et al.*, 1990, Wileman *et al.*, 1990). However, it is known that ST is released from cells *in vivo* by proteolytic cleavage with the amino-terminus of the cleavage product being residue 64 (Weinstein *et al.*, 1987). Although the ST constructs used here contain at most the first 44 residues of ST and so do not include this cleavage site, the mechanism of cleavage is unknown and so it is possible that the release seen here is in some way related to the process which occurs *in vivo*.

How might Golgi enzymes be retained?

Retention of ST apparently involves recognition of the membrane spanning domain and the sequences adjacent to it. As discussed above, the extent of the retention signal suggests that the Golgi enzyme is recognized by another protein arranged perpendicular to the bilayer. Any model for Golgi retention must provide for the Golgi enzymes to have access to their substrates, account for the ability of Golgi proteins to leave the endoplasmic reticulum after synthesis, and account for the rapid and reversible return of Golgi enzymes to the ER in the presence of brefeldin A. Various models to explain the retention of Golgi enzymes have been suggested (reviewed in Farquar, 1985 and Pfeffer and Rothman, 1987). These include the binding of the Golgi enzymes to a structural framework in the Golgi or alternatively the formation of small aggregates of the enzymes, triggered by the lipid or ionic environment of the Golgi, which cannot enter transport vesicles. In the latter case the protein recognising ST would be ST itself, possibly in conjunction with other Golgi enzymes. It is also possible that instead of being immobilized, the Golgi proteins are transport competent but recycle back through the Golgi. A system of recycling of proteins back from the Golgi to the ER has been demonstrated for the retention of soluble ER proteins (Munro and Pelham, 1987; Dean and Pelham, 1990). There is some evidence to suggest that sugar structures are found further back in the Golgi than the enzymes that produce them (Lucocq *et al.*, 1987; Yuan *et al.*, 1987; Gonatas *et al.*, 1989, Lee *et al.*, 1989). However, in few studies has the location of the enzyme and its product been determined in the same cell type (reviewed in Roth, 1987).

At present sufficiently little is known about the Golgi to allow all of the above models to be adapted to fit the known requirements. Certainly it is possible to imagine that recognition in the plane of the bilayer could be involved in aggregation, in binding to an as yet unidentified matrix, or in assembly into vesicles. However, by using the fusion proteins described here it should be possible to look for specific interactions by cross-linking and immunoprecipitation as well as assess the effect on retention of treatments which perturb the internal environment of the Golgi.

Materials and methods

Plasmid construction

All the lysozyme fusion protein expressing plasmids used in this study were derived using standard techniques (Ausubel *et al.*, 1987; Sambrook *et al.*,

1989) from the previously described SAY1 which contains a cDNA for chicken lysozyme under the control of the adenovirus major late promoter as well as an SV40 replication origin (Munro and Pelham, 1987). SAY1 also contains a *Hin*dIII site at the 5′ end of the lysozyme cDNA, and the polymerase chain reaction (PCR) was used to replace the sequence between this site and the first codon of mature lysozyme with two different sequences. In plasmid U16 this sequence ends with a *Bst*BI site next to the first codon of mature lysozyme (TTCGAAA, where the last three bases comprise the first, lysine, codon). In plasmid GNK1 the sequence ends TTCGAAAAACGTGACCGGCTCGAAA, again, the last three bases being the first codon of the mature lysozyme. The sequence between the *Bst*BI site and the AAA codon encodes a potential site for N-linked glycosylation.

Lysozyme fusions were then constructed by inserting synthetic oligonucleotides between the *Hin*dIII site and the *Bst*BI site of either U16 or GNK1. Pairs of complementary oligonucleotides were synthesized to encode either the cytoplasmic, transmembrane or lumenal sections of ST and of DPPIV or the leader peptide of lysozyme. The coding sequences used were those of the original genes (Weinstein *et al.*, 1987; Hong and Doyle, 1987; Jung *et al.*, 1980) with 13 bp of 5′-untranslated region from ST added before the initiator methionine codon in the oligonucleotides encoding the ST and DPPIV cytoplasmic domains and the lysozyme leader peptide. All oligonucleotide pairs had overhanging ends with *Hin*dIII and *Bst*BI compatible ends at the outside of the sets and the internal junctions formed with AAG overhangs (5′ and 3′ respectively on the central transmembrane segment) corresponding to the lysine codons at either end of both ST and DPPIV transmembrane segments (see figure 1) . Thus the ST oligo set was cloned into U16 and GNK1 to make USSS and GSSS respectively. UDDD and GDDD were constructed using the DPPIV set in U16 and GNK1 respectively. To make LPS and LPD the oligos encoding the lumenal sections of ST and DPPIV respectively were ligated with those encoding the lysozyme leader peptide into GNK1.

The ST and DPPIV chimeric constructs were made by ligating the two sets of three oligo pairs in all possible combinations into GNK1. Plasmids DKSD and DKSS were made using an ST transmembrane encoding pair of oligos with two extra lysine codons at the 5′ end. Plasmids S17S , S17D, DK17S, DK17D, S23S and D23D were made using lumenal and cytoplasmic oligo pairs as above plus an oligo pair encoding either L_{17} or KKL_{17} or L_{23} with L_{17} encoded by CTCCTCCTGCTGTTGCTTTTGCTCCTGCT-CCTGCTTCTTCTGCTGCTCCTC and L_{23} encoded by the same sequence followed by TTGCTACTGCTTCTCCTC.

A full-length cDNA for rat α-2,6-sialyltransferase was amplified by PCR from cDNA reversed transcribed from total RNA isolated from Rat2 cells (provided by T.H.Rabbitts). Sequencing of the cDNA confirmed that no PCR mutations had been introduced. Amino acid 367 of ST is shown as a histidine in the published sequence and consistent with this a CAC codon was found in this position in three independent PCR products (Weinstein *et al.*, 1987). However, the published nucleotide sequence of this codon is GAC, suggesting that the nucleotide sequence in the original figure contains a typographical error. *Hin*dIII and *Mae*I sites at the ends of the PCR primers were used to insert the ST cDNA between the *Hin*dIII and *Nco*I sites of SAGM2 (Munro and Pelham, 1987). This COS cell expression vector is related to SAY1 also having an adenovirus promoter followed by a unique *Hin*dIII site. The fusion of the *Mae*I site to the *Nco*I site (both filled in with Klenow) attaches sequences encoding a c-myc derived peptide tag to the exact C-terminus of the ST coding region. The C-terminal amino acids encoded by the resulting fusion are ...IRCYMEQKLISEEDLN where IRC is the original ST C-terminus. The *Hin*dIII site and an *Eco*RI site in the sequences encoding the lumenal section of ST, were used to insert *Hin*dIII–*Eco*RI fragments from GSSS or GSDS to produce plasmids encoding ST with either its own transmembrane segment (STM) or that of DPPIV (DTM).

Antibodies

Rabbit antisera raised against chicken lysozyme and human β-1,4-galactosyltransferase were generously given by Sally Ward and Eric Berger respectively. F10 is a mouse monoclonal which recognizes chicken lysozyme (R.Poljiak, personal communication). 9E10 is a mouse monoclonal which recognizes a peptide epitope from human c-myc (Munro and Pelham, 1987). Fluorescein- and Texas Red-conjugated species-specific antisera were from Amersham.

Transfection of cells and analysis of [^{35}S]Met-labelled proteins

COS cells were transfected with DEAE–dextran/chloroquine as described (Munro and Pelham, 1987). For metabolic labelling, cells in 9.4 cm^2 wells were rinsed in PBS and then labelled in methionine-free DMEM containing 200 μCi/ml [^{35}S]Met (NEN) for 20 min. The cells were then either rinsed in ice-cold PBS and lysed immediately or lysed following a chase in complete medium. Lysis was by scraping into 400 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, 1 mM PMSF, 2 μg/ml leupeptin, 2 mM benzamidine). Lysozyme fusion proteins were immunoprecipitated at 4°C from the post-nuclear supernatant or from the medium by addition of rabbit anti-lysozyme sera followed by incubation with protein A–Sepharose for 2 h. The beads where then washed twice with lysis buffer and twice with RIPA buffer and the precipitated proteins were eluted with SDS sample buffer, separated by SDS–PAGE and fluorographed using Amplify (Amersham). For digestion with endoglycosidases (endo) H or F (Boehringer Mannheim), the precipitated proteins were eluted with 1% SDS and diluted 5-fold into 50 mM sodium acetate, pH 5.2, 1% NP40, 0.2 mg/ml BSA, 0.5% mercaptoethanol, 1 mM PMSF and then incubated overnight at 37°C with either 1 mU endo H or 0.2 U endo F or with no further addition. Following acetone precipitation the proteins were resuspended in SDS sample buffer and analysed as above.

Immunofluorescence

Transfected COS cells were trypsinized 12–24 h post-transfection and transfered to 4- or 8-well slides [C.A Hendley (Essex) Ltd]. Fixation with 2% paraformaldehyde/0.1% glutaraldehyde, immunofluorescence and mounting were as previously described (Munro and Pelham, 1987). Methanol/acetone fixation was for 5 min/45 s respectively at −20°C. For lectin labelling the cells were blocked with Blocking Reagent (Boehringer Mannheim) and treated with primary and secondary antibody in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$. 10 μg/ml fluorescein-conjugated *S.nigra* agglutinin (Vector Labs) was included with the secondary antibody. After washing the cells were mounted in PBS and photographed immediately. All photography was with XP1 film.

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