The EMBO Journal vol.13 no.3 pp.562-574, 1994

# Kin recognition between *medial* Golgi enzymes in HeLa cells

**Tommy Nilsson, Mee H.Hoe, Paul Slusarewicz, Catherine Rabouille, Rose Watson, Felicia Hunte, Gabriele Watzele[1], Eric G.Berger[1] and Graham Warren[2]**

Cell Biology Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, UK and [1]Physiologisches Institut, Universität Zürich, CH-8057 Zürich, Switzerland
[2]Corresponding author

Communicated by G.Warren

**The *medial* Golgi enzymes, *N*-acetylglucosaminyltransferase I (NAGT I) and mannosidase II (Mann II), and the *trans* Golgi enzyme, β-1,4-galactosyltransferase (GalT) were each retained in the endoplasmic reticulum (ER) by grafting on the cytoplasmic tail of the p33 invariant chain. Transient and stable expression of p33/NAGT I in HeLa cells caused relocation of endogenous Mann II to the ER and transient expression of p33/Mann II had a similar effect on endogenous NAGT I. Neither of these endogenous *medial* enzymes were affected by transient expression of p33/GalT. These data provide strong evidence for kin recognition between *medial* Golgi enzymes and suggest a role for them in the organization of the Golgi stack.**

*Key words:* *N*-acetylglucosaminyltransferase I/endoplasmic reticulum/β-1,4-galactosyltransferase/Golgi/mannosidase II

## Introduction

Resident proteins of the secretory pathway contain information in their primary sequence which determines their precise location. One type of information is contained within a short stretch of amino acids at the C-terminus of both soluble and membrane proteins of the endoplasmic reticulum (ER) (Munro and Pelham, 1987; Pääbo *et al.*, 1987). Signals of this kind, when transplanted onto reporter molecules, are sufficient to locate them in the ER (Munro and Pelham, 1987; Nilsson *et al.*, 1989; Jackson *et al.*, 1990). However, the reporter molecules acquire modifications to their bound oligosaccharides showing that they have been exposed to Golgi enzymes (Pelham, 1989; Dean and Pelham, 1990; Jackson *et al.*, 1993). This argues strongly that they have left the ER and been retrieved from the Golgi apparatus through a retrograde pathway (for reviews see Pelham, 1989; Klausner *et al.*, 1992). Isolation of the receptor for soluble ER proteins (Lewis and Pelham, 1990) confirms retrieval from the Golgi apparatus (Lewis and Pelham, 1992) and identifies this type of signal as one that operates only once a protein has been lost from the compartment in which it normally functions.

It is, however, difficult to imagine a retention mechanism that operates entirely by salvaging lost proteins. In fact, careful examination of the oligosaccharides of ER membrane proteins has shown that they do not leave the ER (Brands *et al.*, 1985; Yamamoto *et al.*, 1985). Furthermore, removal of the retrieval signal from soluble (Munro and Pelham, 1987) and membrane (Jackson *et al.*, 1993) proteins of the ER causes them to be transported only very slowly, strongly suggesting that there must be additional retention information elsewhere in the protein.

A clue to the location of such information comes from work on the retention of membrane proteins in the Golgi apparatus. When the membrane-spanning domain of a reporter molecule is replaced by that from a Golgi protein, the hybrid molecule not only resides in the Golgi apparatus (Munro, 1991; Nilsson *et al.*, 1991; Swift and Machamer, 1991; Aoki *et al.*, 1992; Burke *et al.*, 1992; Colley *et al.*, 1992; Russo *et al.*, 1992; Tang *et al.*, 1992; Teasdale *et al.*, 1992; Wong *et al.*, 1992) but also in the correct cisternae (Nilsson *et al.*, 1991; Burke *et al.*, 1992).

The question then arises as to the mechanism by which a membrane-spanning domain could cause retention in the correct Golgi cisterna and perhaps elsewhere along the secretory pathway. The simplest model is to suggest that Golgi enzymes interact with their neighbours through their membrane-spanning domains forming oligomers so large that they cannot enter the vesicles budding from the dilated cisternal rims (Nilsson *et al.*, 1991, 1993b). The density of membrane proteins throughout the secretory pathway ranges from 30 000 to 42 000 molecules/$\mu m^2$ (Quinn *et al.*, 1984), which means that each transport vesicle (70 nm diameter; Orci *et al.*, 1986) could accommodate between 450 and 650 proteins. The oligomers would have to be much larger than this. There are many glycosyltransferases and glycosidases in the Golgi apparatus, but calculation from the purification tables for Golgi membranes (Leelavathi *et al.*, 1970) and *medial* Golgi enzymes (Nishikawa *et al.*, 1988; Moremen *et al.*, 1991) shows that none constitute more than a few per cent of the cisterna(e) in which they reside. This, taken together with qualitative immunocytochemical studies suggesting that these enzymes are evenly distributed throughout the cisterna(e) (Burke *et al.*, 1982; Dunphy *et al.*, 1985; Nilsson *et al.*, 1993a), argues that the oligomers must comprise a mixture of different enzymes. We term such structures kin oligomers.

In this study we tested two critical features of this model. We focused on the *medial* Golgi enzymes, *N*-acetylglucosaminyltransferase I (NAGT I) and mannosidase II (Mann II) because there is considerable biochemical data (Dunphy *et al.*, 1981; Dunphy and Rothman, 1983; Goldberg and Kornfeld, 1983; Balch and Keller, 1986) suggesting that they reside in the same cisternae in the stack. For comparison we used the *trans* Golgi enzyme, β-1,4-galactosyltransferase (GalT) (Roth and Berger, 1982). By attaching an ER retention signal to each of these proteins, thereby retaining each, in turn, in the ER, we showed that

there is an interaction between NAGT I and Mann II, that this interaction is specific and that it involves the membrane-spanning domain and perhaps part of the stalk region.

## Results

### *Retention of p33 hybrid proteins in the ER*

In contrast to the human invariant chain (Ii), p31, which chaperones major histocompatibility class II antigens to the endocytic pathway (Bakke and Dobberstein, 1990; Lotteau *et al.*, 1990), Iip33 resides in the ER (Lotteau *et al.*, 1990). Translation of Iip33 is initiated 16 amino acids upstream of the more predominantly used start codon for Iip31 (Strubin *et al.*, 1984), so this additional, cytoplasmic sequence must be responsible for ER retention. By grafting the cytoplasmic tail of Iip33 onto resident Golgi enzymes we hoped to retain the hybrid proteins in the ER.

cDNAs encoding human NAGT I, murine Mann II and human GalT were modified as described in Materials and methods and the predicted sequence and topology of the hybrid proteins are depicted in Figure 1. Each chimeric cDNA was transferred into the eukaryotic expression vector pCMUIV (Nilsson *et al.*, 1989) and transfected into HeLa cells. After 72 h the cells were either metabolically labelled with [$^{35}$S]methionine or prepared for immunofluorescence or immunoelectron microscopy.

Metabolically labelled cells were chased for up to 2 h, solubilized with NP40 and then incubated with the appropriate antibody. p33/NAGT I was tagged with a myc epitope at the C-terminus (Figure 1), so was immunoprecipitated using the mAb 9E10 (Evan *et al.*, 1985). Both p33/Mann II and p33/GalT were immunoprecipitated using polyclonal antibodies specific for the catalytic (lumenal) domain (Berger *et al.*, 1986; Moremen *et al.*, 1991). The immunoprecipitates were fractionated by SDS−PAGE.

As shown in Figure 2A (left panel), the p33/NAGT I hybrid protein had an apparent molecular weight of 54 000 which did not change during the chase period and was not affected by treatment with neuraminidase, indicating the absence of sialic acid residues. A slightly smaller minor form of the protein was also seen. For comparison, a stable cell line expressing a myc-tagged form of NAGT I (NAGT I-HeLa) (Nilsson *et al.*, 1993a) was also pulsed and chased (Figure 2A, right panel). Immediately after the pulse there were two forms of the protein with apparent molecular weights of 50 000 and 54 000. The lower form could be chased into the upper form and all were sensitive to treatment with neuraminidase. There are no N-linked glycosylation sites in the primary sequence of human NAGT I (Kumar *et al.*, 1990) so the addition of sialic acid must be to O-linked oligosaccharides. Because this addition occurs prior to the *trans* Golgi network (TGN) (Locker *et al.*, 1992) and NAGT I is present in the *medial* and *trans* cisternae of the Golgi stack (Nilsson *et al.*, 1993a), we can conclude that p33/NAGT I did not reach the *medial* Golgi cisterna. A slightly larger form of p33/NAGT I was observed after 2 h of chase (arrow in Figure 2A, left panel) which probably represents the addition of *N*-acetylgalactosamine. This is the first step in the construction of O-linked oligosaccharides and occurs in a late ER compartment (Pathak *et al.*, 1988; Tooze *et al.*, 1988).

Metabolically labelled p33/Mann II had an apparent molecular weight of 120 000 which did not change during the chase period (Figure 2B, left panel). Immediately after

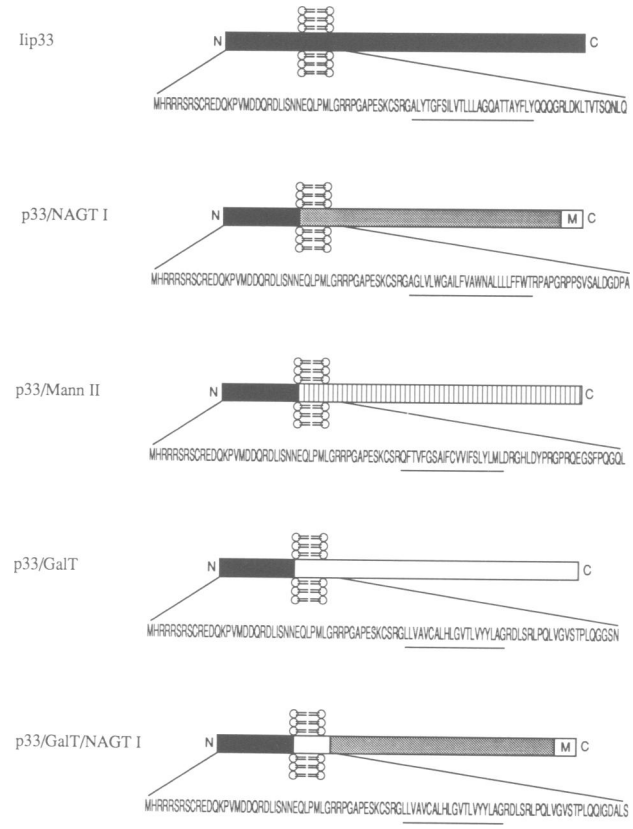


**Fig. 1.** Iip33 and p33/hybrid proteins. The cytoplasmic tail of Iip33 was fused to the membrane-spanning and lumenal domains of NAGT I, Mann II and GalT. The N-terminal sequences are shown. Note the myc epitope (M) at the C-terminus of p33/NAGT I and p33/GalT/NAGT I used to detect the protein.

labelling, the protein was sensitive to endoglycosidase H (Endo H) but less so with increasing chase time. However, even after 2 h of chase, the protein was not fully resistant to Endo H. This was in marked contrast to murine Mann II expressed stably in a HeLa cell line (Mann II-HeLa, see Materials and methods). As shown in Figure 2B (right panel), Mann II became resistant to Endo H with a half time of ~1 h, consistent with earlier studies on the biosynthesis of Mann II in 3T3 cells (Moremen and Touster, 1985). Had the p33/Mann II reached the *medial* cisternae we would have expected complete resistance to Endo H (Tarentino and Maley, 1974; Dunphy *et al.*, 1985). The simplest explanation is that the oligosaccharides are being modified by some of the Golgi enzymes that appear in the ER as a result of expressing this hybrid protein (see Discussion).

The antibody used for these experiments has been shown to cross-react with human Mann II but did not do so under the conditions used here. Human Mann II would be predicted to migrate at a position corresponding to the apparent molecular weight of 134 000−136 000 following 2 h chase (Moremen and Touster, 1985), and would easily have been observed and distinguished from murine Mann II.

p33/GalT had an apparent molecular weight of 50 000 throughout the chase period and remained sensitive to Endo H (Figure 2C, left panel). A proteolytic fragment of ~33 000 apparent molecular weight was also observed which was most likely a soluble fragment of the lumenal domain. The sensitivity of p33/GalT to Endo H was in marked contrast to endogenous GalT in untransfected HeLa

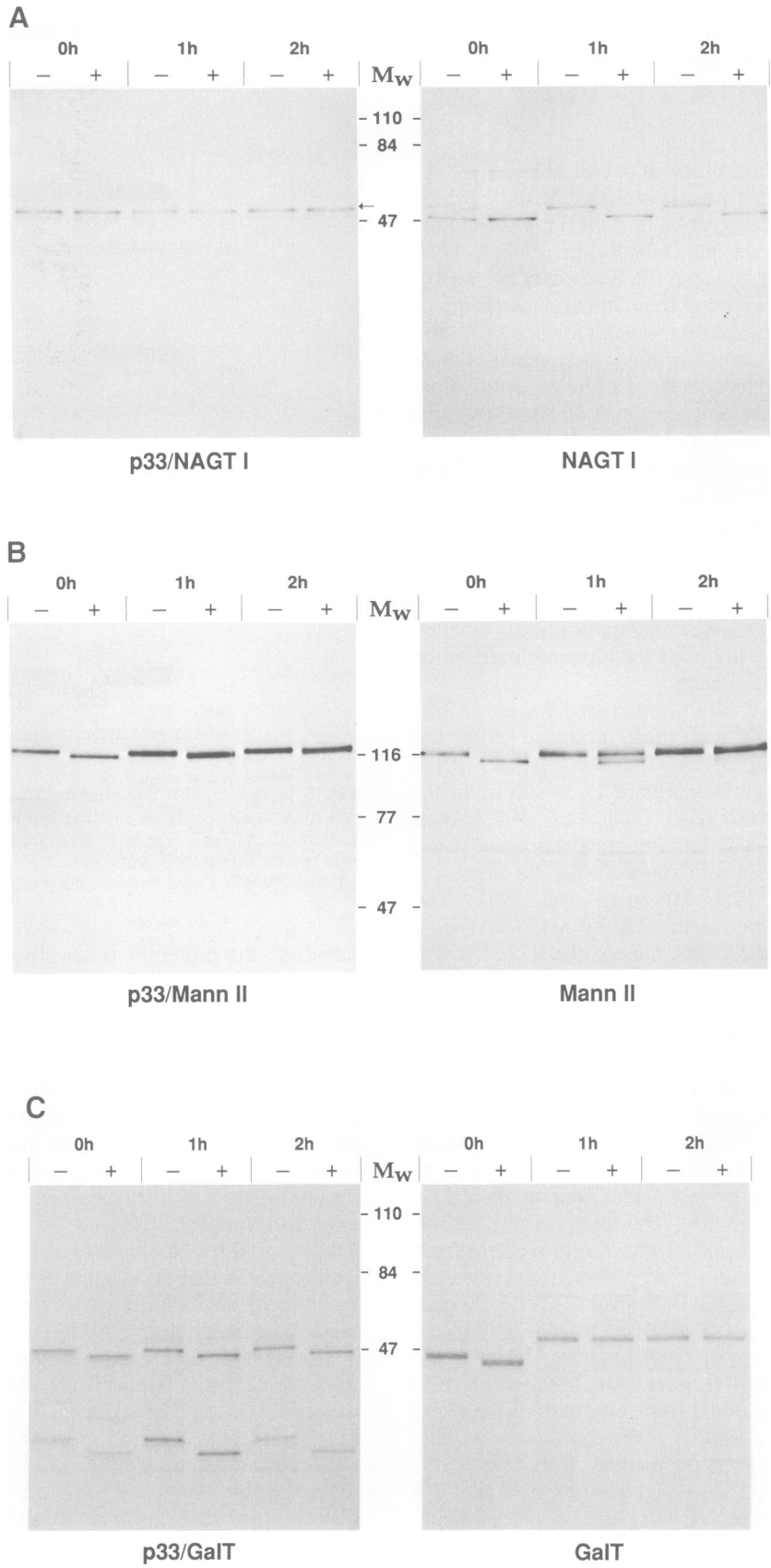


**Fig. 2.** Pulse–chase labelling of Golgi enzymes and p33/hybrid proteins. Cells were incubated with [$^{35}$S]methionine for either 20 min (**C**) or 1 h (**A** and **B**), and chased for up to 2 h before lysis and immunoprecipitation. Immunoprecipitated material was treated with or without either neuraminidase (A) or Endo H (B and C) and subsequently fractionated by SDS–PAGE. (A) p33/NAGT I from transfected HeLa cells and NAGT I from NAGT I-HeLa cells. (B) p33/Mann II from transfected HeLa cells and endogenous Mann II from HeLa cells. (C) p33/GalT from transfected HeLa cells and endogenous GalT from HeLa cells. The molecular weights are shown in kDa.

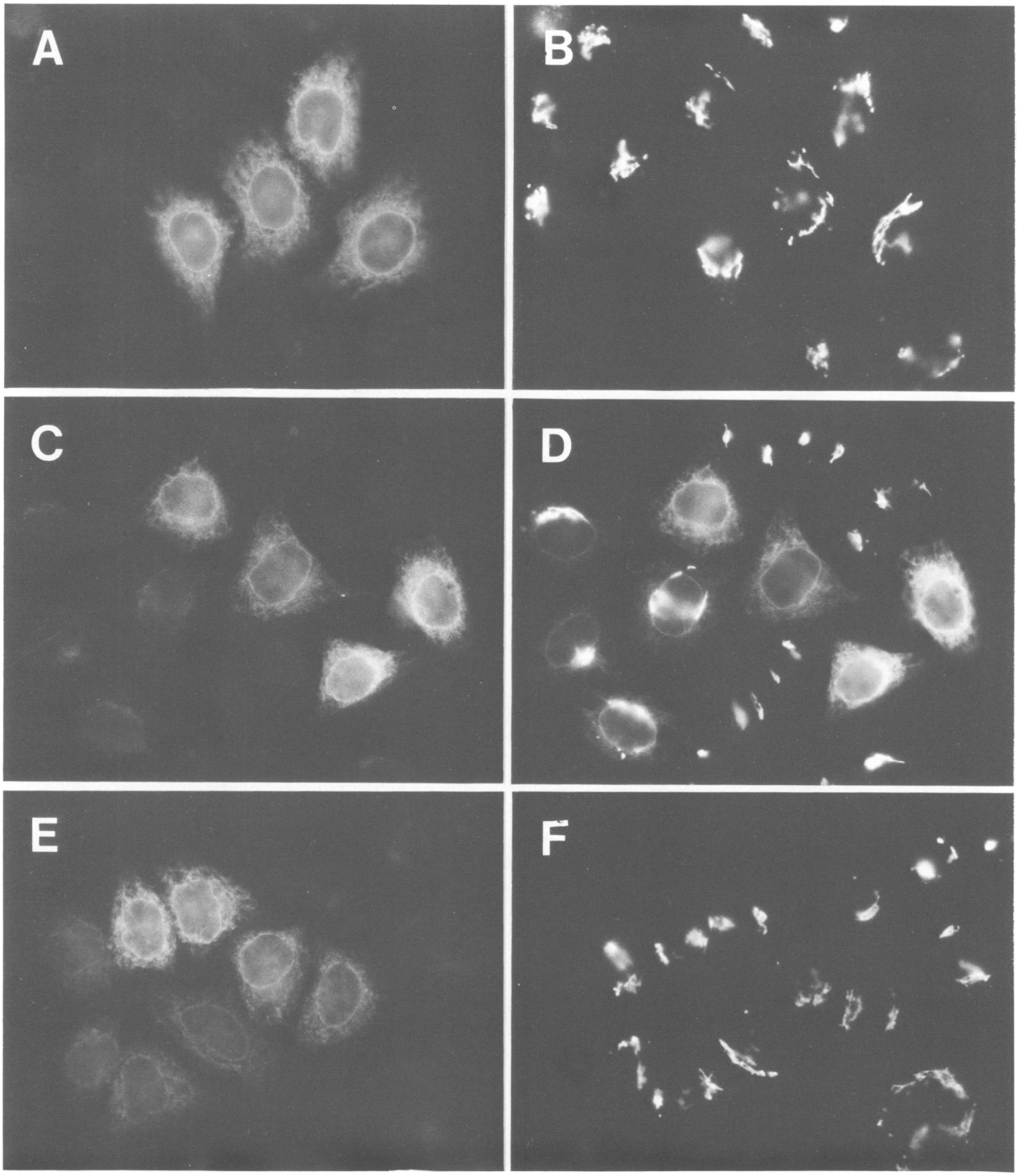


**Fig. 3.** Immunofluorescence microscopy of Mann II-HeLa cells transfected with cDNAs encoding Iip33, p33/NAGT I and p33/Gal T. Mann II-HeLa cells transfected with cDNAs encoding Iip33 (**A** and **B**), p33/NAGT I (**C** and **D**) and p33/GalT (**E** and **F**) were fixed after 72 h and double labelled with rabbit polyclonal antibody to Mann II (B, D and F) and mouse mAbs to either Iip33 (A), p33/NAGT I (C) or p33/GalT (E) followed by secondary antibodies coupled to FITC (B, D and F) and Texas Red (A, C and E). Magnification: ×700.

cells (Figure 2C, right panel). At 0 chase time, a doublet with the expected molecular weights of 45 000 and 47 000 was observed (Strous and Berger, 1982). After 1 h chase, a doublet with the molecular weight of 52 000 appeared which was completely resistant to Endo H.

Having shown that the p33 hybrid proteins did not reach the *medial* Golgi, their location was narrowed down further by microscopic studies. Iip33 alone (Figures 3A and 7A), p33/NAGT I (Figure 3C), p33/Mann II (Figure 7C) and p33/GalT (Figures 3E and 7E) gave the same immunofluorescence staining pattern which had all the hallmarks of the ER. The nuclear envelope was labelled as well as associated cisternal elements which were continuous with a tubulo-reticular network pervading the entire cell cytoplasm. Immunoelectron microscopy provided the final confirmation. As an example, Mann II-HeLa cells transfected

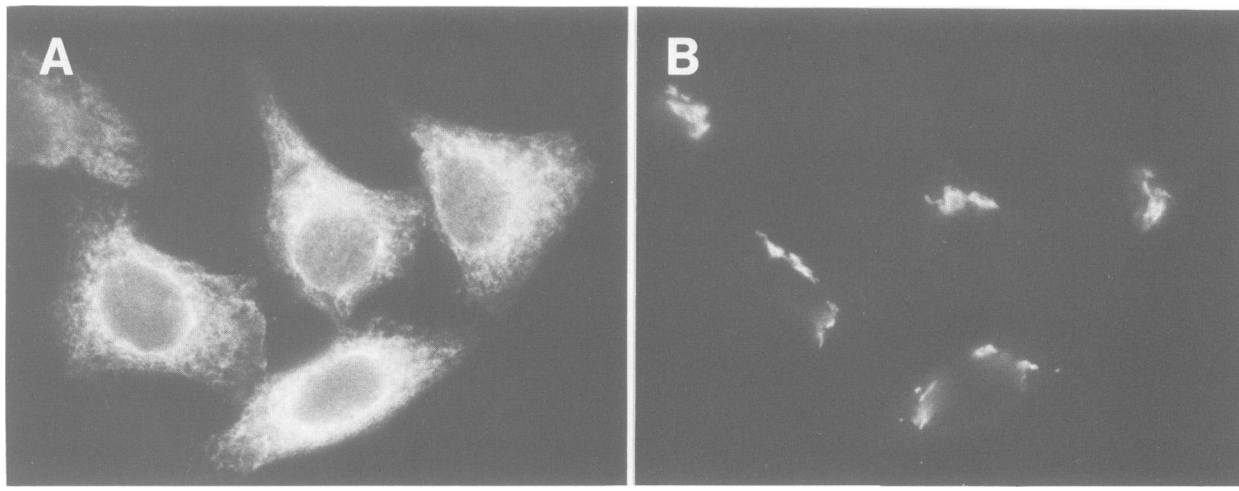


**Fig. 4.** Immunofluorescence microscopy of Mann II-HeLa cells transfected with cDNA encoding p33/GalT/NAGT I. Mann II-HeLa cells transfected with cDNA encoding p33/GalT/NAGT I were fixed after 72 h and double labelled with rabbit polyclonal antibody to Mann II (**B**) and mouse mAbs to the myc-tagged p33/GalT/NAGT I (**A**), followed by secondary antibodies coupled to FITC (B) and Texas Red (A). Magnification: ×850.

with p33/NAGT I were fixed and cryo-sections labelled with anti-myc antibodies followed by secondary antibodies coupled to gold. As shown in Figure 5B and C, labelling for p33/NAGT I was almost entirely restricted to the nuclear envelope and peripheral ER membranes. Little, if any, labelling was observed over Golgi cisternae.

### *Kin recognition*

Having established the location of each of the p33 hybrid proteins, we looked next at their effect on the distribution of Golgi enzymes that were not modified by the p33 cytoplasmic tail.

Mann II-HeLa cells were transfected with either Iip33, p33/NAGT I or p33/GalT (Figure 3). Iip33 provided a control for the transfection procedure. The presence of this protein in the ER (Figure 3A) had no effect on the distribution of Mann II (Figure 3B) which was restricted to a compact juxta-nuclear reticulum, characteristic of the Golgi apparatus in many cultured cell lines (Louvard *et al.*, 1982).

The distribution of Mann II (Figure 3F) was also unaffected by p33/GalT (Figure 3E), but p33/NAGT I (Figure 3C) caused it to appear in the ER (Figure 3D). This effect depended on the level of expression. At relatively low levels of p33/NAGT I some of the Mann II appeared in the ER, but a substantial portion was still found in a juxta-nuclear reticulum. At high levels essentially all of the Mann II codistributed with the p33/NAGT I.

This effect was not the consequence of an interaction between the catalytic domains of Mann II and NAGT I. When the membrane-spanning domain and part of the stalk region of p33/NAGT I were replaced by those of GalT, the resulting construct, p33/GalT/NAGT I (see Figure 1), was present in the ER (Figure 4A) but had no effect on the distribution of Mann II (Figure 4B). This strongly suggests that the observed interaction between p33/NAGT I and Mann II is mediated through the membrane-spanning domain and perhaps part of the stalk region.

The codistribution of Mann II and p33/NAGT I was confirmed by immunoelectron microscopy in the Mann II-HeLa cell line. In untransfected cells, labelling for Mann II could be detected in one or two Golgi cisternae, most likely representing the *medial* and perhaps *trans* cisternae (Figure 5A). In transfected cells, p33/NAGT I was found only in the nuclear envelope and the peripheral ER (Figure 5B). Labelling for Mann II was mostly absent from the Golgi stack but present in the ER colocalizing with p33/NAGT I. The identity of the ER was confirmed using an mAb, 1D3 (Vaux *et al.*, 1990), against protein disulfide isomerase (PDI), a resident ER protein. Separate double labelling experiments showed that this protein colocalized with both Mann II and p33/NAGT I (data not shown). As the expression level of p33/NAGT I increased the Golgi first appeared to become smaller (compare Figure 5A and B), and at even higher levels they disappeared altogether (Figure 5C). At high levels of expression, Mann II colocalized completely with p33/NAGT I, and both were found in extensive tubulo-vesicular networks surrounding the nucleus.

This effect on Golgi structure was quantitated as shown in Figure 6. Increased levels of p33/NAGT I led to the complete disappearance of Golgi stacks as measured by the width of Golgi stacks in transverse sections. In marked contrast, increasing expression of p33/GalT/NAGT I had very little effect on Golgi structure, showing that the disappearance of the Golgi stacks was related to the relocation of Mann II to the ER.

A reciprocal set of experiments was also carried out transfecting NAGT I-HeLa cells with either Iip33 (Figure 7A), p33/Mann II (Figure 7C) or p33/GalT (Figure 7E). The distribution of NAGT I in the Golgi apparatus was unaffected by either Iip33 (Figure 7B) or p33/GalT (Figure 7F), but was changed markedly by p33/Mann II (Figure 7D). NAGT I codistributed with p33/Mann II in the ER but some of the NAGT I often remained in a juxta-nuclear structure except at the highest levels of expression.

### *Stable expression of p33/NAGT I*

Having established an interaction between *medial* Golgi enzymes by immunofluorescence and immunoelectron microscopy, we wanted to obtain complementary biochemical data. Transiently transfected cell lines are unsuitable because not all cells are transfected and those that are express varying amounts of protein. Therefore we

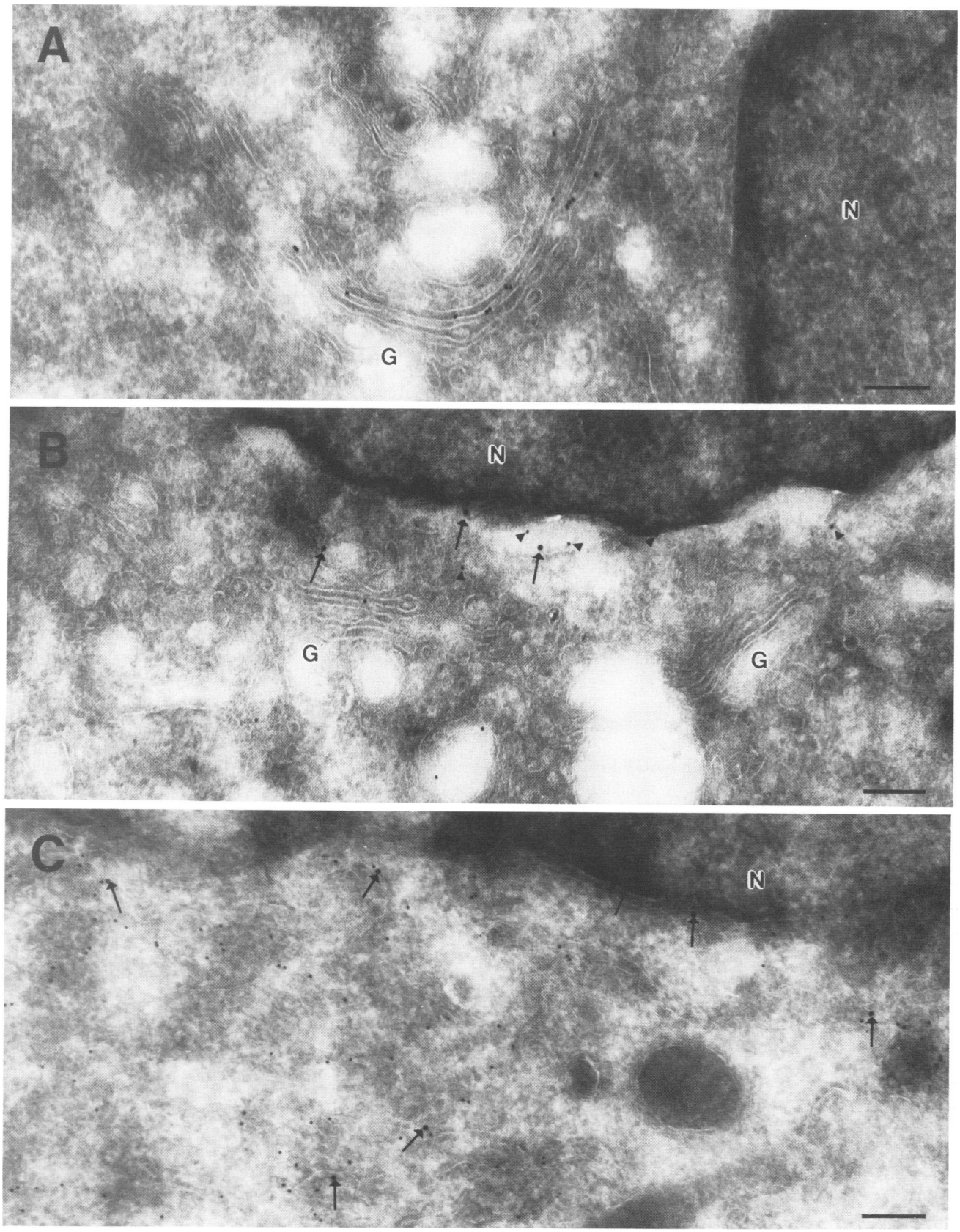


**Fig. 5.** Immunoelectron microscopy of Mann II-HeLa cells transfected with cDNA encoding p33/NAGT I. Mann II-HeLa cells were fixed and prepared for cryo-sectioning and immunolabelling either before (**A**) or after transfection with p33/NAGT I (**B** and **C**). Mann II was visualized using a rabbit polyclonal antiserum and protein A gold (A, 10 nm; B and C, 15 nm). p33/NAGT I was visualized using the 9E10 mouse mAb to the myc epitope followed by goat anti-mouse conjugated to 10 nm gold (B and C). Note that Mann II (arrows in B and C) colocalized with NAGT I (arrowheads in B) in the ER of transfected cells (B and C), but was only present in *medial* and perhaps *trans* Golgi cisternae in untransfected cells (A). G = Golgi apparatus; N = nucleus; bar = 0.2 $\mu$m.

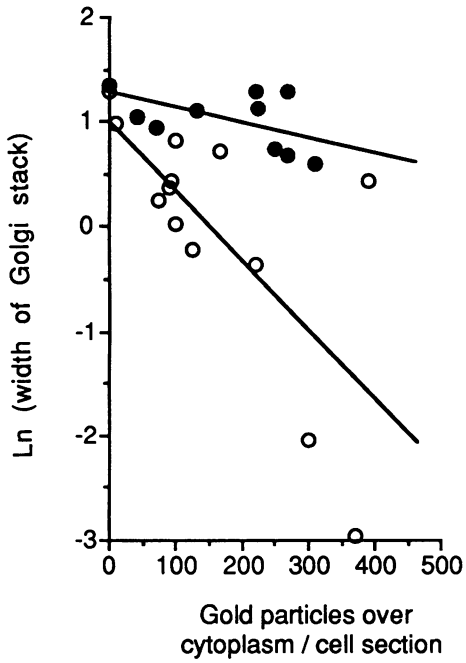


**Fig. 6.** The effect on Golgi structure of increasing expression levels of p33/NAGT I and p33/GalT/NAGT I in Mann II-HeLa cells. Mann II-HeLa cells transiently transfected with p33/NAGT I (○) and p33/GalT/NAGT I (●) were fixed and prepared for cryo-sectioning and immunolabelling. Both hybrid proteins were visualized using the 9E10 mouse mAb to the myc epitope followed by goat anti-mouse conjugated to 10 nm gold. The width of stacked Golgi cisternae and the total amount of gold over the cell cytoplasm were estimated in randomly chosen cell sections.

generated a stable HeLa cell line expressing p33/NAGT I, as described in Materials and methods.

This cell line was compared with NAGT I-HeLa cells for the distribution of Golgi markers by immunofluorescence microscopy, as shown in Figure 8. The *cis* Golgi network (CGN) marker, p53 (Schweizer *et al.*, 1988) (Figure 8G and H), and the *trans* Golgi marker, GalT (Figure 8E and F), had the same distribution in both cell lines. The *medial* markers, however, had very different distributions. NAGT I and Mann II were present in the Golgi in the NAGT I-HeLa cells (Figure 8A and C) but in the ER in the p33/NAGT I-HeLa cell line (Figure 8B and D). However, Mann II in the ER was barely detectable, so the distribution was confirmed by cell fractionation on sucrose gradients as shown in Figure 9. NADH-cytochrome *c* reductase and GalT were used as markers for the ER and Golgi, respectively. Both markers had the same distribution in both cell lines except that ~25% of GalT was present in the ER in the p33/NAGT I-HeLa cells (Figure 9A). Both Mann II (Figure 9B) and NAGT I (Figure 9C) cofractionated with GalT in the NAGT I-HeLa cells, but mostly with the ER in the p33/NAGT-HeLa cells. Approximately 30% of Mann II and 20% of NAGT I still cofractionated with GalT.

Having redistributed two *medial* Golgi enzymes and perhaps others to the ER, it was of interest to compare the Golgi in the two stable cell lines. There was no significant difference in the surface density of the Golgi membranes measured as the sum of two components, the stacked membrane profiles and the associated tubulo-vesicular profiles. The values, relative to cytoplasmic volume, were $1.48 \pm 0.65\ \mu m^{-1}$ for the NAGT I-HeLa cell line and $1.54 \pm 0.70\ \mu m^{-1}$ for the p33/NAGT I-HeLa cell line. Though the sum of the two components did not differ significantly, their relative contribution did. The ratio of the surface density of stacked membrane profiles to associated tubulo-vesicular profiles was 1.39 ± 0.70 for the NAGT I-HeLa cell line, but only 0.73 ± 0.35 for the p33/NAGT I-HeLa cell line. This 2-fold change in the ratio meant that the p33/NAGT I-HeLa cell line had 20% more Golgi vesicles or fenestrated cisternal rims. The remaining stacks were smaller, having a 30% lower volume density than the Golgi stacks in the NAGT I-HeLa cells (0.039 ± 0.022 versus 0.052 ± 0.025), but had a similar number of cisternae (data not shown). In addition, ~25% of the stack profiles in the p33/NAGT I-HeLa cell line showed evidence of slight cisternal dilation, often, though not always, restricted to the *medial* cisternae. All of the observed differences were presumably caused by the removal of *medial* enzymes.

## Discussion

Kin oligomerization is a simple, yet effective, way of retaining proteins along the secretory pathway (Figure 10). Newly synthesized Golgi enzymes would assemble in the ER-forming homodimers that interact through their catalytic domains (Khatra *et al.*, 1974; Moremen *et al.*, 1991) and perhaps their stalk regions (Munro, 1991; Colley *et al.*, 1992; Tang *et al.*, 1992). These would then move by default to the correct cisterna where they would bind specifically through their membrane-spanning domains to pre-existing oligomers, thereby preventing further movement.

There is some evidence for the formation of oligomers. The E1 protein of an avian corona virus resides in *cis* Golgi membranes because of information in the first of the three membrane-spanning domains in the protein (Swift and Machamer, 1991). In detergent extracts this protein exists as a large oligomer. Purified Mann II exists as a dimer in Triton X-100 (Moremen *et al.*, 1991), but removal of this detergent causes the protein to aggregate (Tulsiani *et al.*, 1977). The problem with this type of observation is that it is difficult to distinguish between oligomerization and non-specific aggregation. Here we have taken a different approach and looked for specific interactions between two *medial* Golgi enzymes. The results were unequivocal. Stable or transient expression of p33/NAGT I caused endogenous Mann II to appear in the ER. This was demonstrated by immunofluorescence microscopy, immunoelectron microscopy and cell fractionation. The converse experiment also worked. Transient expression of p33/Mann II caused endogenous NAGT I to appear in the ER, though the effect was not quite so dramatic. Both sets of experiments clearly point to the formation of hetero-oligomers and unpublished experiments (T.Nilsson) show that homo-oligomers can also form. These data strongly support the concept of kin recognition.

The specificity of kin recognition was tested using both *cis* and *trans* Golgi markers. Transient expression of p33/GalT had no significant effect on the distribution of either Mann II or NAGT I, as judged by immunofluorescence microscopy. Furthermore, in the p33/NAGT I-HeLa cell line, fractionation showed that only ~25% of the GalT was in the ER compared with 70% of Mann II and 80% of NAGT I. The distribution of the CGN marker, p53, as judged by immunofluorescence microscopy was also unaffected in transient transfections (data not shown), as well

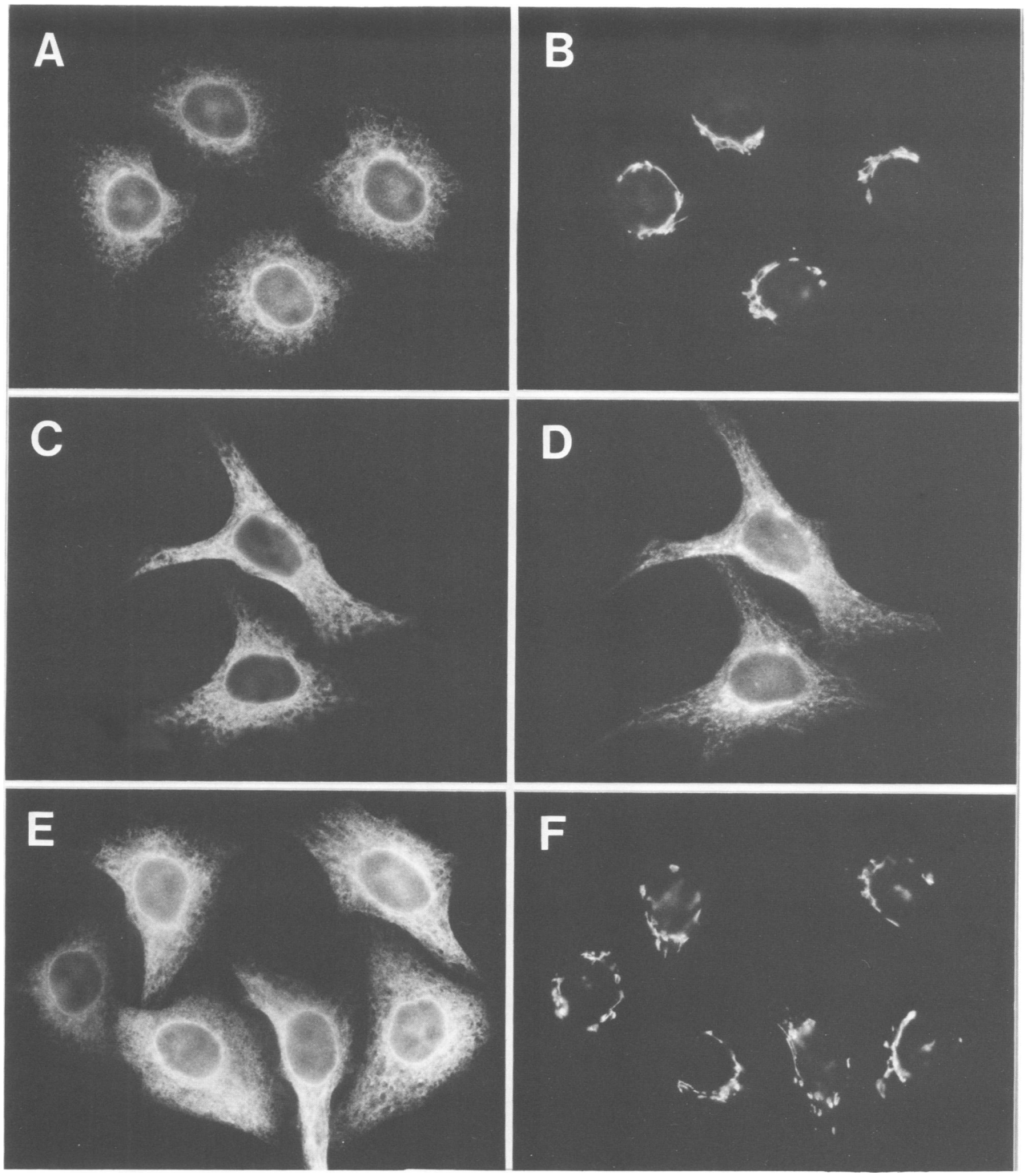


**Fig. 7.** Immunofluorescence microscopy of NAGT I-HeLa cells transfected with cDNAs encoding Iip33, p33/Mann II and p33/Gal T. NAGT I-HeLa cells transfected with cDNAs encoding Iip33 (**A** and **B**), p33/Mann II (**C** and **D**) and p33/GalT (**E** and **F**) were fixed after 72 h and double labelled with mouse mAb to the myc-tagged NAGT I (B, D and F) and rabbit polyclonal antibodies to either Iip33 (A), p33/Mann II (C) or p33/GalT (E), followed by secondary antibodies coupled to Texas Red (B, D and F) and FITC (A, C and E). Magnification: ×700.

as in the stable line, and we can infer that the same was true for the *cis* Golgi enzymes. This is because the p33 hybrid proteins had oligosaccharide modifications showing that they had not reached the *medial* cisternae. If, for example, the *cis* Golgi enzyme, mannosidase I, had been present in the ER in amounts comparable with NAGT I and Mann II, p33/Mann II should have acquired complete resistance to Endo H. In fact, only some, but not all, of the oligosaccharides on a given molecule became resistant even after 2 h of chase. This partial resistance to Endo H may suggest that a small amount of mannosidase I is present in the ER. Alternatively, another *medial* Golgi mannosidase (Bonay and Hughes, 1991), known to bypass the action of mannosidase I, could act on the high mannose oligosaccharides generating intermediates that would serve as substrates for NAGT I and Mann II. The final product

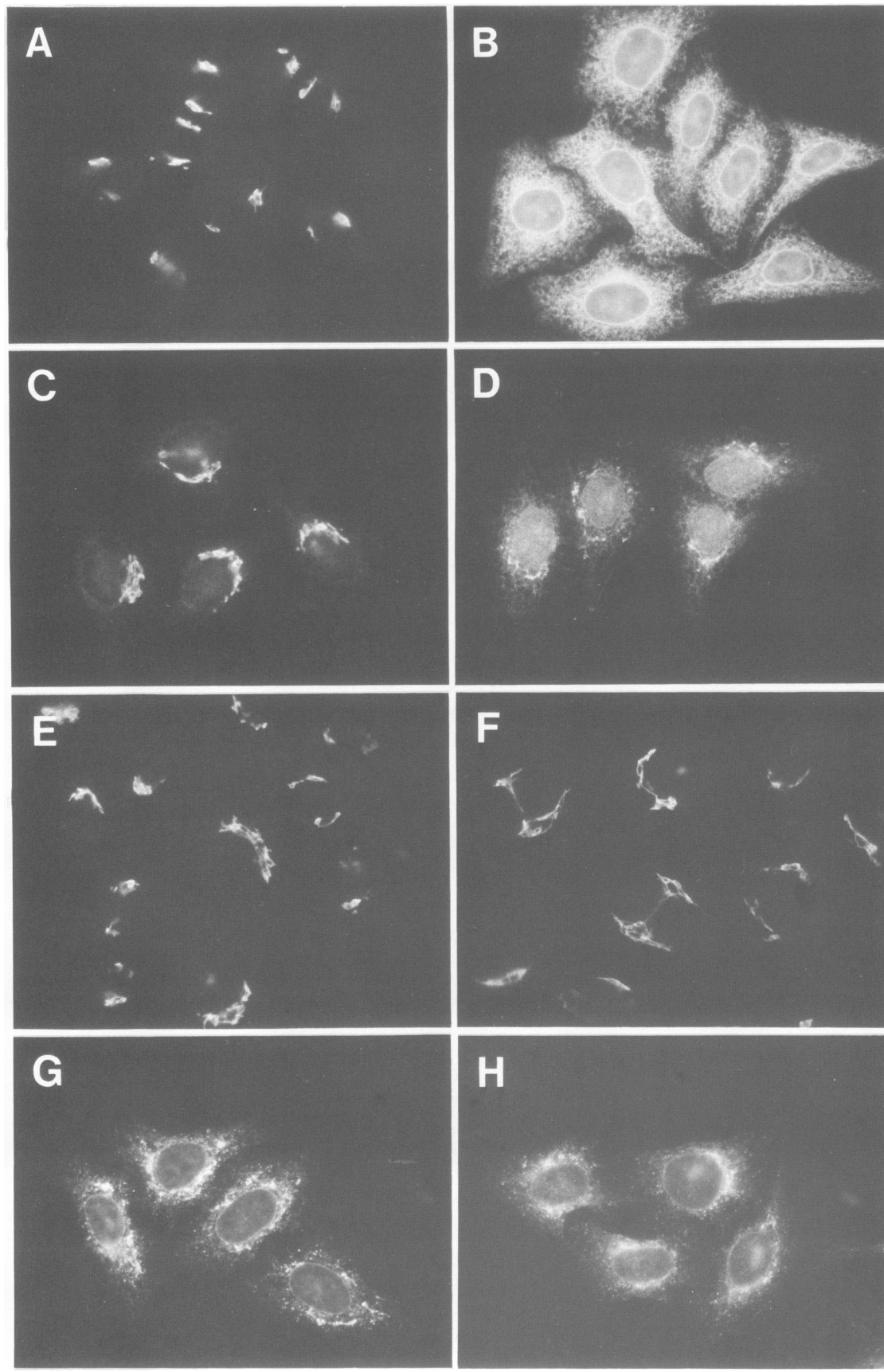


**Fig. 8.** Immunofluorescence microscopy of NAGT I- and p33/NAGT I-HeLa cells. HeLa cells were fixed, permeabilized and labelled with mouse mAb to the stably expressed NAGT I (**A**) and p33/NAGT I (**B**); rabbit polyclonal antibody against endogenous Mann II (**C** and **D**); rabbit polyclonal antibody against GalT (**E** and **F**); and mouse mAb against p53 (**G** and **H**) followed by secondary antibodies coupled to Texas Red. Note the reduced Mann II labelling in the p33/NAGT I cell line (D). Magnification: ×700.

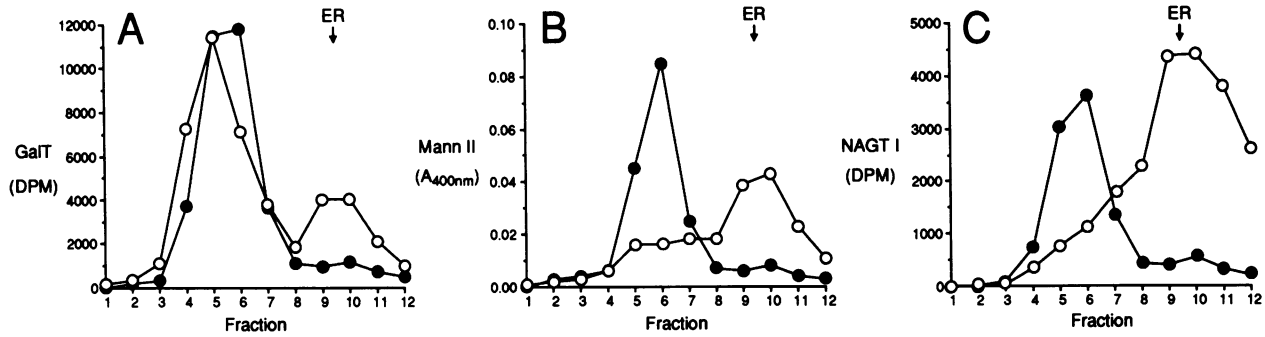


**Fig. 9.** Fractionation of NAGT I- and p33/NAGT I-HeLa cells on sucrose gradients. NAGT I-HeLa or p33/NAGT I-HeLa cells were homogenized and post-nuclear supernatants applied to 0.6–1.8 M sucrose gradients and centrifuged to equilibrium. Fractions from NAGT I-HeLa (●) and p33/NAGT I-HeLa (○) cells were assayed for GalT (**A**), Mann II (**B**) and NAGT I (**C**). NADH cytochrome *c* reductase was used to determine the peak position of ER membranes (arrows).

of this mannosidase is, however, sensitive to Endo H but is not a substrate for NAGT I. This would explain the remaining sensitivity, even at long chase times. In either case, the kin recognition observed would be specific for *medial* Golgi enzymes.

The route by which the *medial* enzymes appear in the ER is far from clear. Transfection takes 72 h, well in excess of the half-life of 20 h which is typical for Golgi enzymes (Strous and Berger, 1982; Moremen and Touster, 1985). So the most likely explanation is that the resident Golgi enzymes are degraded and all of the *medial* enzymes in the ER are newly synthesized. An alternative explanation is that the *medial* enzymes are brought back to the ER either because Golgi enzymes recycle (see Nilsson *et al.*, 1993a for a discussion) or because the p33 cytoplasmic tail can act as a retrieval signal (M.Jackson, personal communication) and fetch the enzymes from the Golgi. This, however, is unlikely because both the modifications to the bound oligosaccharides show that they have not reached the *medial* Golgi (see above) and lectins specific for a variety of oligosaccharides found in the Golgi do not label the ER in transfected cells (T.Nilsson, unpublished experiments).

We have assumed that the interaction between *medial* Golgi enzymes is through the membrane-spanning domain and perhaps part of the stalk region. Replacing these regions in the p33/NAGT I hybrid protein with those of GalT resulted in a hybrid protein which did not retain Mann II in the ER as judged by immunofluorescence microscopy. This argues strongly that these regions are involved in the observed kin recognition between *medial* Golgi enzymes. However, we have also made several constructs based on the invariant chain, the transferrin receptor and GalT in which the corresponding regions were replaced by those of either Mann II or NAGT I. None of these constructs retained the *medial* Golgi enzymes in the ER. One must therefore conclude that, whilst the membrane spanning domain and part of the stalk region are necessary for the observed interaction, they alone are not sufficient to mediate kin recognition. Since the hetero-oligomers form when the entire cytoplasmic tail is replaced by that from p33, the additional information must reside elsewhere in the lumenal domain. It is unlikely that the information resides in the catalytic domains. Mann II acts on the product of NAGT I but there is no evidence for any interaction between the catalytic domains. Immunoprecipitation of either enzyme in non-ionic detergents, such as Triton, does not bring down the other. The additional information, therefore, is most likely present

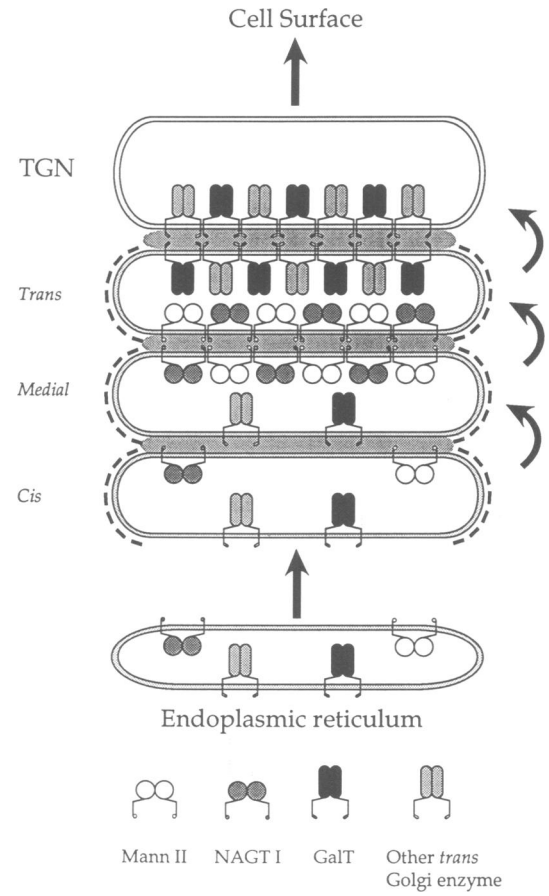


**Fig. 10.** Model for kin recognition. Synthesis and assembly of Golgi enzymes in the ER is followed by transport along the default pathway until the homodimers interact with the pre-existing kin oligomers through their membrane-spanning domains and stalk regions. The kin oligomers are retained because their size prevents them entering the vesicles budding from the cisternal rims (dashed lines). The intercisternal matrices are shown as interacting with the cytoplasmic tails of Golgi enzymes.

in other parts of the stalk region which has been implicated in the retention of several Golgi enzymes (Munro, 1991; Colley *et al.*, 1992; Tang *et al.*, 1992). Experiments are in progress to test this possibility.

Though we have assumed that the *medial* and *trans* enzymes used in this study are present in separate compartments, our recent work (Nilsson *et al.*, 1993a) shows that they are not. Most of the NAGT I is present in the *medial*

cisterna but approximately one-third is present in the *trans* cisterna together with about half of the GalT. The rest of the GalT is present in the TGN. No interaction between GalT and NAGT I was observed by transient expression. Further, we have recently isolated an intercisternal matrix which binds both NAGT I and Mann II but not GalT, showing that they have different biochemical properties as well (Slusarewicz *et al.*, 1994). The simplest solution is indicated in Figure 10 where we assume that GalT forms separate kin oligomers to NAGT I and Mann II, on opposite faces of the *trans* cisterna. Such a distribution would provide a simple model for the stacking of Golgi cisternae, with intercisternal matrices binding to the cytoplasmic tails of the same enzymes in adjacent cisternae (Nilsson *et al.* 1993a). The only evidence against this interpretation is that obtained by fractionating the p33/NAGT I-HeLa cells. Approximately 25% of the GalT was found in the ER, though whether this was interacting with *medial* enzymes has still to be shown.

These studies suggest that *medial* enzymes are involved in maintaining the structure of the Golgi stack. Both NAGT I and Mann II constitute only a few per cent of the enzymes in Golgi cisternae, yet overexpression of p33/NAGT I and relocation of Mann II to the ER led to the complete disappearance of the Golgi stacks. It would be remarkable if we had chosen the two enzymes responsible for maintaining stack structure. More probable is the possibility, predicted by our hypothesis, that NAGT I and Mann II are representative of *medial* enzymes that are relocated to the ER. Complete relocation would then release the cisternae from the intercisternal matrix (Figure 10) explaining the loss of stack structure. The p33/NAGT I-HeLa cell line retains ~20% of the *medial* enzymes in the Golgi stacks and these are slightly more vesiculated than those in the NAGT I-HeLa cell line. This suggests that not more than one-fifth of the enzymes are needed to maintain the morphology of the Golgi and that stacked cisternae are necessary for cell survival.

## Materials and methods

### *Recombinant DNA*

Full-length cDNA encoding human NAGT I (Kumar *et al.*, 1990) tagged with a c-*myc* epitope and subcloned into the eukaryotic expression vector pCMUIV (Nilsson *et al.*, 1989) has been described previously (Nilsson *et al.*, 1993a). For convenience, restriction enzyme sites for *Xba*I and *Sal*I were introduced into the cDNA prior to the start codon and at position 265 (GCAGGC was changed to GTCGAC) respectively. Nested synthetic oligonucleotides (see below) encoding amino acids 45–48 of Iip33 (Strubin *et al.*, 1984) and 8–36 of NAGT I were subcloned between *Xba*I and *Sal*I. A *Hin*dIII–*Sac*II fragment encoding the first 46 cytoplasmic amino acids of Iip33 (see Lotteau *et al.*, 1990) was then subcloned to generate p33/NAGT I. Synthetic oligonucleotides used were: 5′-GCCTGGAATGCATTGCTGCTCCTCTTCTTCTGGACGCGCCCAGCACCTGG, 5′-TCGACCAGGTGCTGGGCGCGTCCAGAAGAAGAGGAGCAGCAATGCATT, 5′-CTAGAGCCGCGGAGCAGGGCTTGTGCTGTGGGGCGCTATCCTCTTTGTG and 5′-CCAGGCCACAAAGAGGATAGCGCCCCACAGCACAAGCCCTGCTCCGCGGCT. PCR (Saiki *et al.*, 1988) was used to introduce a *Xho*I site into position 10 (AGTCGC was changed to TCGAGG) of the Mann II cDNA (Moremen and Robbins, 1991) and into position 141 of the Iip33 cDNA (AGCCGC was changed to TCGAGG) to generate p33/Man II. Primers used were: 5′-GTCGACGGATCCCTCGAGGCAGTTCACCGTGTTTGGCAGC, 5′-GGATCCGGATCCGTAAACATCCAACATCTGCACATCCCGGGG, 5′-GGATCCGGATCCGTCGACATGCACAGGAGGAGAGCAGG and 5′-GAGCTCGGATCCTCGAGCACTTGCTCTCCGGGGCCCC. Both p33/NAGT I and Mann II were also transferred into pSRα (DNAX, Palo Alto, CA) for stable integration into HeLa cells.

Nested synthetic oligonucleotides encoding amino acids 24–58 of human GalT (Watzele and Berger, 1990) were subcloned between *Sac*II and *Pst*I of Iip33. cDNA encoding the lumenal domain of GalT was then joined to the *Pst*I site to generate p33/GalT. Synthetic oligonucleotides used were: 5′-GGACTGCTCGTGGCCGTCTGCGCTCTGCACCTTGGCGTCACCCTCGTTTACTACCTGGCTGGCCGCG, 5′-TCAGGTCGCGGCCAGCCAGGTAGTAAACGAGGGTGACGCCAAGGTGCAGAGCGCAGACGGCCACGAGCAGTCCGC, 5′-ACCTGAGCCGCCTGCCCCAACTGGTCGGAGTCTCCACACCGCTGCA and 5′-GCGGTGTGGAGACTCCGACCAGTTGGGGCAGGCGGC. The 138 bp *Bam*HI–*Pst*I fragment of p33/GalT, encoding the cytoplasmic tail of Iip33 as well as the membrane-spanning domain and part of the stalk region of GalT, was joined to the 1160 bp *Pst*I–*Bam*HI fragment encoding the lumenal domain of NAGT I and subcloned into the *Bam*HI site of pCMUIV to generate p33/GalT/NAGT I.

### *Cell culture, transfection and stable lines*

Monolayer HeLa cells (ATCC CCL185) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal calf serum, penicillin (100 μg/ml) and streptomycin (100 μg/ml). Transient transfection was carried out essentially as described previously (Nilsson *et al.*, 1991). A stable line expressing human NAGT I tagged with a myc epitope (NAGT I-HeLa) has been described elsewhere (Nilsson *et al.*, 1993a). HeLa cells transfected with either murine Mann II (Mann II-HeLa) or p33/NAGT I (p33/NAGT I-HeLa) were selected in the presence of Geneticin (500 μg/ml) (Gibco) for 3 weeks. Clones were picked at random and screened for expression by indirect immunofluorescence (see below). Positive clones were subjected to single cell dilution cloning. Expanded clones were then maintained in the presence of Geneticin (200 μg/ml).

### *Antibodies*

Antibodies used in immunoprecipitation, indirect immunofluorescence and immunoelectron microscopy were as follows: (i) affinity-purified 9E10 mouse mAb recognizing the last 10 amino acids of c-*myc* (Evan *et al.*, 1985) was used to monitor NAGT I and p33/NAGT I, both tagged at their C-terminus with this epitope; and (ii) a rabbit polyclonal antibody raised against the native catalytic domain of rat liver Golgi Mann II (Moremen *et al.*, 1991) (this antibody was shown not to cross-react with NAGT I by transfecting HeLa cells with p33/NAGT I and showing that no staining of the ER occurred); (iii) a rabbit polyclonal peptide antiserum, K2, (V.Quaranta, unpublished results) or a mouse mAb, LN2 (Biotest Ltd, UK), recognizing the lumenal domain of human invariant chain; (iv) an affinity-purified rabbit polyclonal antibody, $N_{11}$, reactive against the deglycosylated lumenal domain of GalT (Watzele *et al.*, 1991) or a mouse mAb raised against human GalT (Berger *et al.*, 1986); (v) a mouse mAb, 1D3 (Vaux *et al.*, 1990), raised against the C-terminus of PDI (KDDDKAVKDEL); (vi) and a mouse mAb, G1/93, recognizing the lumenal domain of p53 (Schweizer *et al.*, 1988).

### *Metabolic labelling and gel fractionation*

Radiolabelling and immunoprecipitation were carried out essentially as described previously (Hoe and Hunt, 1992) except that a mixture of protein A (Boehringer Mannheim) and protein G–agarose (Calbiochem) was used to bind antibody–antigen complexes. Endo H (Boehringer Mannheim) and neuraminidase (Calbiochem) treatments were carried out as described by Pinter and Honnen (1988) and Sege *et al.* (1981) respectively. SDS–PAGE using 8 and 10% gels was performed essentially as described by Blobel and Dobberstein (1975) and fluorography according to Bonner and Laskey (1974).

### *Immunofluorescence microscopy*

Transfected cells were seeded out onto coverslips and, 2 days later, fixed and processed for indirect immunofluorescence essentially as described by Louvard *et al.* (1982), except that 0.2% fish skin gelatin (Sigma) was used as a blocking agent. Secondary antibodies conjugated to Texas Red (Vector Laboratories, UK) or FITC (Tago Inc., Burlingame, CA) were used to visualize the primary antibodies. Cells were viewed using a Zeiss Axiophot microscope and images were recorded on Ilford HP5 Plus film.

### *Subcellular fractionation*

Approximately $10^9$ p33/NAGT I-HeLa cells were harvested, resuspended and incubated for 45 min on ice in fresh medium containing 20 μg/ml cytochalasin B (Sigma) and 20 mM HEPES (pH 7.4). Cells were then washed twice and incubated for 10 min on ice in 25 ml TEA-KCl, pH 7.4 (10 mM triethanolamine–HCl, 150 mM KCl). This was followed by one wash in KEHM, pH 7.4 [50 mM KCl, 10 mM EGTA, 50 mM HEPES–KOH, 1.92 mM $MgCl_2$, 1 mM DTT and a protease inhibitor cocktail comprising aprotinin (1 μg/ml), leupeptin (1 mg/ml), pepstatin (1 μg/ml), antipain (1 μg/ml), benzamide (1 mM) and PMSF (40 μg/ml)]. Cells were finally resuspended in an equal volume of KEHM and homogenized using a ball bearing homogenizer with 10 μm clearance (Balch and Rothman, 1985).

A post-nuclear supernatant (PNS) was then produced by centrifugation at 1800 *g* for 5 min and at 4°C. Aliquots were snap-frozen in liquid nitrogen and stored at −70°C.

The PNS (0.5 ml) was loaded onto a continuous, 0.6−1.8 M sucrose gradient in the same buffer and centrifuged at 40 kr.p.m. for 16 h at 4°C in an SW40.Ti rotor (Beckman). Fractions (1 ml) were taken from the top, snap-frozen in liquid nitrogen and stored at −70°C. Protein was assayed using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

### *Enzyme assays*

NAGT I (E.C. 2.4.1.101) and GalT (E.C. 2.4.1.22) were assayed essentially as described by Vischer and Hughes (1981) and Bretz and Staubli (1977) respectively. Microsomal NADH cytochrome *c* reductase activity was assayed as described by Sottocasa *et al.* (1967) except that assay volumes were reduced to 1 ml. Endogenous Mann II (EC 3.2.1.114) was assayed by mixing 400 μl of each fraction with an equal volume of 2% Triton X-100, 40 mM Tris−HCl (pH 7.4), 0.3 M NaCl, 80 μl protein A−agarose suspension (10% v/v) (Boehringer Mannheim) and 4 μl of anti-Mann II polyclonal antiserum. After incubation at 4°C for 2 h, immunoprecipitates were washed three times with 0.1% Triton X-100, 0.15 M NaCl and 20 mM Tris−HCl (pH 7.4). Immunoprecipitated Mann II was then assayed as described by Bischoff and Kornfeld (1984).

### *Electron microscopy, immunolabelling and stereology*

Monolayer cells were fixed at room temperature in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h. Cells were removed by scraping and processed for cryo-immunoelectron microscopy as described by Rabouille *et al.* (1993). 70 nm ultra-thin sections were cut and incubated with primary antibodies as described by Nilsson *et al.* (1993a), except that gold coupled to protein A (Cell Biology Department, Utrecht School of Medicine, The Netherlands) was used to visualize the polyclonal antibody against Mann II. Protein A gold did not bind to the 9E10 mAb so it could be used for double labelling experiments. Labelled sections were stained with uranyl acetate and embedded in methyl cellulose as described by Tokuyashu (1980). Cells were prepared for Epon sectioning as described by Pypaert *et al.* (1991).

To quantify the effect of transient expression of p33/NAGT I and p33/GalT/NAGT I on the Golgi structure, labelled cell sections were chosen at random and the total number of gold particles over the cell cytoplasm (reflecting ER labelling) was determined. The same antibody was used to detect both proteins so that the expression levels could be directly compared. The width of Golgi stacks in transverse section was measured in the same sections. The width of curved stacks was measured by drawing and summing tangential segments.

For stereological analysis of the Golgi in the NAGT I-HeLa and p33/NAGT I-HeLa stable cell lines, both stacked membrane profiles and associated groups of tubulo-vesicular profiles were defined as Golgi membranes. A stack was defined as two or more cisternal profiles associated for at least half of their length at a distance from each other not exceeding two membrane widths. The minimum length of a cisterna was four times its width. Tubulo-vesicular membrane profiles comprised five or more associated profiles in the Golgi region. Results were processed as described by Nilsson *et al.* (1993a).

## Acknowledgements

We are indebted to the following people for generously providing us with reagents: Dr Kelley Moremen (Athens, GA) for cDNA encoding Mann II and polyclonal antibody against Mann II; Dr Pamela Stanley (Albert Einstein, NY) for cDNA encoding NAGT I; Dr Marilyn Farquhar (UCSD, San Diego, CA) for polyclonal antibody against Mann II; Dr Hans-Peter Hauri (Basel Biozentrum, Switzerland) for mAb against p53; Dr Vito Quaranta (Scripps Clinic and Research Foundation, CA) for polyclonal antibody against human invariant chain; and Dr Stephen Fuller (EMBL) for mAb against PDI. We would also like to thank the oligonucleotide service at ICRF, Clare Hall, for providing us with high-quality synthetic oligonucleotides, Dr Gerard Evan and the hybridoma development unit at ICRF, Clare Hall, for affinity-purified 9E10 mAb, the Photographic Department for high-quality work, Regina Kieckbusch for excellent technical assistance, and both Deborah Mackay and Dr Stella Hurtley for critical reading of the manuscript and useful suggestions.

## References

Aoki,D., Lee,N., Yamaguchi,N., Dubois,C. and Fukuda,M. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 4319−4323.

Bakke,O. and Dobberstein,B. (1990) *Cell*, **63**, 707−716.

Balch,W.E. and Keller,D.S. (1986) *J. Biol. Chem.*, **261**, 14690−14696.

Balch,W.E. and Rothman,J.E. (1985) *Arch. Biochem. Biophys.*, **240**, 413−425.

Berger,E.A., Aegerter,E., Mandel,T. and Hauri,H.-P. (1986) *Carbohydrate Res.*, **149**, 23−33.

Bischoff,J. and Kornfeld,R. (1984) *Biochem. Biophys. Res. Commun.*, **125**, 324−331.

Blobel,G. and Dobberstein,B. (1975) *J. Cell Biol.*, **67**, 835−851.

Bonay,P. and Hughes,R.C. (1991) *Eur. J. Biochem.*, **197**, 229−238.

Bonner,W. and Laskey,R.A. (1974) *Eur. J. Biochem.*, **46**, 83−88.

Brands,R., Snider,M.D., Hino,Y., Park,S.S., Gelboin,H.V. and Rothman,J.E. (1985) *J. Cell Biol.*, **101**, 1724−1732.

Bretz,R. and Staubli,W. (1977) *Eur. J. Biochem.*, **77**, 181−192.

Burke,B., Griffiths,G., Reggio,H., Louvard,D. and Warren,G. (1982) *EMBO J.*, **1**, 1621−1628.

Burke,J., Pettitt,J.M., Schachter,H., Sarkar,M. and Gleeson,P.A. (1992) *J. Biol. Chem.*, **267**, 24433−24440.

Colley,K.J., Lee,E.U. and Paulson,J.C. (1992) *J. Biol. Chem.*, **267**, 7784−7793.

Dean,N. and Pelham,H.R. (1990) *J. Cell Biol.*, **111**, 369−377.

Dunphy,W.G. and Rothman,J.E. (1983) *J. Cell Biol.*, **97**, 270−275.

Dunphy,W.G., Fries,E., Urbani,L.J. and Rothman,J.E. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 7453−7457.

Dunphy,W.G., Brands,R. and Rothman,J.E. (1985) *Cell*, **40**, 463−472.

Evan,G.I., Lewis,G.K., Ramsay,G. and Bishop,J.M. (1985) *Mol. Cell. Biol.*, **5**, 3610−3616.

Goldberg,D.E. and Kornfeld,S. (1983) *J. Biol. Chem.*, **258**, 3159−3165.

Hoe,M.H. and Hunt,R.C. (1992) *J. Biol. Chem.*, **267**, 4916−4923.

Jackson,M.R., Nilsson,T. and Peterson,P.A. (1990) *EMBO J.*, **9**, 3153−3162.

Jackson,M.R., Nilsson,T. and Peterson,P.A. (1993) *J. Cell Biol.*, **121**, 317−333.

Khatra,B.S., Herries,D.G. and Brew,K. (1974) *Eur. J. Biochem.*, **44**, 537−560.

Klausner,R.D., Donaldson,J.G. and Lippincott-Schwartz,J. (1992) *J. Cell Biol.*, **116**, 1071−1080.

Kumar,R., Yang,J., Larsen,R.D. and Stanley,P. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9948−9952.

Leelavathi,D.E., Estes,L.W., Feingold,D.S. and Lombardi,B. (1970) *Biochim. Biophys. Acta*, **211**, 124−138.

Lewis,M.J. and Pelham,H.R.B. (1990) *Nature*, **348**, 162−163.

Lewis,M.J. and Pelham,H.R.B. (1992) *Cell*, **68**, 353−364.

Locker,J.K., Griffiths,G., Horzinek,M.C. and Rottier,P.J.M. (1992) *J. Biol. Chem.*, **267**, 14094−14101.

Lotteau,V., Teyton,L., Peleraux,A., Nilsson,T., Karlsson,L., Schmid,S., Quaranta,V. and Peterson,P.A. (1990) *Nature*, **348**, 600−605.

Louvard,D., Reggio,H. and Warren,G. (1982) *J. Cell Biol.*, **92**, 92−107.

Moremen,K.W. and Robbins,P.W. (1991) *J. Cell Biol.*, **115**, 1521−1534.

Moremen,K.W. and Touster,O. (1985) *J. Biol. Chem.*, **260**, 6654−6662.

Moremen,K.W., Touster,O. and Robbins,P.W. (1991) *J. Biol. Chem.*, **266**, 16876−16885.

Munro,S. (1991) *EMBO J.*, **10**, 3577−3588.

Munro,S. and Pelham,H.R. (1987) *Cell*, **48**, 899−907.

Nilsson,T., Jackson,M. and Peterson,P.A. (1989) *Cell*, **58**, 707−718.

Nilsson,T., Lucocq,J.M., Mackay,D. and Warren,G. (1991) *EMBO J.*, **10**, 3567−3575.

Nilsson,T., Pypaert,M., Hoe,M.H., Slusarewicz,P., Berger,E.G. and Warren,G. (1993a) *J. Cell Biol.*, **120**, 5−13.

Nilsson,T., Slusarewicz,P., Hoe,M.H. and Warren,G. (1993b) *FEBS Lett.*, **330**, 1−4.

Nishikawa,Y., Pegg,W., Paulsen,H. and Schachter,H. (1988) *J. Biol. Chem.*, **263**, 8270−8281.

Orci,L., Glick,B.S. and Rothman,J.E. (1986) *Cell*, **46**, 171−184.

Pääbo,S., Bhat,B.M., Wold,W.S. and Peterson,P.A. (1987) *Cell*, **50**, 311−317.

Pathak,R.K., Merkle,R.K., Cummings,R.D., Goldstein,J.L., Brown,M.S. and Anderson,R.G. (1988) *J. Cell Biol.*, **106**, 1831−1841.

Pelham,H.R.B. (1989) *Cell Biol.*, **5**, 1−23.

Pinter,A. and Honnen,W.J. (1988) *J. Virol.*, **62**, 1016−1021.

Pypaert,M., Mundy,D., Souter,E., Labbe,J.-C. and Warren,G. (1991) *J. Cell Biol.*, **114**, 1159−1166.

Quinn,P., Griffiths,G. and Warren,G. (1984) *J. Cell Biol.*, **98**, 2142−2147.

Rabouille,C., Strous,G.J., Crapo,J.D., Geuze,H.J. and Slot,J.W. (1993) *J. Cell Biol.*, **120**, 897−908.

Roth,J. and Berger,E.G. (1982) *J. Cell Biol.*, **93**, 223−229.

Russo,R.N., Shaper,N.L., Taatjes,D.J. and Shaper,J.H. (1992) *J. Biol. Chem.*, **267**, 9241−9247.

Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T.

and Erlich,H.A. (1988) *Science*, **239**, 487–491.
Schweizer,A., Fransen,J.A.M., Bächi,T., Ginsel,L. and Hauri,H.-P. (1988) *J. Cell Biol.*, **107**, 1643–1653.
Sege,K., Rask,L. and Peterson,P.A. (1981) *Biochemistry*, **20**, 4523–4530.
Slusarewicz,P., Nilsson,T., Hui,N., Watson,R. and Warren,G. (1994) *J. Cell Biol.*, in press.
Sottocasa,G.L., Kuylenstierna,B., Ernster,L. and Bergstrand,A. (1967) *J. Cell Biol.*, **32**, 415–438.
Strous,G.J. and Berger,E.G. (1982) *J. Biol. Chem.*, **257**, 7623–7628.
Strubin,M., Mach,B. and Long,E.O. (1984) *EMBO J.*, **3**, 869–872.
Swift,A.M. and Machamer,C.E. (1991) *J. Cell Biol.*, **115**, 19–30.
Tang,B.L., Wong,S.H., Low,S.H. and Hong,W. (1992) *J. Biol. Chem.*, **267**, 10122–10126.
Tarentino,A.L. and Maley,F. (1974) *J. Biol. Chem.*, **249**, 811–817.
Teasdale,R.D., D'Agostara,G. and Gleeson,P.A. (1992) *J. Biol. Chem.*, **267**, 4084–4096.
Tokuyasu,K.T. (1980) *Histochem. J.*, **12**, 381–403.
Tooze,S.A., Tooze,J. and Warren,G. (1988) *J. Cell Biol.*, **106**, 1475–1487.
Tulsiani,D.R.P., Opheim,D.J. and Touster,O. (1977) *J. Biol. Chem.*, **252**, 3227–3233.
Vaux,D., Tooze,J. and Fuller,S. (1990) *Nature*, **345**, 495–502.
Vischer,P. and Hughes,R.C. (1981) *Eur. J. Biochem.*, **117**, 275–284.
Watzele,G. and Berger,E.G. (1990) *Nucleic Acids Res.*, **18**, 7174.
Watzele,G., Bachofner,R. and Berger,E.G. (1991) *Eur. J. Cell Biol.*, **56**, 451–458.
Wong,S.H., Low,S.H. and Hong,W. (1992) *J. Cell Biol.*, **117**, 245–258.
Yamamoto,A., Masaki,R. and Tashiro,Y. (1985) *J. Cell Biol.*, **101**, 1733–1740.

*Received on September 30, 1993*