

Overlapping Distribution of Two Glycosyltransferases in the Golgi Apparatus of HeLa Cells

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Abstract. Thin, frozen sections of a HeLa cell line were double labeled with specific antibodies to localize the *trans*-Golgi enzyme, β 1,4 galactosyltransferase (GalT) and the *medial* enzyme, *N*-acetylglucosaminyltransferase I (NAGT I). The latter was detected by generating a HeLa cell line stably expressing a *myc*-tagged version of the endogenous protein. GalT was found in the *trans*-cisterna and *trans*-Golgi network

but, contrary to expectation, NAGT I was found both in the *medial*- and *trans*-cisternae, overlapping the distribution of GalT. About one third of the NAGT I and half of the GalT were found in the shared, *trans*-cisterna. These data show that the differences between cisternae are determined not by different sets of enzymes but by different mixtures.

THE construction of N-linked, bi-antennary oligosaccharides in the Golgi apparatus is a three-stage process. The first stage, thought to take place in the *cis*-Golgi network (CGN)/*cis*-cisternae, continues the trimming of mannose residues started in the ER. The second stage, in *medial*-cisternae, involves addition of *N*-acetylglucosamine, the removal of a further two mannose residues and the addition of another *N*-acetylglucosamine. Fucose may also be added at this stage. The third and last stage, in the *trans*-cisternae and *trans*-Golgi network (TGN), involves addition of galactose followed by sialic acid (for review see Kornfeld and Kornfeld, 1985).

These activities have been assigned to the different cisternae and networks based on localization of the appropriate enzymes and their products (for reviews see Dunphy and Rothman, 1985, and Roth, 1987). The first stage enzyme, mannosidase I, is thought to reside in the CGN/*cis*-cisternae because it acts on oligosaccharides attached to proteins that have just left the ER (Balch et al., 1986) and before the second stage enzyme, *N*-acetylglucosaminyltransferase I (NAGT I), which has been immunolocalized to *medial*-cisternae (Dunphy et al., 1985). Other second stage enzymes (mannosidase II and NAGT II) cofractionate with NAGT I on sucrose gradients and, since they can be partially separated from third-stage enzymes (β 1,4 galactosyltransferase [GalT] and α 2,6 sialyltransferase [SialylT]) on the same gradients (Dunphy et al., 1981; Dunphy and Rothman,

1983; Goldberg and Kornfeld, 1983) they are assumed to occupy the *medial*-cisternae. The third stage enzymes have been immunolocalized to the *trans*-cisternae and TGN (Roth and Berger, 1982; Strous et al., 1983; Slot and Geuze, 1983; Roth et al., 1985) and though these membranes have not been cleanly separated either biochemically or cytochemically, there is data suggesting that SialylT, unlike GalT, might be concentrated more in the TGN than the *trans*-cisternae (Roth et al., 1985).

These data have led to the view that enzymes involved in the three stages of construction are physically separated in cisternae that are ordered in a stack. There have been occasional reports of Golgi residents occupying more than one group of cisternae (Novikoff et al., 1983; Roth et al., 1986; Hendricks et al., 1991) but they have generally been regarded as exceptions to the rule.

Despite the wide acceptance of this model there is no direct evidence in support of it. The most compelling evidence would come from immunolabeling studies, showing that the enzymes of each stage are physically restricted to cisternae distinct from the others. The main barrier to such an analysis has been the lack of antibodies that recognize enzymes in the different stages within the same species. The problem has been compounded by the unresolved difficulty of producing antibodies useful for quantitative immuno-electron microscopic studies.

We have overcome this problem by generating a stable HeLa cell line expressing a *medial*-Golgi enzyme, NAGT I, tagged with a *myc* epitope, which is recognized by the high affinity mAb, 9E10. This has been used, in double-label experiments, with polyclonal antibodies to a *trans*-Golgi enzyme, GalT. The results show that the distributions are not discrete but overlap substantially.

Tommy Nilsson and Marc Pypaert contributed equally to this work.

1. *Abbreviations used in this paper:* CGN, *cis*-Golgi network; GalT, galactosyltransferase; NAGT I, *N*-acetylglucosaminyltransferase I; PCR, polymerase chain reaction; TGN, *trans*-Golgi network.

Materials and Methods

Recombinant DNA

The human NAGT I cDNA (Kumar et al., 1990), inserted into pGEM (Promega Corp., Madison, WI), was used as a template in a polymerase chain reaction (PCR) (Saiki et al., 1988). PCR primers were as follows (bases encoding the myc epitope recognized by the 9E10 mAb [Evan et al., 1985] are underlined):

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5'GGATCCGGATCCTCTAGACCATGATCAAGAAGCAGTCTGCTGGCCTGGT  
5'GGATCCGGATCCTTACAGGTCTTCTTCAGAGATCAGTTTCTGTTCCGGATT  
CCAGCTAGGATCATAGCCCTC
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To introduce a Kozak consensus sequence (see Kozak, 1983) for proper initiation of translation, the first codon after the ATG was changed from CTG (encoding leucine) to ATC (encoding isoleucine). The PCR product was next digested with BamHI and cloned into pCMUTV (Nilsson et al., 1989). After sequencing and transient expression, the mycNAGT I cDNA was transferred into pSR α (DNAX, Palo Alto, CA) for stable integration into HeLa cells.

Cell Culture and Transfection

Monolayer HeLa cells (ATCC CCL185) were grown in DME (Northumbria Biologicals Limited, Cramlington, UK) supplemented with 10% FCS, penicillin, and streptomycin at 100 μ g/ml. After transfection with the cDNA encoding mycNAGT I (Nilsson et al., 1989), cells were maintained in the above medium containing 450 μ g/ml Geneticin (G-418 sulphate) (Gibco Laboratories, Inc., Grand Island, NY) for 3 wk before isolation of individual clones. Clones were screened by indirect immunofluorescence using the 9E10 mAb (see below) and diluted to single cells for a second round of cloning. The mycNAGT I HeLa cells were maintained in the presence of 200 μ g/ml Geneticin.

Preparation of Golgi Membranes

Golgi membranes were prepared from the parental and stable cell lines as described by Balch et al. (1984) using $\sim 10^9$ cells. Harvested Golgi membranes were purified 25–30-fold over the crude homogenate (as assayed for by GalT activity). Protein was assayed using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

Assays for GalT and NAGT I

GalT (E.C.2.4.1.22) was assayed as described by Bretz and Stäubli (1977) and NAGT I (E.C.2.4.1.101) as described by Visher and Hughes (1981) except that reactions were incubated for 2.5 h, stopped with ice-cold 1% phosphotungstic acid/0.5 M HCl, the precipitates washed once with the same mixture and once with ice-cold 95% EtOH. The pellets were solubilized in 0.4 M unbuffered Tris/4% SDS for 1–2 h before the addition of scintillation fluid.

Western Blotting

After SDS-PAGE, proteins were transferred onto Hybond-C super membranes (Amersham Corp., Arlington Heights, IL) using a semi-dry blotter. Filters were next blocked overnight using 5% nonfat milk and 0.2% Tween-20 in PBS. First antibodies were used at a dilution of 1:1,000 followed by secondary antibodies conjugated to peroxidase (Tago, Inc., Burlingame, CA). The bands were visualized using the ECL detection reagents (Amersham Corp.).

Indirect Immunofluorescence and Confocal Microscopy

The stable cell line was processed for indirect immunofluorescence (Louvart et al., 1982; Warren et al., 1984) using 0.2% fish skin gelatin instead of bovine gelatin to minimize nonspecific binding. Secondary antibodies conjugated to Texas red (Vector Laboratories, Inc., Burlingame, CA) or FITC (DAKOPATTS, Copenhagen) were used to visualize the primary antibodies. Both primary and secondary antibodies were preadsorbed onto cells fixed with paraformaldehyde and permeabilized with Triton X-100 before staining. Cells were visualized using a Bio-Rad MRC-600 Laser scanning confocal imaging system (Bio-Rad Laboratories, Richmond, CA). Bleed-through from the fluorescein channel into the Texas red channel amounted to 10% and was corrected for in the images scanned. Final images were merged using Photoshop on a Macintosh computer and photographed directly from the screen.

Immunoelectron Microscopy

Monolayer HeLa cells were fixed for 1 h at room temperature (RT) with 0.5% (vol/vol) glutaraldehyde (Fluka BioChemika, Glossop, England) in 0.2 M Pipes buffer, pH 7.2. They were washed twice in the same buffer, scraped from the dish and spun at 13,000 g for 10 min at RT. The pellets were infiltrated with a solution of 2.1 M sucrose in PBS, pH 7.4, for 30 min at RT, then mounted onto aluminium studs and quickly frozen in liquid nitrogen.

100-nm-thick sections were cut at -90°C using an ultracut E microtome with FC4E cryoattachment, and then transferred onto formvar- and carbon-coated 100-mesh grids. Before immunolabeling, free aldehyde groups were quenched by incubation for 10 min at RT with 0.1 M ammonium chloride in PBS. The sections were washed for 5 min with 0.5% fish skin gelatin in PBS (PBS/gelatin), pH 7.4, then incubated in 8 μ l of antibody (rabbit anti-GalT) diluted in PBS/gelatin in a moist chamber for 30 min at RT. After six washes in PBS/gelatin (15 min total), the grids were incubated for 20 min at RT in 8 μ l of goat anti-rabbit IgG conjugated to 5- or 10-nm gold particles (Biocell Research Laboratories, Cardiff, UK) diluted 20-fold in PBS-gelatin. The grids were finally washed six times in PBS (30 min total). For the double-labeling experiments, the grids were first incubated in a mixture of anti-GalT and anti-myc (9E10) antibodies followed by goat anti-mouse IgG-gold and then the anti-rabbit IgG-gold with appropriate washes in between.

Sections were contrasted and embedded in epoxy resin using a modification of the method of Keller et al. (1984), essentially as described by Lucoq et al. (1989). They were stained for 10 min with lead citrate according to the method of Reynolds (1963), and then examined at 80 kV in a Phillips CM10 electron microscope (Phillips Scientific, Mahwah, NJ).

Immunogold Quantitation

Golgi profiles were sampled at random, photographed, and printed at a final magnification of 98,000. Only those profiles which were sectioned transversely and showed polarized labeling with anti-GalT were selected for quantitation. For the double-labeling experiments, another condition was that the profiles selected were labeled with at least one gold particle of each size.

Cisternae were assigned as described in the text and the density of gold in each cisterna was calculated by counting the number of gold particles and dividing it by the surface area of the cisternal profile expressed in μm^2 . The latter was measured using a square lattice grid with 5-mm point-point spacing. The surface area of a cisternal profile = $\Sigma P \times d^2$ where ΣP is the number of points falling over it and d is the distance between lines of the grid. When a gold particle was found between two cisternae, it was assigned to the one which was the closest. The same principle was used to assign points from the grid which fell over the intracisternal space. Background was calculated by counting the number of gold particles over large areas of cytoplasm, and dividing this number by the surface estimated using a square lattice grid with 20-mm spacing. The significance of the labeling density was assessed using the t test and a probability level of 0.05.

Linear densities were estimated by dividing the number of gold particles by the length of cisternal membrane measured by counting intersections of membrane with lines of a grid with a 10-mm point-point spacing.

During the course of these experiments, a different batch of anti-rabbit IgG-gold complex was used which nearly doubled the level of signal and background. To compare data using these different antibodies, a correction factor was applied which was calculated using a simple test system. Formvar- and carbon-coated grids were floated on PBS/gelatin for 5 min, then incubated with 8 μ l of anti-GalT antibodies as described above. After six short washes in PBS/gelatin (5 min total), the grids were labeled for 20 min with either batch of anti-rabbit IgG-gold complex diluted 20-fold in PBS/gelatin. The grids were washed six times in PBS (5 min total), air dried after a short wash in distilled water, and the gold particles then counted at the electron microscope. The new batch of gold complex gave, on average, 1.67 times more gold particles than the older batch, and this value was used to correct the data.

Stereology

Cells were sampled at random, then photographed, and the pictures printed at a final magnification of 16,000. Golgi stacks within these cells were sampled, photographed, and the pictures printed at a final magnification of 90,000.

Surface densities of Golgi membrane per unit volume of cytoplasm ($S_{\text{go}}/V_{\text{cyto}}$) and mitochondria ($S_{\text{go}}/V_{\text{mito}}$) were calculated using the following equations:

$$S_{go}/V_{cyto} = S_{go}/V_{go} \times V_{go}/V_{cyto} \quad (1)$$

and

$$S_{go}/V_{mito} = S_{go}/V_{go} \times V_{go}/V_{mito} \quad (2)$$

where S_{go}/V_{go} is the surface density of Golgi membrane within individual Golgi stacks, V_{go}/V_{cyto} is the fraction of cytoplasmic volume occupied by Golgi stacks, and V_{go}/V_{mito} is the ratio of the volume of Golgi stacks per volume of mitochondria.

S_{go}/V_{go} was estimated from the higher magnification (90,000) pictures using a square lattice grid with 20- μm spacings. The equation $\Sigma I/\Sigma P \times d$ gave S_{go}/V_{go} , where ΣI is the sum of vertical and horizontal intercepts of the lattice grid with Golgi membranes, ΣP is the sum of the points falling over Golgi cisternae or associated vesicles as well as over the intracisternal space, and d is the distance between lines of the grid.

V_{go}/V_{cyto} and V_{go}/V_{mito} were estimated from the lower magnification (16,000) pictures using the same lattice grid as above, by counting the number of points falling over Golgi areas and dividing it by the number of points falling over the cytoplasm or mitochondria.

Results

Distribution of GalT by Immuno-EM

Thin, frozen sections of the parental HeLa cell line were labeled with polyclonal antibodies specific for the polypeptide chain of GalT (Watzel et al., 1991), followed by anti-rabbit IgG coupled to 10 nm gold. The specificity of the antibody was confirmed by showing that the signal over the Golgi was 37 times that over the cytoplasm.

GalT was found in two cisternae on one side of the stack (Fig. 1) in agreement with earlier results on the same cell line (Roth and Berger, 1982; Lucocq et al., 1989). To provide a consistent framework for quantitation, the *trans*-cisterna (T) was defined as the last continuous cisterna labeled by antibodies to GalT (see Fig. 1). The high level of labeling meant that most transversely sectioned Golgi stacks were counted. The *medial*-cisterna would be T-1 and the *cis*-cisterna T-2. The next cisterna, T-3, could represent the CGN but this was only present in 30–50% of the sections depending on the experiment. The tubulo-vesicular cisterna on the other side of the T cisterna, T+1, most probably represented the TGN.

Labeling for GalT was quantified and the results are presented as the number of gold particles per μm^2 of cisternal profile (Fig. 2). Labeling was almost entirely restricted

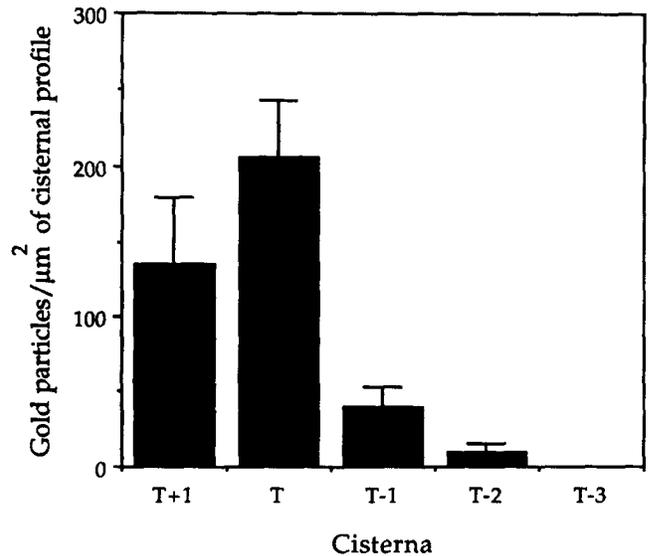


Figure 2. Quantitative distribution of GalT. Images such as that presented in Fig. 1 were used to calculate the density of gold particles over each cisternal profile. Results are presented as the mean \pm SEM ($n = 10$). Background labeling was 2.6 gold particles per μm^2 of cytoplasm.

to the T and T+1 cisternae. Labeling of the other cisternae was not significantly above background with the exception of the T-1 cisterna though this was not a consistent observation (see below). Differences were considered significant at the $p < 0.05$ level using the t test. This level of significance was used in all subsequent experiments.

Characterization of a Stable Cell Line Expressing mycNAGT I

The localization of NAGT I was made possible by generating a HeLa cell line stably expressing human NAGT I tagged with a *myc* epitope. This was placed at the extreme COOH terminus of the protein, which resides in the Golgi lumen. The mycNAGT I cDNA (Kumar et al., 1990) was transferred into pSR α (see Materials and Methods) and stable transformants were selected in the presence of geneticin. The clone

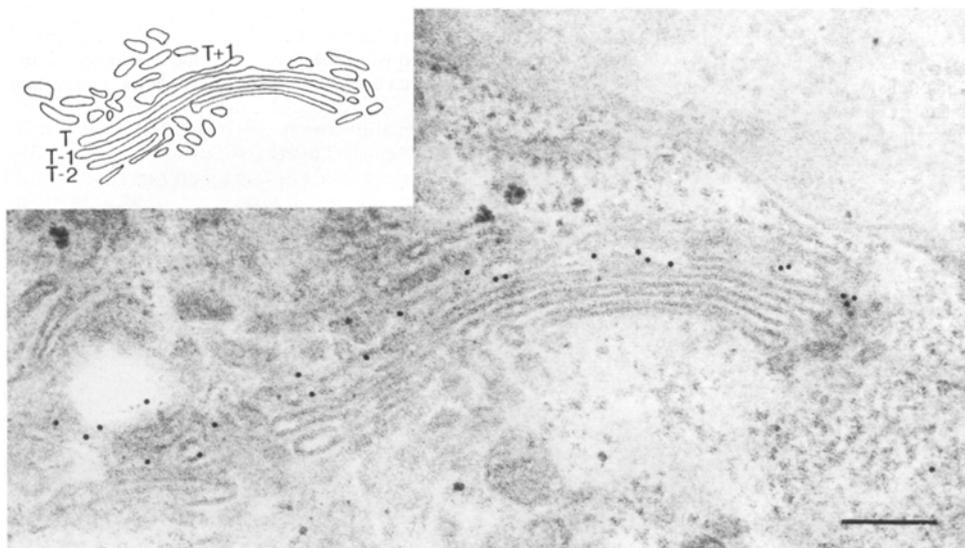


Figure 1. Distribution of GalT by immuno-EM. Thin, frozen sections of parental HeLa cells were labeled with polyclonal anti-GalT followed by anti-rabbit IgG coupled to 10 nm gold. The T cisterna (see inset) was defined as the last continuous cisterna that labeled for GalT. The others are indicated and most probably represent the TGN (T+1), the medial-cisterna (T-1), and the cis-cisterna (T-2). Note that labeling for GalT was restricted to the T and T+1 cisternae. Bar, 0.2 μm .

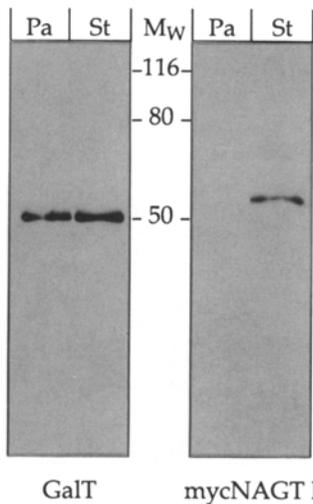


Figure 3. GalT and mycNAGT I in isolated Golgi membranes. Golgi membranes were isolated from both the parental (Pa) and stable (St) cell lines, fractionated by SDS-PAGE, blotted, and probed with antibodies to either GalT or mycNAGT I.

selected for this work expressed mycNAGT I in every cell at approximately equivalent levels, allowing for the phase of the cell cycle. This was assessed by indirect immunofluorescence microscopy.

The mycNAGT I had the expected molecular weight of 55,000 (Fig. 3) and was enzymatically active as shown by the fourfold higher activity of isolated Golgi membranes when compared with those from the parental cell line (Fig. 4). GalT was used as an internal control in these experiments to correct for differences in the purity of the Golgi membranes. This was a valid control since the specific activity of GalT was almost the same in the two cell lines (see legend to Fig. 4) as was the amount of protein (Fig. 3). Enzyme activity was

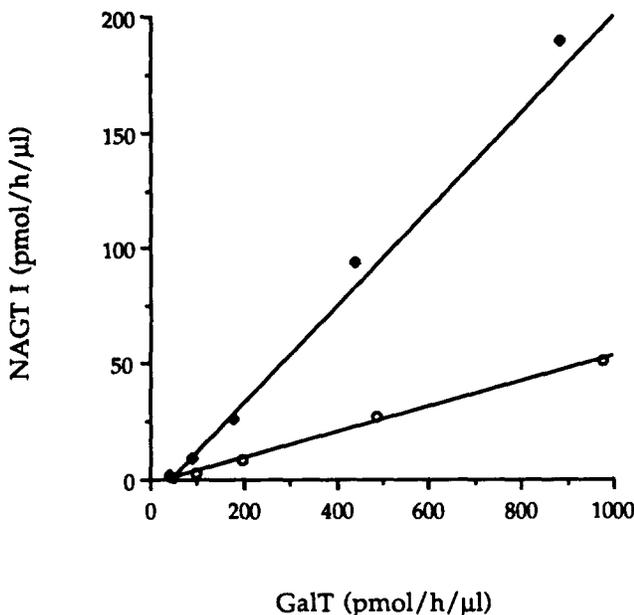


Figure 4. Activity of NAGT I in the parental and stable cell lines. Isolated Golgi membranes were assayed for NAGT I activity. The results are expressed relative to the activity of GalT, which had almost the same specific activity in both the parental (○) (43 nmoles/h/mg protein) and stable (◆) (51 nmoles/h/mg protein) cell lines so could be used to correct for differences in purity of the isolated membranes. The results are the mean of two experiments and the ratio of the slopes is 4.1.

Table I. The Volume and Surface Densities of Golgi in the Stable and Parental Cell Lines

Cell Line	Cytoplasm		Mitochondria	
	V _v	S _v (μm ⁻¹)	V _v	S _v (μm ⁻¹)
Parental	0.054 ± 0.024	1.68 ± 0.76	0.40 ± 0.21	12.5 ± 6.8
Stable	0.045 ± 0.014	1.49 ± 0.47	0.44 ± 0.18	14.4 ± 6.0

The volume density (V_v) and surface density (S_v) of the Golgi for both cell lines were estimated relative to either cytoplasm or mitochondria. Results are presented as the mean ± SEM (n = 8). The differences between the stable and parental cell lines were not significant at the p = 0.05 level using the t test.

used to monitor the level of over-expression since estimates of the protein level require antibodies to the endogenous NAGT I which are not yet available. The expression of mycNAGT I had no obvious effects on the morphology of the Golgi apparatus nor on its volume and surface area relative to either cytoplasm or mitochondria (Table I).

Distribution of GalT and mycNAGT I by Immuno-EM

Thin, frozen sections of the stable cell line were labeled with a mixture of antibodies to localize both GalT and mycNAGT I, followed by anti-rabbit and anti-mouse second antibodies coupled to different sizes of gold. The signal for mycNAGT I over the Golgi was 17 times that over the cell cytoplasm. This confirms the high specificity of the antibody suggested by Fig. 3. As shown in Fig. 5, A-C, mycNAGT I was mostly found in two cisternae, T-1 and T. This was confirmed by quantitation (Fig. 6 A) and showed that there was no significant difference in density over these two cisternae. The density of labeling over the other cisternae was not significantly different from background.

The density distribution of GalT (Fig. 6 A) was very similar to that seen in the parental cell line (Fig. 2) though the signal over the Golgi was now 48 times that over the cytoplasm and the density in all cisternae other than T and T+1 was not significantly above background.

The same distributions were obtained when the results were presented as linear densities (Fig. 6 B). This format does not allow comparison with background labeling but is not distorted by differences in the volume of each cisterna.

The distribution of GalT clearly overlapped with that of mycNAGT I in the T cisterna. This overlap was not a rare event revealed only by collecting and summing the results from individual Golgi profiles. Fig. 5, (A-C) shows typical examples of Golgi stacks used for counting purposes. The high level of labeling with both antibodies clearly revealed double labeling of the T cisterna. In fact, of the 42 Golgi stacks used for counting, 36 showed double labeling of the T cisterna. Overlap was also observed when the sizes of the gold were reversed (Fig. 5, D and E).

The approximate amount of each enzyme in the shared, trans-cisterna was estimated by combining the density values with the estimated sizes of the cisternae. The T cisterna contained 53% of the GalT and 35% of the mycNAGT I. These figures assume that the labeling efficiency was the same for each cisterna.

Distribution of GalT and mycNAGT I by Confocal Microscopy

Given the precision with which these two enzymes could be

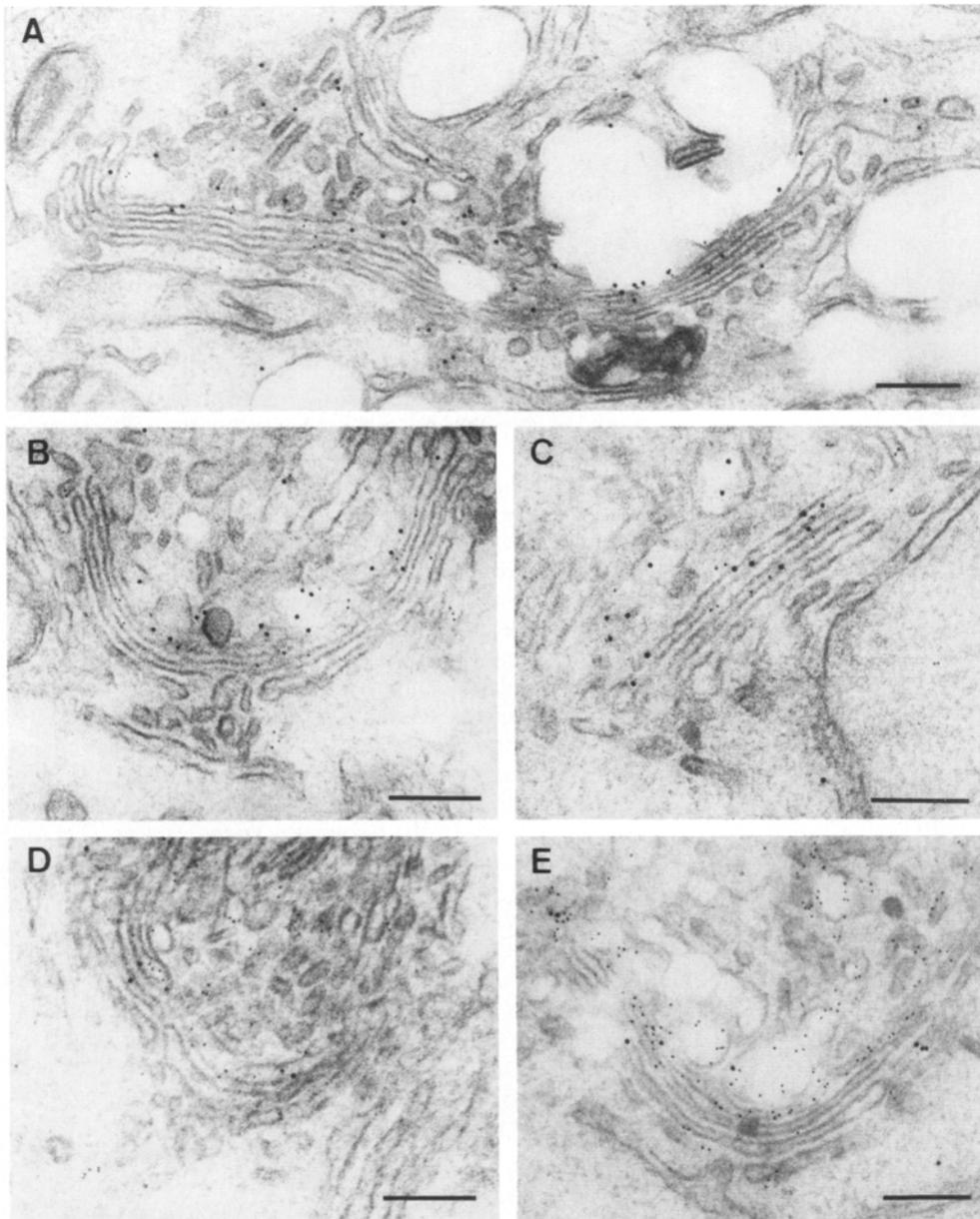


Figure 5. Distribution of GalT and mycNAGT I by immuno-EM. Thin, frozen sections of the stable HeLa cell line were labeled with both polyclonal anti-GalT and 9E10 antibodies followed by appropriate second antibodies coupled to different sizes of gold. (A-C) GalT = 10 nm gold, mycNAGT I = 5 nm gold; (D and E) GalT = 5 nm gold, mycNAGT I = 10 nm gold. Bars, 0.2 μm .

localized by immuno-EM, it was of interest to determine their distribution by confocal microscopy. Work by Rambourg and Clermont (1990) suggests that the Golgi apparatus in three dimensions comprises a series of stacks linked by tubules to form a ribbon, the tubules linking equivalent cisternae in adjacent stacks. The cisternae themselves are in register, that is they sit one on top of the next in the stack. With this in mind, Fig. 7 shows a series of sections through the nuclear region of the stable HeLa cell line labeled so as to reveal GalT in green (FITC) and mycNAGT I in red (Texas red). The two columns on the left show the unmerged pictures, that on the right the merged ones. Overlap registers as yellow.

There are three clear types of image in the merged pictures. The first is a broad area of a single color which changes as one moves through the sections. The green area in Fig. 7 C (*arrowhead*) changes to red in Fig. 7 L (*arrow-*

head) passing through intermediates of all three colors (Figs. 7, F and I, *arrowheads*). This is most easily interpreted as an en face section through the Golgi moving from the TGN (Fig. 7 C) through the *trans* (Fig. 7, F and I) to the *medial* (Fig. 7 L). The GalT labeling appears stronger and more even than that of the mycNAGT I but this may simply reflect the differences in sensitivity of the optical system rather than a change to a more fenestrated structure.

The second type of image is a compact series of three colors with yellow in the middle (Fig. 7, I and L, *arrows*). The observed width and thickness is consistent with a transverse section through a stack. The third and last type shows the same distribution of the three colors (Fig. 7 L, *open arrowhead*) but they are more spread out suggesting a tangential section through a stack.

As a positive control to show that the separation of labels was not an artefact of the procedure or a limitation of the ma-

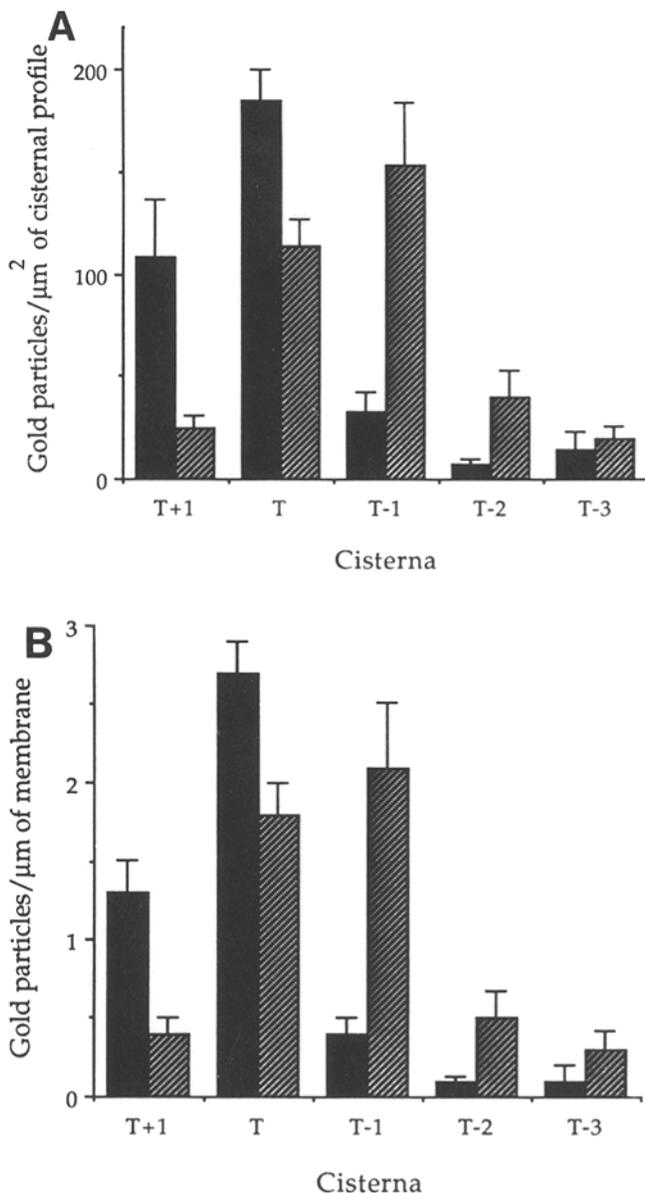


Figure 6. Quantitative distribution of GalT (■) and mycNAGT I (▨). Images such as those presented in Fig. 5, A–C were used to calculate: (A) the density of the two different sizes of gold over each cisternal profile. Results are presented as the mean number of gold particles per $\mu\text{m}^2 \pm \text{SEM}$ ($n = 42$). Background labeling was 1.9 (GalT) or 5.4 (mycNAGT I) gold particles/ μm^2 of cytoplasm; (B) the linear density of the two different sizes of gold along each cisternal membrane. Results are presented as the mean number of gold particles per μm length of membrane $\pm \text{SEM}$ ($n = 42$).

chine, GalT was double labeled using a monoclonal (Berger et al., 1986) as well as the polyclonal antibody. The labels were superimposable (data not shown).

Discussion

The technical success of these double-label experiments relied on a stable HeLa cell line expressing NAGT I tagged with a myc epitope. The cDNA used encoded human NAGT I so there was no species incompatibility. The myc tag was

placed at the extreme COOH terminus, well away from the retention signal at the other end of the protein (Tang et al., 1992). The myc tag itself is only 11 amino acids in length, <2.5% of the NAGT I protein, and has been used on many occasions with no discernible effect on the location of the protein (for example see Munro and Pelham, 1986). The tag had no apparent effect on the folding of the protein since a fourfold increase in activity was registered by enzyme assay. Enzymatic activity is probably the most sensitive measure of proper folding and lessens the possibility that the observed distribution is the consequence of a misfolded form of the protein. Endogenous NAGT I is a minor protein of the Golgi constituting <0.2% of Golgi membrane protein in rabbit liver (Nishikawa et al., 1988). A fourfold increase in amount would not then be expected to change the distribution of the protein nor the structure of the Golgi and in fact no significant changes in Golgi morphology, volume, or surface area were detected. There was also no change in the distribution or amounts of the other Golgi enzyme, GalT. Taken together these data argue very strongly that the observed distribution of NAGT I is not the consequence of expressing a myc-tagged version of the protein.

An overlapping distribution of these two Golgi enzymes does not conflict with any of the published data. Fractionation experiments showed that the peak of GalT activity could be separated from NAGT I but the distributions on the gradient overlapped leaving open the possibility that they shared a cisterna. All immunocytochemical observations of medial- and trans-Golgi enzymes have been single-label experiments and their locations attributed using morphological criteria. The fact that different cell types from different species were used made it almost impossible to predict that overlap might have been present.

In earlier experiments NAGT I was mostly found in two adjacent cisternae (Dunphy et al., 1985) suggesting duplication of the cisterna containing the second-stage oligosaccharide-modifying enzymes. The same was true for GalT (Roth and Berger, 1982) suggesting duplication of the trans-cisterna each containing third stage enzymes. Our data argue strongly against cisternal duplication. Overlapping distributions of these two enzymes generates three distinct cisternae not two sets of duplicate cisternae. This also means that each cisterna differs from its neighbors not by having completely different sets of enzymes but by having different mixtures. The large number of oligosaccharide-modifying enzymes in the Golgi could generate many mixtures, enough that even in those rare cases where the Golgi comprises stacks of a score or more cisternae (for example see Grassé and Carasso, 1957), each cisterna could have a different mixture and therefore have a different set of functions.

There has been a recent suggestion (Mellman and Simons, 1992) that the cisternae in the stack are physically interconnected with each cisterna representing a domain within the overall structure. There is a precedent for such a structure in the ER which has both rough and smooth domains that are physically connected but these connections are frequently seen in marked contrast to those postulated for the Golgi stack. Only further work will determine whether each cisterna is discrete or part of an interconnected whole.

The presence of each enzyme in two adjacent cisternae might be relevant to cisternal stacking. Golgi residents are retained in their appropriate cisternae by retention signals in

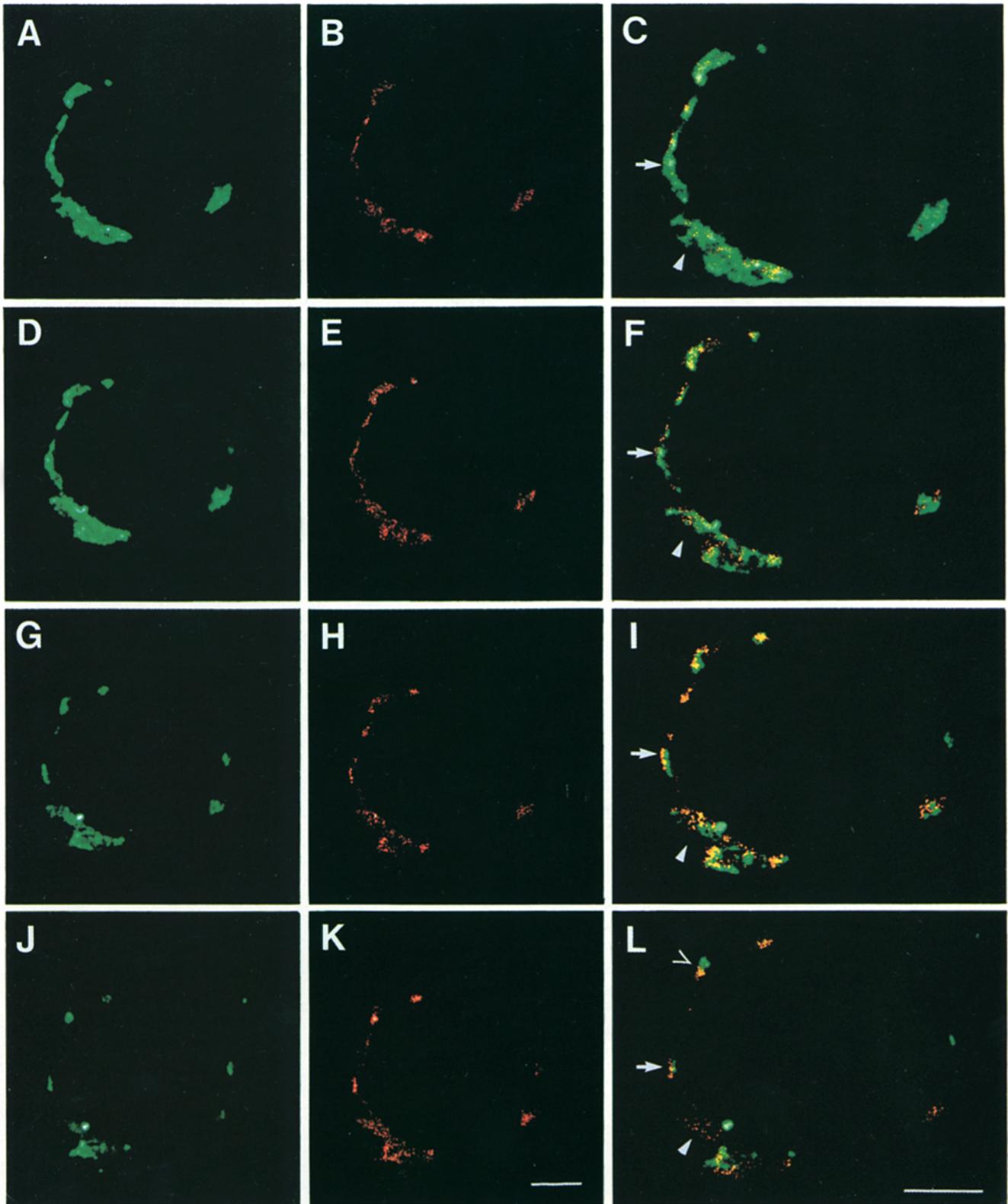


Figure 7. Distribution of GalT and mycNAGT I by confocal microscopy. Cells were fixed, permeabilized, and labeled with anti GalT and 9E10 antibodies followed by appropriate second antibodies to reveal GalT (*green*) and mycNAGT I (*red*). Z sections were taken every 0.5 μm , from top to bottom, parallel to the coverslip, and nine were needed to encompass the whole cell. Sections 4 (A-C), 5 (D-F), 6 (G-I), and 7 (J-L) are shown with the cell nucleus in the middle. The unmerged pictures are shown in the two columns on the left, the merged picture in the column on the right. The indicated structures are discussed in Results. Bar, 7 μm .

the membrane spanning domain (Swift and Machamer, 1991; Munro, 1991; Nilsson et al., 1991; Aoki et al., 1992; Colley et al., 1992; Russo et al., 1992; Teasdale et al., 1992; Wong et al., 1992). The flanking regions and cytoplasmic tail may play an accessory role (Munro, 1991; Nilsson et al., 1991). The retention mechanism is unclear but an attractive possibility is that the residents of each cisterna form heterooligomers so large that they cannot enter budding vesicles and so are retained. If such oligomers were present in adjacent cisternae, but only on one face such that they could interact across the intercisternal gap, then this would provide a stacking mechanism and explain why they are present in two cisternae. A linking protein would be needed to join the short cytoplasmic tails because the intercisternal space is ~ 100 Å in width. Such a mechanism could accommodate any number of cisternae and provide a template for reassembling the Golgi both at the end of mitosis (Lucocq et al., 1989) or after treatment with drugs such as brefeldin A (for review see Klausner, 1992). Unfortunately, it has not been possible to obtain evidence for such a distribution of enzymes within the cisternae. They are too narrow, relative to the size of the antibodies and gold conjugates, to determine which side the labeling is on and all efforts to enlarge the cisternae selectively have, so far, failed.

Although most of each enzyme is present in two adjacent cisternae there is also some in the cisternae on either side. Though statistically insignificant (with one exception) at the $p = 0.05$ level, the levels are many-fold higher than background and they tail off in the cisternae furthest from the labeled two. They could indicate a recycling pathway which makes up for any deficiencies in the Golgi retention mechanism. NAGT I, leaving the *trans*-cisterna, would end up in the TGN and be returned to an earlier part of the stack, perhaps the *cis*-cisterna. This retrograde pathway might be similar to one of those already described (for reviews see Pelham, 1989; Klausner, 1992) and would imply the presence of a retrieval signal on Golgi enzymes as has been shown for both soluble and membrane proteins of the ER. The rate of recycling should ensure that each molecule of NAGT I passes through the TGN at least once within its lifetime so one would expect to see evidence of such passage from examination of bound oligosaccharides. The absence of N-linked oligosaccharides from NAGT I precludes analysis of this protein but mannosidase II, which should codistribute with NAGT I, exhibits *trans*-Golgi modifications such as sulphation of N-linked oligosaccharides (Moremen and Touster, 1985), consistent with a recycling pathway.

Stable cell lines such as that described here should solve the problem of localizing Golgi enzymes. Available antibodies to resident Golgi proteins are either unsuitable for immuno-EM or have a very restricted species specificity. This paper, in fact, represents the first double labeling of Golgi enzymes originally thought to be in different cisternae. Earlier comparisons had to rely on those features of the Golgi that could be measured in different cell types such as cisternal number, morphology, and cytochemical reactivity. Unfortunately, these criteria were inadequate as evidenced by the present work though the generality of our observations must await the production of other stable lines expressing mycNAGT I. There are several tags, other than myc, which work by immuno-electron microscopy so as more and more Golgi enzymes are cloned and sequenced, they can be tagged

and expressed, either stably or transiently, in the mycNAGT I line. Double labeling would then allow precise localization relative to mycNAGT I, with no need to consider morphology beyond the ability to determine the physical boundary of the cisterna being measured. This approach is not restricted to cell type and should, in time, allow a complete map of the Golgi enzymes to be built up.

A panel of stable cell lines expressing different, tagged Golgi enzymes would also be useful for assigning locations to other proteins. There are many antibodies which appear to stain the Golgi region but do not work by immuno-EM, so that the precise location cannot be determined. Our results using confocal microscopy show that different parts of the Golgi apparatus can be distinguished. The resolution of this technique must be of the order of 0.05 μm , comprising the width of the cisterna (~ 0.04 μm) and the intercisternal space (~ 0.01 μm), which is slightly better than the theoretical resolution of 0.1 μm . It should not only be possible to assign proteins to the Golgi apparatus but even to particular parts of it. This would remove much of the present uncertainty and provide a firmer foundation upon which to test models of Golgi function.

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