Immunocytochemical Localization of Galactosyltransferase in HeLa Cells: Codistribution with Thiamine Pyrophosphatase in *trans*-Golgi Cisternae

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ABSTRACT An affinity-purified, monospecific rabbit antibody against soluble human milk galactosyltransferase was used to localize the enzyme in HeLa cells by immunofluorescence and by the protein A-gold technique at the electron microscope level. Specific immunofluorescence was observed in a juxtanuclear cytoplasmic region which was identified, on immunostained thin sections of low-temperature Lowicryl K4M-embedded HeLa cells, as Golgi apparatus. Label by gold particles was limited to two to three *trans* cisternae of the Golgi apparatus, indicating a compartmentalization of galactosyltransferase in the cisternal stack. Combination of preembedding thiamine pyrophosphatase cytochemistry with postembedding immunostaining for galactosyltransferase proved codistribution of the two enzymes. However, the acid phosphatase-positive, *trans*-most cisterna was negative for galactosyltransferase. The close topological association of both galactosyltransferase and thiamine pyrophosphatase (or nucleoside diphosphatase) suggests a concerted action of both enzymes in glycosylation.

Biosynthesis of complex type heteroglycans proceeds in two distinct stages in different cellular organelles: first, *N*-glycosylation involving the en bloc transfer of a lipid-linked oligosaccharide takes place in the rough endoplasmic reticulum (for recent review, see reference 27) where part of it is processed by glucosidases (6); thereafter, the glycoproteins move to the Golgi apparatus where *N*-linked glycans are further processed (10) before being elongated by sequential action of terminal glycosyltransferases which include *N*-acetyl-D-glucosaminyl-, galactosyl-, fucosyl-, and sialyltransferases (for review, see reference 26). On the basis of cell fractionation studies (3, 5, 11, 15) and autoradiographic (1, 19) evidence, chain elongation is assumed to occur in the Golgi apparatus. Accordingly, galactosyltransferase activity has frequently been selected as marker for Golgi fractions (see 3, 5, 8, 11, 14, 15, 26 for selected references). In order to obtain conclusive *in situ* evidence of the Golgi-association of galactosyltransferase (UDP-galactose: β-D-*N*-acetylglucosaminyl-protein β (1–4) transferase, EC 2.4.1.22) and to get insight into the organization of this organelle with respect to the glycosylation mechanism, an immunocytochemical study in HeLa cells was conducted. For this purpose the protein A–gold (pAg) technique as developed by Roth et al. (20, 22) was chosen. This postembedding staining method does not have the drawback of limited antibody penetration into cells characteristic of the preembedding labeling techniques. In addition, the use of gold particles as marker results in excellent and clear localization of the antigen to cellular organelles (23, 24). We report here the first ultrastructural localization of a glycosyltransferase, namely galactosyltransferase, and demonstrate its presence in a distinct Golgi subcompartment which is composed of two to three thiamine pyrophosphatase-positive *trans*-Golgi cisternae.

MATERIALS AND METHODS

Galactosyltransferase was purified from pooled human milk (9). The immunization schedule included six subcutaneous injections of 0.2 mg of galactosyltransferase emulsified in complete Freund's adjuvant into rabbits. Antibody production was monitored by an enzyme-linked immunosorbent assay (ELISA) (2). Monospecific antibodies were obtained by affinity purification on immobilized galactosyltransferase. Their specificity towards purified galactosyltransferase was checked by ELISA (2) and the immunoreplica technique essentially according to Towbin et al. (29), except that the antigen on the nitrocellulose sheet was developed using a protein A–peroxidase conjugate as described in reference 2.

HeLa cells (Microbiological Associates, Bethesda, MD) were grown in culture dishes in medium F11 (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum until confluence of the monolayer was reached.

Confluent monolayers were fixed with chilled acetone for 5 min, rinsed five times for 5 min each with PBS (0.15 M NaCl, 0.05 M phosphate buffer pH 7.3), and stored in PBS overnight at 4°C. The cells were incubated *in situ* with the galactosyltransferase antibody (100 μg/ml) for 2 h at room temperature, rinsed three times with PBS for 5 min each, and exposed to fluorescein isothiocyanate (FITC)–labeled protein A (0.1 mg/ml; Pharmacia Fine Chemicals, Uppsala,

Sweden) for 1 h at room temperature. The cells were counterstained with 0.01% Evans blue and observed in a Leitz Ortholux fluorescence microscope equipped with a Ploemopak L.2 illuminator. Specificity controls included (*a*) incubation with the galactosyltransferase antibody preabsorbed with an excess of purified galactosyltransferase followed by FITC-labeled protein A and (*b*) omission of the antibody incubation step.

For antigen localization on thin sections of resin-embedded cells by the pAg technique (20, 22), confluent monolayer cultures were fixed in 0.5% glutaraldehyde or in 2% formaldehyde with 0.1% glutaraldehyde in PBS for 30 min at room temperature, rinsed several times with PBS, and treated with 0.5 M NH_4Cl in PBS for 45 min in order to block free aldehyde groups. After further rinses in PBS, the cells were harvested using a rubber policeman, pelleted by centrifugation, and enclosed in 1% agar. Since we were unable to localize the antigenic sites on thin sections of Epon-embedded cells, we performed embedding according to the recently developed low-temperature embedding procedure using the polar methacrylate mixture Lowicryl K4M (4, 12). This embedding protocol has been shown to provide better conditions for the preservation of antigenicity and cellular fine structure than Epon embedding (21).

Thin sections (60–80 nm) were mounted on nickel grids having a Parlodion-carbon film and processed for the pAg technique. Nickel grids with the attached thin sections were floated on a drop of PBS for 5 min, transferred to a drop of galactosyltransferase antibody (100 μg/ml) for 2 h at room temperature in a moist chamber, carefully washed with PBS, and further incubated with the pAg complex for 1 h at room temperature. After rinses with PBS and distilled water, sections were counterstained with 2% uranyl acetate (7 min) and lead citrate (45 s) and examined in a Philips EM 300 electron microscope at 60 kV.

In addition, monolayer cultures were labeled before embedding in order to visualize cell-surface galactosyltransferase. Unfixed cells or prefixed cells (fixed in 2% newly prepared formaldehyde in PBS for 5 min) were incubated with the galactosyltransferase antibody (100 μg/ml) for 1 h at 4°C, followed by pAg for 1 h at 4°C. After rinses with cold PBS, fixation with 2% glutaraldehyde in PBS for 30 min at 4°C and 1% OsO_4 for 1 h was performed. Cells were embedded in Epon.

Cytochemical controls included: (*a*) replacement of the antibody by an antibody preabsorbed with purified galactosyltransferase followed by the pAg complex, (*b*) omission of the antibody incubation step, and (*c*) incubation with the antibody followed by 1-h incubation with nonlabeled pA (0.1 mg/ml) and then by pAg complex.

The intensity of labeling expressed as number of gold particles/μm^2 of labeled *trans*-Golgi cisternae, remaining Golgi apparatus, lysosomes, mitochondria, cytoplasm (without lysosomes and mitochondria), and nucleus was evaluated on a total of 45 HeLa cells from three different confluent monolayers. Micrographs were taken on 70-mm film at the primary magnification of 25,000 (for the Golgi apparatus) or of 11,000 (for the other cellular compartments) calibrated with a carbon grating replica (2,160 lines/mm). The surface of the different compartments as well as the number of gold particles present over them was recorded on a graphic tablet (Tektronix, type 4973) connected to a microprocessor system (IMSAI, type 8080) programmed to calculate the number of particles/μm^2 of the compartment. (D. Bertrand and M. Amherdt, manuscript in preparation). Statistical comparison of the values was done with the Student's *t* test.

Confluent cell cultures were fixed in 2% formaldehyde with 0.1% glutaraldehyde in PBS for 30 min, washed several times with 7% sucrose in PBS, and stored in 7% sucrose overnight at 4°C. Cytochemical reactions were performed on monolayer cultures that had been frozen and thawed. Thiamine pyrophosphatase (TPPase) activity was demonstrated by the method of Novikoff and Goldfischer (17) (incubation time, 40 min at 37°C). Substrate omission and the addition of 10 mM sodium fluoride served as the cytochemical controls.

Acid phosphatase was localized as described by Novikoff (16) with cytidine-5′-monophosphate as substrate (incubation time, 15–40 min at 37°C). Controls consisted of incubation in a substrate-free medium or in medium containing 10 mM sodium fluoride. Afterwards, the cultures were washed with 7% sucrose, fixed with OsO_4, and embedded in Epon.

Cells were incubated *in situ* for TPPase as described above. After washes with sucrose and without OsO_4 fixation, low-temperature Lowicryl K4M embedding followed. Finally, thin sections were prepared and processed for galactosyltransferase localization as already described.

RESULTS

Galactosyltransferase purified from human body fluids by sequential affinity chromatography on *N*-acetylglucosamine-agarose and α-lactalbumin agarose yields a family of charge heteromorphs with similar molecular weights, all of them exhibiting comparable enzyme activity and substrate specificity (9). The structural basis of charge heterogeneity as revealed by analytical isoelectric focusing is unknown; the pattern produced by this technique is shown in Fig. 1: in the absence of specific galactosyltransferase zymogram techniques, the focusing pattern may be used as "fingerprint equivalent" to identify the enzyme, as it is characteristic and highly reproducible (compare Fig. 1*A* with pattern published in reference 9). The affinity-purified rabbit antibody against human milk galactosyltransferase was tested for its specificity by several methods including enzyme inhibition, double immunodiffusion (our unpublished observations), and ELISA (2). The immunoreplica technique (29) was applied to purified and crude galactosyltransferase (Fig. 1 *B* and *C*) resolved on isoelectric focusing: replication of the galactosyltransferase pattern using crude human milk confirmed the specificity of the antibody used and showed that it is directed against all charge heteromorphs of galactosyltransferase.

Fig. 2 shows the immunofluorescence pattern of HeLa cells

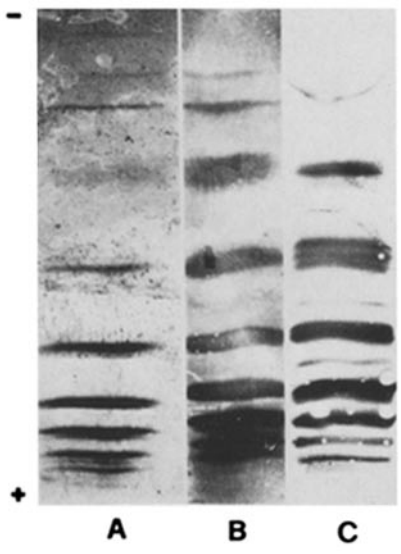


FIGURE 1 Immunoreplica of human milk galactosyltransferase resolved on isoelectric focusing. Purified enzyme was subjected to analytical isoelectric focusing in a pH gradient 3.5–9 as previously described (9). Lane *A* shows a Coomassie Blue stain of purified enzyme which is composed of at least nine charge heteromorphs. Lane *B* show its corresponding immunoreplica (from a different gel). Lane *C* shows a replica from crude human milk resolved on isoelectric focusing.

FIGURE 2 Immunofluorescence for galactosyltransferase in HeLa cells. Brightly fluorescent reticular or ribbonlike structures are located in a juxtanuclear region (arrows). The white spots correspond to the positive structures out of the plane of focus. × 330.

FIGURE 3 Control for specificity of immunofluorescence. Replacement of the antibody by antigen-preabsorbed antibody results in abolition of cellular fluorescence. × 330.

FIGURE 4 Lowicryl K4M thin section showing part of a HeLa cell. Specific labeling for galactosyltransferase as indicated by gold particles is present over a horizontally cut Golgi apparatus. Small vesicles in the Golgi region, the remaining cytoplasm and part of the nucleus is negative. The arrowhead points to a sticky microaggregate of pAg complexes that is nonspecifically adsorbed to the section. *Ly*, lysosomes (verified by positive reaction for acid phosphatase); arrows point out rough endoplasmic reticulum. × 31,000.

FIGURES 5–7 In a perpendicular cut (along the *cis-trans* axis) Golgi apparatus, galactosyltransferase immunoreactive sites are present in two to three *trans* cisternae. Note the absence of label over the other Golgi cisternae including the *trans*-most cisterna (*TC* in Fig. 7). Since cells were only aldehyde fixed, membranes appear as white lines. (Fig. 5) × 48,000; (Figs. 6 and 7) × 66,000.

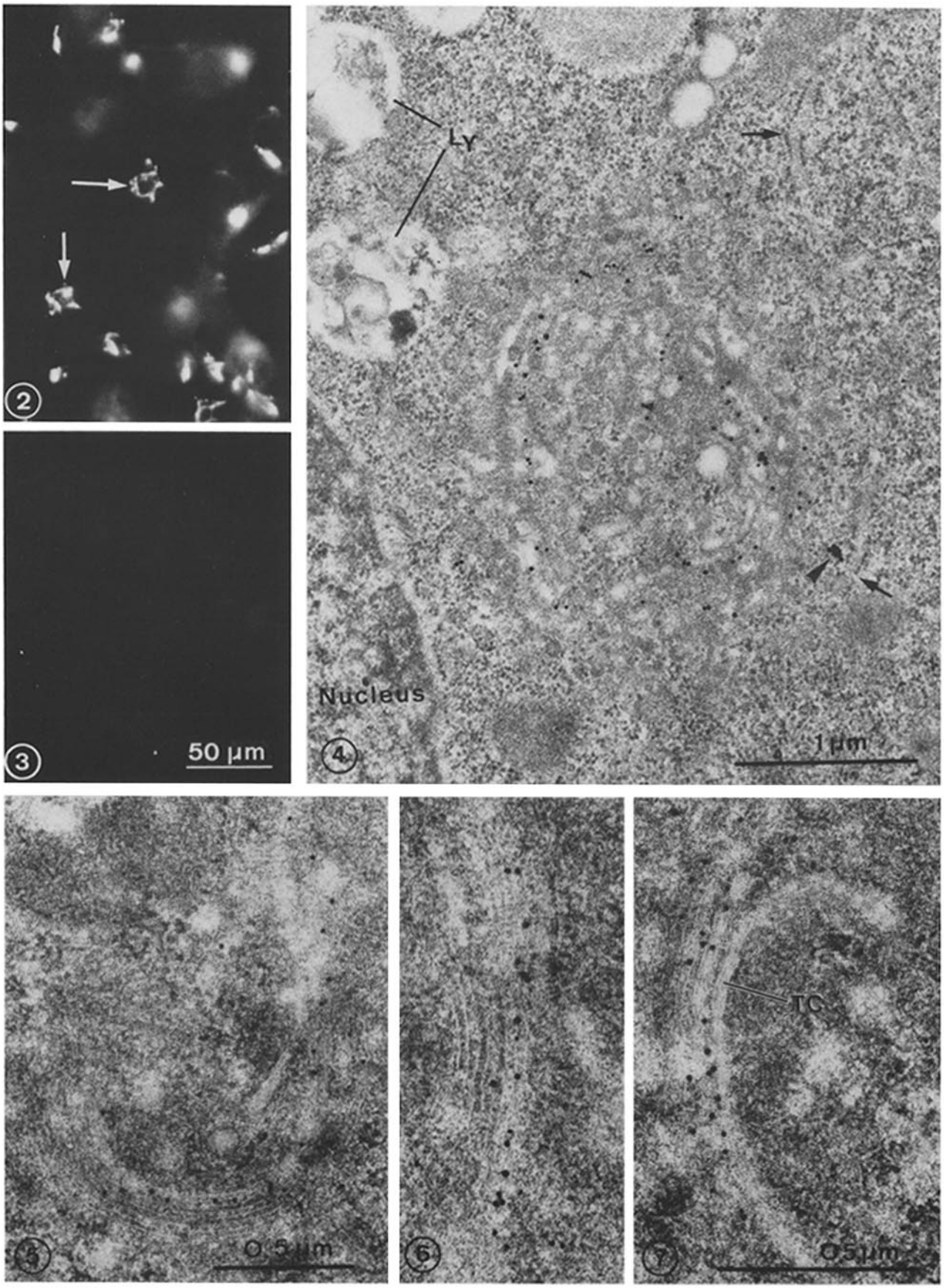

2
3
50 μm
LY
Nucleus
4
1 μm
0.5 μm
6
TC
7
0.5 μm

TABLE I

Density of Labeling for Galactosyltransferase over Cellular Compartments of HeLa Cells, gold particles/μm^2 ± SEM

	Trans-Golgi cisternae	Remaining Golgi apparatus	Lysosomes	Mitochondria	Remaining cytoplasm	Nucleus
Antigalactosyltransferase*	96.28 ± 4.75	0.87 ± 0.48	1.13 ± 0.52	2.01 ± 0.41	1.02 ± 0.59	1.89 ± 0.29
Antigen-adsorbed antibody	0.46 ± 0.29	0.49 ± 0.27	0.29 ± 0.18	0.28 ± 0.09	0.10 ± 0.07	0.93 ± 0.22
pAg alone	0.18 ± 0.05	0.16 ± 0.07	0.15 ± 0.07	0.31 ± 1.1	0.08 ± 0.06	0.07 ± 0.03
Antigalactosyltransferase-pA-pAg	0.21 ± 0.09	0.17 ± 0.05	0.14 ± 0.03	0.30 ± 0.9	0.10 ± 0.06	0.1 ± 0.04

* Data based on evaluation of a total of 45 cells from three different cultures. Student's *t* test analysis of data indicates significant differences ($P < 0.001$) in labeling intensity over *trans*-Golgi cisternae compared to other cellular compartments and control values.

stained *in situ*. In each cell a fluorescent reticular or ribbonlike structure was seen in the juxtanuclear region. The immunofluorescence was specific for galactosyltransferase since it could be completely abolished under the different conditions of the cytochemical controls (Fig. 3).

The fine structure of HeLa cells after mild aldehyde fixation and low-temperature embedding in Lowicryl K4M was generally well-preserved. As shown in Fig. 4, a specific labeling for galactosyltransferase as revealed by the presence of gold particles was present only over the Golgi apparatus. Small vesicles present in this region were practically devoid of label. Sometimes, a low number of gold particles was scattered over the nucleus and the cytoplasm. However, as judged from the cytochemical controls, these gold particles and especially small-particle aggregates were due to a low degree of nonspecific background staining. In controls, as defined in Materials and Methods, the labeling by gold particles over the Golgi apparatus was abolished (Table I).

In cases where the Golgi apparatus was cut along the *cis-trans* axis, the gold particle label was found only over two to three Golgi cisternae at the *trans* (inner maturing) side of the Golgi apparatus (Figs. 5–7). The cisternae in intermediate regions and at the *cis* side (Figs. 5 and 6) as well as the *trans*-most cisterna[1] (Fig. 7) were not labeled. This labeling pattern was consistently found in all Golgi apparatus examined. No definite decision could be made as to whether the gold particle labeling was related to membranes or to the cisternal space of Golgi cisternae, since the best lateral resolution of the pAg technique as performed in this study is ~18 nm. Cell-surface galactosyltransferase immunoreactive sites were not detectable by light or electron microscopy.

In HeLa cells, reaction product for thiamine pyrophosphatase activity was found only in two to three Golgi cisternae at the *trans* (inner maturing) side (Fig. 8). Acid phosphatase activity was found only in the *trans*-most cisterna adjacent to the Golgi stack and in lysosomes (Fig. 9). In Figs. 10–12, the double staining for TPPase and galactosyltransferase is illustrated. The gold particle label revealing galactosyltransferase immunoreactive sites was associated only with TPPase-positive *trans*-Golgi cisternae. Both enzymes were absent in the *trans*-most cisterna (Figs. 10 and 11).

DISCUSSION

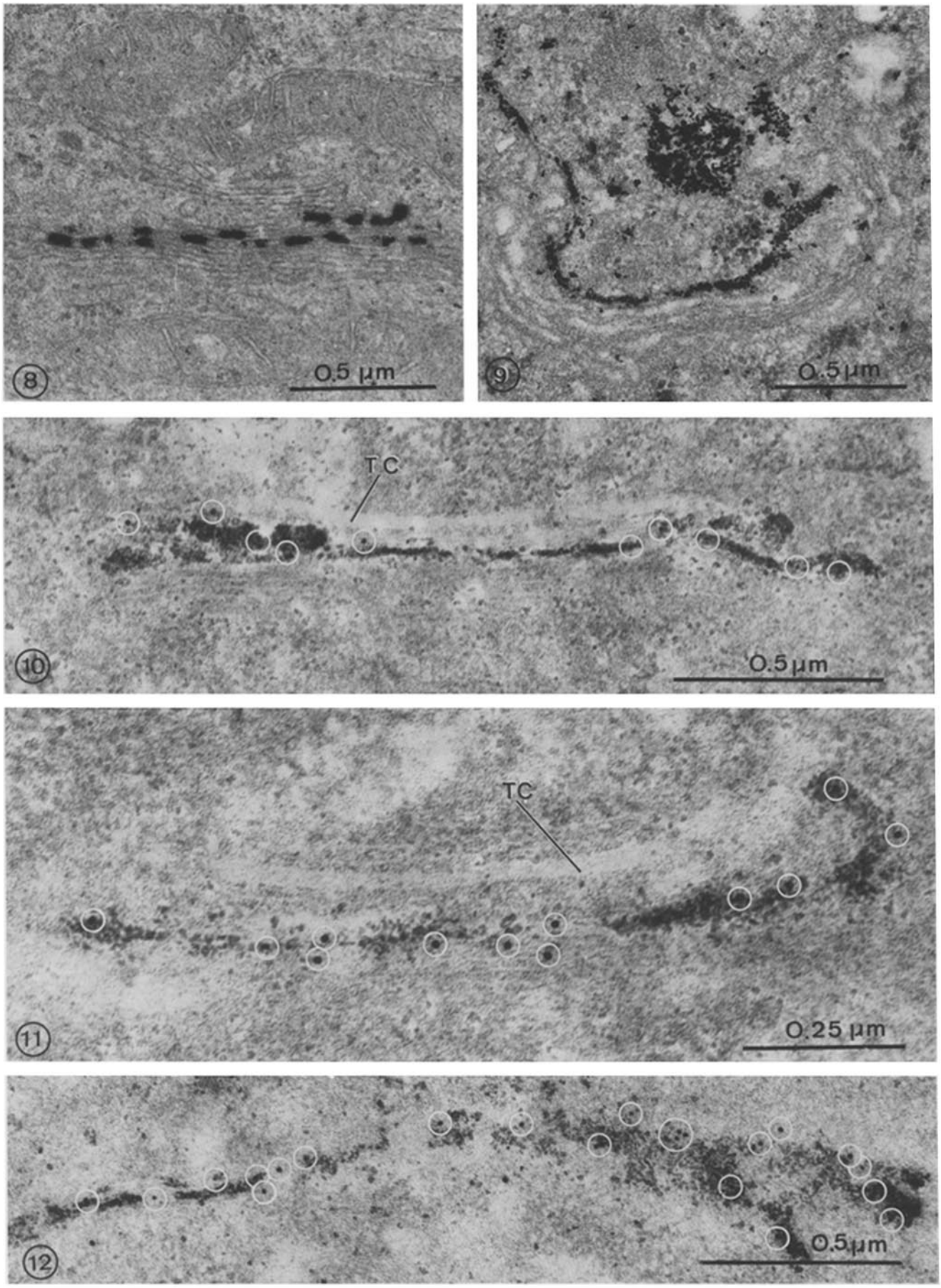
Functional and structural heterogeneity of membranes of the Golgi apparatus has been demonstrated by biochemical studies on Golgi subfractions, measurement of lipid and enzyme content in isolated Golgi cisternae, by different histochemical techniques such as osmium impregnation, enzyme or carbohydrate histochemistry, and by thin-section and freeze-fracture electron microscopy (for comprehensive literature, see references 7, 16, 18, 28). This diversity suggests the existence of functional subcompartments within this complex organelle (25, 28). In the present work, we provide *in situ* evidence by immunoelectron microscopy that, in HeLa cells, galactosyltransferase immunoreactive sites are localized in two to three cisternae of the *trans* side of the Golgi apparatus. It should be borne in mind that all antigenic sites may not be preserved after aldehyde fixation and resin embedding for subsequent immunocytochemical localization with the pAg technique or might be present in amounts too low to be immunocytochemically demonstrable. With respect to possible antigen denaturation due to organic solvents and resins, the low-temperature embedding procedure employed in this study provides favorable conditions for antigen preservation and is superior to Epon embedding which was unsuccessfully applied for immunocytochemical localization of galactosyltransferase. Since the Golgi labeling pattern for galactosyltransferase was consistent and reproducible, we believe we are justified to assume that the observed concentration of gold particles on *trans* cisternae represents meaningful topographic specialization and an example of subcompartmentalization within the stack of Golgi cisternae.

[1] The term "*trans*-most cisterna" refers to a cisterna closely adjacent to the Golgi stack. This cisterna is acid phosphatase positive and distal to the TPPase-positive *trans*-Golgi cisternae. Therefore, the *trans*-most cisterna is representing an element of GERL.

FIGURE 8 Demonstration of TPPase activity in single cisternae at the *trans* side of a Golgi apparatus. × 55,000.

FIGURE 9 Acid phosphatase activity is seen in a *trans*-most cisterna and in lysosomes. Note the absence of reaction product in the Golgi stack. × 50,000.

FIGURES 10–12 Double staining for TPPase activity (revealed by the amorphous electron-dense reaction product) and galactosyltransferase immunoreactive sites (indicated by gold particles) by combination of preembedding and postembedding staining, respectively. In this procedure, osmium fixation had to be omitted; therefore, membranes are negatively stained in contrast to their appearance in Figs. 8 and 9. Gold particles (○) are present over TPPase-positive cisternae, whereas other cisternae including the *trans*-most cisterna (*TC*) are free of gold particle label. In Fig. 12, gold particles are scattered over an obliquely sectioned TPPase-positive cisterna. (Fig. 10) × 79,000; (Fig. 11) × 120,000; (Fig. 12) × 73,000.


8
0.5 µm
9
0.5 µm
TC
10
0.5 µm
TC
11
0.25 µm
12
0.5 µm

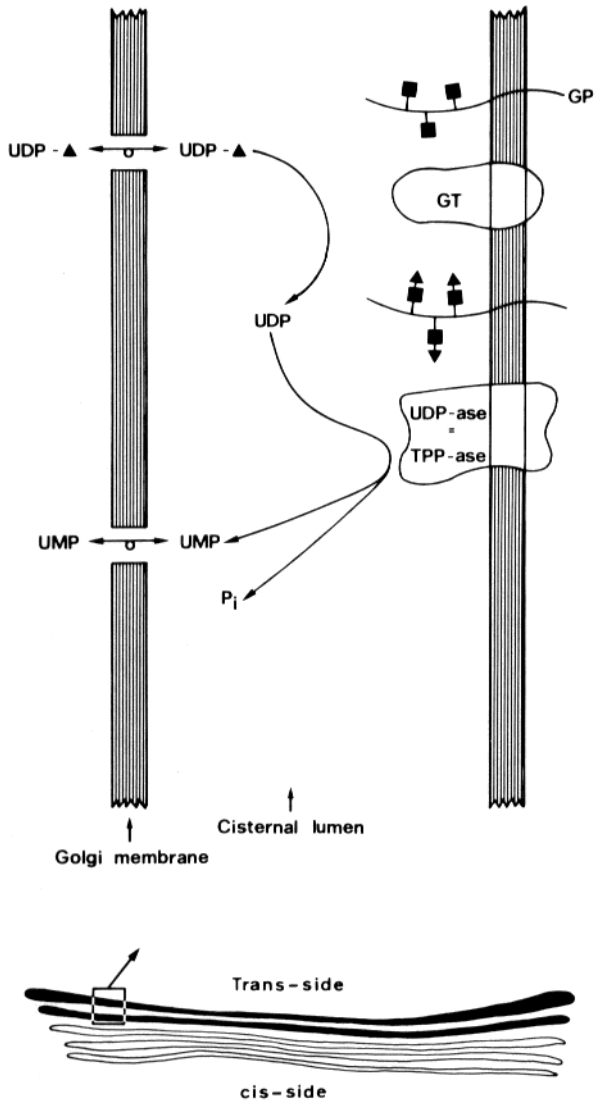


FIGURE 13 Schematic localization of both galactosyltransferase and TPPase (or nucleoside diphosphatase) in *trans* cisternae of the Golgi apparatus in HeLa cells. Codistribution of both enzymes supports a model of glycosylation proposed by Kuhn and White (13) for lactose biosynthesis in the mammary gland: On the basis of kinetic evidence, a carrier-mediated transport mechanism for glucose, UDP-galactose, and UMP through Golgi membranes and a diffusion barrier for UDP were postulated. Galactosyltransferase (*GT*) catalyzes the transfer of galactose (▲) to glycoprotein acceptors containing GlcNAc (■) at the nonreducing end of the heteroglycan. This reaction generates free UDP, which is immediately hydrolyzed by UDPase (or TPPase). This prevents the build-up of inhibiting concentrations of UDP.

The codistribution of galactosyltransferase with TPPase was directly demonstrated by a double-staining approach. The presence of immunolabel for galactosyltransferase in *trans* cisternae corresponding to the distribution of TPPase activity supports the assumption that glycosyltransferases are localized in specific Golgi subcompartments. Our morphological results confirm biochemical evidence obtained by Hino et al. (11) that galactosyltransferase activity is associated with TPPase in Golgi subfractions which probably correspond to the secretory (*trans*) side of the organelle, whereas Golgi subfractions enriched for several endoplasmic reticulum "marker" enzymes are depleted in glycosyltransferase activities. The close relationship between galactosyltransferase and TPPase strongly supports a model of glycosylation proposed by Kuhn and White (13) which we have extended as shown in Fig. 13. In this context it is of particular interest that Yamazaki et al. (30) demonstrated that nucleoside diphosphatase paralleled TPPase activity over the whole purification procedure applied to bovine liver microsomes and that both activities are manifested by a single enzyme. The membrane-association of galactosyltransferase has not been demonstrated by our immunocytochemical results due to the limited lateral resolution of the pAg technique. Evidence for it is based on detergent activation of the Golgi-membrane enzyme reported by numerous investigators (2, 3, 15). In conclusion, the data demonstrate the presence of galactosyltransferase in *trans* (inner maturing) Golgi cisternae that are positive for TPPase activity and are in accordance with a model that proposes a concerted action of both galactosyltransferase and nucleoside diphosphatase in chain elongation of maturing glycoproteins.

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