Immunocytochemical Demonstration of
Ecto-galactosyltransferase in Absorptive Intestinal Cells

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ABSTRACT Galactosyltransferase immunoreactive sites were localized in human duodenal enterocytes by the protein A-gold technique on thin sections from low temperature Lowicryl K4M embedded biopsy specimens. Antigenic sites detected with affinity-purified, monospecific antibodies were found at the plasma membrane of absorptive enterocytes with the most intense labeling appearing along the brush border membrane. The lateral plasma membrane exhibited a lower degree of labeling at the level of the junctional complexes but the membrane interdigitations were intensely labeled. The labeling intensity decreased progressively towards the basal part of the enterocytes and reached the lowest degree along the basal plasma membrane. Quantitative evaluation of the distribution of gold-particle label proved its preferential orientation to the outer surface of the plasma membrane. In addition to this membrane-associated labeling, the glycocalyx extending from the microvillus tips was heavily labeled. Occasionally, cells without plasma membrane labeling were found adjacent to positive cells. The demonstration of ecto-galactosyltransferase on membranes other than Golgi membranes precludes its general use as a marker for Golgi membrane fractions. The possible function of galactosyltransferase on a luminal plasma membrane is unclear at present, but a role in adhesion appears possible on the basolateral plasma membrane.

In 1970, Roseman (24) put forward a hypothesis on a possible role of cell surface glycosyltransferases in cell adhesion and recognition. Two reviews on this subject have been published (18, 32) and give a comprehensive account of all experimental evidence supporting the original hypothesis. In summary, evidence for a cell surface location of glycosyltransferases is still based on indirect biochemical and autoradiographic data. Glycosyltransferase activities were found associated with plasma membranes upon fractionation (6, 12, 38), and orientation to the external face was assumed by their ability to glycosylate nonpermeable substrates such as glycoprotein acceptors and sugar-derivatized agarose beads (16, 36, 37). Autoradiographic evidence that consisted of the electron microscopic demonstration of radioactive substrates incorporated into the plasma membrane also suggested the presence of these enzymes on the cell surface but did not formally prove it (20). The conclusions based on the evidence summarized in the above cited reviews were challenged by Keenan and Morré (13) and Deppert et al. (8). At the present time, location of glycosyltransferase at the plasma membrane is still an open question since no clear-cut in situ demonstration has been provided. Evidence for a putative role in adhesion and recognition, for which a conclusive demonstration of glycosyltransferases on the outer side of the plasma membrane is a prerequisite, remains circumstantial (18).

Recently, monospecific antibodies against human galactosyltransferase (E.C. 2.4.1.22, 2.4.1.38, 2.4.1.90) became available and were used to localize this enzyme by immunofluorescence (3) and with the protein A-gold technique (28) in HeLa cells. The enzyme was found to be located exclusively in two to three trans-Golgi cisternae. In accordance with a previous report from Hagopian et al. (11) dealing with fractionation of HeLa cells, no immunocytochemical evidence for a cell surface location of galactosyltransferase was found in HeLa cells. However, galactosyltransferase does not seem to be absent from all cell surfaces. Recent light microscopical immunohistochemical studies on intestinal cells (17) and the fallopian tube epithelium (7) strongly suggest the presence of galactosyltransferase on the apical membrane. Due to the limited resolution of this approach it remains unclear whether cell surface-related staining truly reflected ecto-galactosyltransferase associated with the outer side of the plasma membrane.
brane, or alternatively, enzyme trapped into the mucus or associated with intracellular structures. To answer this question, we carried out an immunocytochemical study on intestinal cells that allowed us to demonstrate cell surface galactosyltransferase immunoreactive sites.

**MATERIALS AND METHODS**

**Tissue Processing for Electron Microscopy:** Human duodenal biopsies were obtained from 4- to 6-yr-old children who underwent this procedure for diagnostic purposes. Specimens from 12 donors that appeared normal upon routine histologic examination were used in this study. After aspiration, biopsy material from 12 donors was fixed in 2% paraformaldehyde/0.1% glutaraldehyde in isonicopic phosphate-buffered saline (pH 7.4) for 2 h at room temperature. After several rinses in phosphate-buffered saline, free aldehyde groups were blocked by incubation with 0.1 mol/liter NH4Cl in phosphate-buffered saline for 1 h. Tissue pieces were dehydrated in ethanol at progressively lowered temperature down to -35°C. Infiltration with Lowicryl K4M and UV-polymerization (for 24 h) was done at -35°C followed by a 2-d room temperature UV-polymerization. Thin sections were mounted on Parlodion carbon-coated nickel grids.

**Tissue Processing for Galactosyltransferase Measurements:** Approximately 20 cm duodenum obtained on abdominal surgery of a 30-yr-old female (type A blood type) was immediately frozen and stored for several months at -20°C. After thawing, mucosal scrapings were obtained and suspended in 0.05 mol/liter Tris-HCl (pH 7.4) and 2 mmol/liter phenylmethylsulfonyl fluoride. The suspension was further homogenized in a loose-fitting Dounce homogenizer and then sonicated for 30 s at 4°C at amplitude 6. The homogenate was used for the demonstration of enzyme inhibition by antibodies (Fig. 1). Galactosyltransferase activity was measured as previously described (10) using ovalbumin as acceptor substrate.

**Partial Purification of Intestinal Galactosyltransferase:** 50 ml homogenate containing 3 mmol/liter N-acetylglucosamine was applied to an α-lactalbumin-Sepharose column (250 ml) at a flow rate of 1 ml/min. The column was washed with 4 column volumes of a buffer containing 0.05 mol/liter Tris-HCl and 3 mmol/liter N-acetylglucosamine. The enzyme was eluted by omitting N-acetylglucosamine. Fractions containing activity were pooled and concentrated by pressure dialysis and Minicon to 0.25 ml. This single purification step resulted in a 1,000-fold purification of galactosyltransferase.

**Antigalactosyltransferase Antibody:** Previously used antibodies were purified by conventional affinity chromatography on immobilized antigen (3) and tested for specificity by immunoblotting (28) or ELISA (3). In this study, an alternative method for affinity purification of polyclonal rabbit antisera was applied. 1 mg galactosyltransferase prepared from milk of a blood-type 0 donor as previously described (10) was further purified by PAGE in presence of 0.1% SDS in a 12.5% gel (14). The enzyme was layered on the top of a 1 ml column (100 x 1.5-mm) of electrophorated at 4°C and electroblotted onto a sheet of Gene Screen (New England Nuclear, Boston, MA) which was "sandwiched" between the gel and a sheet of nitrocellulose (Millipore Continental Water Systems, Bedford, MA). Galactosyltransferase protein was identified on nitrocellulose by amidohydrazin staining according to Towbin et al. (34); the corresponding strip of the Gene Screen was cut out and incubated with 3% bovine serum albumin in Tris-buffered saline at 37°C for 1 h. After three washes with Tris-buffered saline, the strip was incubated with whole rabbit antisera against galactosyltransferase for 5 min at room temperature, washed three times with Tris-buffered saline, and placed for 2 min into a solution of 0.1 mol/liter glycine adjusted to a pH of 2.5 with HCl. Solid Triton X-100 was immediately added to the tube containing the eluted antibody to obtain a pH of 8.5. One incubation of this sorbent with 5 ml whole antiserum removed 10% of the antibodies as determined by ELISA.

**Immunocytochemical Procedures:** Colloidal gold was prepared as a monodisperse sol of 3-nm particles or a polydisperse sol of 3-6-nm particles with white phosphorus in ether as reducing agent and used for the preparation of protein A-gold complexes (25, 26, 29) or Ricinus communis lectin 1-gold complexes (27).

To demonstrate galactosyltransferase immunoreactive sites, we incubated thin sections with the galactosyltransferase antibody (20-200 μg/ml) for 2 h, followed by protein A-gold complex for 1 h at room temperature. Lectin binding sites were visualized by incubation of thin sections with R. communis lectin 1-gold (60 μg/ml) for 30 min at room temperature. Thin sections were counterstained with uranyl acetate and lead acetate.

**Cytotoxic Controls:** Controls for the specificity of galactosyltransferase localization were (a) the use of antigen-preabsorbed antibodies followed by protein A-gold, and (b) the omission of the antibody incubation step. Specificity of lectin labeling was checked by (a) addition of the inhibitor sugar (0.1 mol/liter galactose) to the R. communis lectin 1-gold complex, and (b) a first incubation step with an excess of nonlabeled R. communis lectin 1-gold complexes (200 μg/ml) followed by R. communis lectin 1-gold complexes for 30 min, respectively.

**Evaluation of the Gold Particle Distribution on Microvilli:** Micrographs from three biopsy specimens were taken on 70-nm film (×30,000) calibrated with a carbon grating replica (2,160 lines/mm). Distribution of gold particles over cross sections of 50 microvilli from 10 enterocytes of each biopsy specimen was determined on photographs (×100,000). Gold particles were classified as follows: (a) on the outer side of the plasma membrane, (b) overlying the plasma membrane, (c) on the inner side of the plasma membrane associated with the inner dense leaflet, and (d) over the microvillus core.

**RESULTS**

Affinity-purified antibodies were prepared from a rabbit anti-human milk galactosyltransferase antisera using a protocol detailed in the Materials and Methods section. Briefly, 1 mg human milk galactosyltransferase was subjected to SDS PAGE and electroblotted onto a nylon sheet; the strip containing the enzyme was cut and dipped into the native antiserum; antibodies were eluted from the nylon sheet by low pH and immediately neutralized. To ascertain the reactivity of these antibodies with human intestinal galactosyltransferase, we solubilized enzyme activity by sonication from mucosal scrapings of surgical specimens of duodenum, incubated with antibody and tested for activity. Fig. 1 shows the inhibition of enzyme activity with increasing amounts of antibody, thus demonstrating cross-reactivity of the intestinal enzyme with the antibody prepared against milk galactosyltransferase. Furthermore, we used a suspension of mucosal scrapings containing soluble galactosyltransferase activity to partially purify the enzyme by affinity chromatography on an α-lactalbumin-Sepharose column as described by Andrews (1). We subjected this preparation to immunoblotting (34) to ascertain homology of the intestinal immunoreactive enzyme with the milk enzyme that was used to obtain the antisera. As shown in Fig. 2, the partially purified intestinal enzyme (lane B) co-migrated on SDS PAGE with the purified milk.

**Figure 1** Inhibition of galactosyltransferase activity from intestinal mucosa using monospecific antibodies. 75 μl of homogenized mucosal scrapings brought to a protein concentration of ~10 mg/ml in Tris buffer (0.05 mmol/liter [pH 7.4] + 2 mmol/liter phenylmethylsulfonyl fluoride) was prepared as described in Materials and Methods and was incubated with increasing amounts of antagalactosyltransferase IgG (0.34 mg/ml) supplemented with nonimmune IgG of the same concentration to an equal-end volume. Incubation was at 4°C over night. Measurements of galactosyltransferase activity were carried out before and after centrifugation at 12,000 rpm in an Eppendorf desk top centrifuge. Results were identical before and after centrifugation, indicating the absence of sedimentable immune complexes.

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The present immunocytochemical study demonstrates galactosyltransferase immunoreactive sites at the outer side of the plasma membrane of absorptive enterocytes. This finding raises the question concerning the nature of their membrane-association, their intracellular transport, their possible function as ecto-enzyme, and their application as marker enzyme for Golgi fractions.

Monospecificity of the antibodies was assumed on the basis of the following evidence. The antiserum strongly inhibited intestinal galactosyltransferase as demonstrated earlier for the milk enzyme (4); this indicated specificity towards this enzyme but did not exclude presence of contaminating antibodies as previously described (39). Partially purified intestinal enzyme was shown to co-migrate on SDS PAGE/immunoblotting with the soluble milk enzyme, which demonstrates presence and homology of the intestinal antigen with the milk antigen (Fig. 2, lane B). Using this technique, no consistent evidence for other cross-reacting material could be found. Different antibody preparations, i.e., affinity purified according to conventional methods (3) as well as the one recovered from blotted enzyme, gave the same staining pattern and a comparable intensity of labeling. Thus, the immunostaining described in earlier work (17) corresponds to the findings presented here and represents bona fide galactosyltransferase antigenic sites. The immunocytochemical technique employed permits a lateral resolution that is at the best the size of the antibody molecule. Upon quantitation of the gold particle distribution, it was apparent that most of them were on the outer side of the plasma membrane or overlying it. This finding strongly suggests some sort of a direct anchorage of galactosyltransferase to the plasma membrane that could explain its accumulation at this site. In contrast, in HeLa cells where plasma membrane-associated galactosyltransferase was not detectable (28), expression of the enzyme at this site is of short duration, preventing its build-up to a cytochemically
detectable level before its release into the culture medium (33). This assumption is supported by the finding that the enzyme recovered from the surface of HeLa cells already appeared to be proteolytically processed (33). The enzyme was immunocytochemically detectable along the whole surface of enterocytes, but predominantly at the apical plasma membrane. The reasons for this inhomogeneity in density of labeling will be the subject of a more detailed study on the
FIGURES 7 and 8  Same material as in Fig. 3. The immunolabeling for galactosyltransferase (Fig. 7) in the brush border corresponds to the distribution of galactose residues (Fig. 8) as visualized with the \textit{R. communis} lectin I-gold complex. Some bacteria (asterisks) overlying (but not in contact with) the brush border show only a low background labeling. The arrow points to a bar indicating the thickness of the glyocalyx. Bar, 0.5 \textmu m. \times 45,000.

FIGURES 9 and 10  An intense labeling for galactosyltransferase is present along the interdigitations of the lateral plasma membrane of adjacent enterocytes. The tight and intermediate junctions (arrows in Fig. 9) appear less intensely stained. Note the labeling over the intercellular space of the two desmosomes (arrowheads in Fig. 9). MV, basal part of microvilli. Bar, 0.25 \textmu m. (Fig. 9) \times 60,000; (Fig. 10) \times 49,000.

Intracellular distribution of galactosyltransferase. We observed that the entire plasma membrane possesses galactosyltransferase immunoreactivity that is presumably derived from \textit{trans}-Golgi cisternae (J. Roth, M. J. Lentze, and E. G. Berger, manuscript in preparation). This suggests that it receives components from \textit{trans}-Golgi cisternae and that sorting between basolateral and apical membrane occurs at a site distal from \textit{trans}-Golgi membranes. This assumption was recently
proposed on the basis of co-localization in the Golgi apparatus of vesicular stomatitis virus G glycoprotein and influenza hemagglutinin in doubly infected MDCK cells (23). Our data appear fully compatible with the conclusion that divergence of hemagglutinin and G glycoprotein on the passage to their predominant site of cell surface expression occurs at a post-Golgi stage.

The presence of galactosyltransferase on lateral plasma membranes of absorptive enterocytes raises the question about a possible interaction with carbohydrate structures exposed on the surface of the adjacent cell that could be important in mediating adhesion. The demonstration of galactosyltransferase immunoreactive sites in the cement substance of desmosomes is in favor of such an assumption but provides no proof for such a function. The same interpretation is valid for the other regions of the lateral and the basal plasma membrane that were positive for both galactosyltransferase and galactose residues. Recently, evidence was obtained that galactosyltransferase may function as a surface receptor for poly-N-acetyllactosamine compounds in mouse carcinoma cells (31). Rauvala et al. (22) have provided data that fucosyltransferase immobilized on a support can function in adhesion of cells. At present, the function (if any) of galactosyltransferase in the brush border remains completely unclear. Functions pertinent to its catalytic activity appear unlikely as this enzyme is co-localized with a β-galactosidase (35) and nucleotide pyrophosphatase (15). Perhaps other properties of galactosyltransferase that may be related to its carbohydrate binding site are of relevance in mediating bacterial adherence (30).

An intriguing earlier observation (17) concerned the dramatic differences of brush-border labeling intensity among adjacent enterocytes. Recently, this observation was extended to the ciliated cells of the fallopian tube (7). An analogous “all or none” distribution of fucose among adjacent enterocytes was observed after in vivo administration of [3H]fucose to rats and its subsequent autoradiographic detection (2). These data were interpreted as the result of different functional states of enterocytes. On the basis of our observations, we assume that differential metabolic labeling by sugar precursors among neighboring cells may be due to the presence or absence of corresponding glycosyltransferases. The impli-
cations of this finding with respect to the “functional state of enterocytes” need to be further explored.

Galactosyltransferase activity measurements have been routinely carried out for the determination of fractions of Golgi membranes (9). The results presented here suggest that galactosyltransferase activity measurements may not discriminate between Golgi, basolateral plasma membrane, and brush-border fractions. Previous reports based on carefully worked out fractionation methodology demonstrated the presence of galactosyltransferase activity in enterocyte membrane fractions that were assigned to the basolateral portion by virtue of their Na⁺, K⁺-ATPase activity (38). Other studies using the same method (21) have also detected galactosyltransferase activity in brush-border membrane preparations. These measurements probably were not optimized as they were based upon an assay devised for serum galactosyltransferase (19). Further work is needed to establish whether galactosyltransferase can be used as bona fide marker for Golgi membrane fractions on other cells such as the hepatocytes (5).

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