Cytochemical Localization of Terminal N-Acetyl-D-galactosamine Residues in Cellular Compartments of Intestinal Goblet Cells: Implications for the Topology of O-Glycosylation

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ABSTRACT   The O-linked oligosaccharides of mucin-type glycoproteins contain N-acetyl-D-galactosamine (GalNAc) that is not found in N-linked glycoproteins. Because *Helix pomatia* lectin interacts with terminal GalNAc, we used this lectin, bound to particles of colloidal gold, to localize such sugar residues in subcellular compartments of intestinal goblet cells. When thin sections of low temperature Lowicryl K4M embedded duodenum or colon were incubated with *Helix pomatia* lectin-gold complexes, no labeling could be detected over the cisternal space of the nuclear envelope and the rough endoplasmic reticulum. A uniform labeling was observed over the first and several subsequent *cis* Golgi cisternae and over the last (duodenal goblet cells) or the two last (colonic goblet cells) *trans* Golgi cisternae as well as forming and mature mucin droplets. However, essentially no labeling was detected over several cisternae in the central (medial) region of the Golgi apparatus. The results strongly suggest that core O-glycosylation takes place in *cis* Golgi cisternae but not in the rough endoplasmic reticulum. The heterogenous labeling for GalNAc residues in the Golgi apparatus is taken as evidence that termination of certain O-oligosaccharide chains by GalNAc occurs in *trans* Golgi cisternae.

Glycoproteins can be classified according to the nature of the linkage between the oligosaccharide chains and the polypeptide. The two main families are those having oligosaccharide chains linked N-glycosidically from *N*-acetyl-D-glucosamine to the amide nitrogen of asparagine and those possessing oligosaccharide chains O-glycosidically linked from *N*-acetyl-D-galactosamine (GalNAc)[1] to the hydroxyl groups of serine and/or threonine (22, 28, 35, 36, 72). The biosynthesis of N-linked glycoproteins is currently considered to be a single pathway involving the assembly of a lipid linked oligosaccharide (1, 41, 42), the en bloc transfer of the oligosaccharide from the lipid carrier to the nascent peptide chain (31, 32, 55), the processing of the oligosaccharide chains by glycosidases (12, 18, 24, 27, 38, 63–66) and the addition of terminal sugars by glycosyltransferases (5, 44, 57). The establishment of the carbohydrate to protein linkage in N-glycosylation seems to be a cotranslational process located in the rough endoplasmic reticulum and later steps of the assembly are thought to occur mainly in the Golgi apparatus (2, 22, 28). Much less information is available about the assembly of the

oligosaccharide chains of O-linked glycoproteins and its topology. The initial glycosylation reaction in the synthesis of O-linked glycoproteins involves the direct transfer of GalNAc from uridine diphosphate (UDP)-GalNAc to the polypeptide by a UDP-GalNAc: polypeptide transferase and does not require oligosaccharide preassembly nor lipid intermediates (22, 23, 25, 36, 37, 58, 67). Opinions vary among investigators, however, regarding the subcellular localization of the site of this initial glycosylation reaction. Most found evidence that smooth microsomes were the main submicrosomal loci for the enzyme responsible for the transfer of GalNAc to the polypeptide and to additional, more distal, sites of the oligosaccharide chains (19, 23, 30, 33, 34, 56). Kinetic studies are consistent with such a view (71). In contrast, Strous (62), by using an in vitro approach, has reported that the initial O-glycosylation event occurs at the ribosomal level on nascent

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[1] *Abbreviations used in this paper:* GalNAc, *N*-acetyl-D-galactosamine; PBS, 0.15 M NaCl-0.01 M phosphate buffer; HPL-gold, *Helix pomatia* lectin-gold; UDP, uridine diphosphate.

peptide chains, which implies that the rough endoplasmic reticulum is the subcellular site of this process.

Despite the inherent problems in applying subcellular fractionation methods to tissues rich in mucins, the evidence presently available indicates that O-glycosylation probably occurs posttranslationally in membrane fractions that could correspond in intact cells to smooth endoplasmic reticulum and/or Golgi apparatus. To further substantiate such a notion, we applied a newly developed cytochemical approach for the localization of sugar residues with lectins on thin sections of intact cells by electron microscopy (46, 48) to intestinal goblet cells which synthesize large amounts of O-linked glycoproteins (7, 8, 61). For this purpose the lectin from the snail *Helix pomatia*, which interacts specifically with terminal (nonreducing) α-N-acetyl-D-galactosamine residues (20, 21, 68), was bound to particles of colloidal gold and the complex used to determine the intracellular compartments to which this lectin binds. The observed labeling pattern strongly suggested that the Golgi apparatus is the initial site where attachment of GalNAc to proteins occurs. Furthermore, the observed differential labeling pattern for GalNAc within the Golgi apparatus provides further evidence for subcompartmentalization of this organelle.

MATERIALS AND METHODS

Reagents: Helix pomatia lectin was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetylneuraminic acid were from Sigma Chemical Co. (St. Louis, MO). Chloroauric acid (HAuCl₄ × 2 H₂O), β-D-galactose, L-fucose, trisodium citrate and polyethylene glycol (20,000 mol wt) were from Merck (Darmstadt, Federal Republic of Germany). The Lowicryl K4M kit came from Balzers (Balzers, FL).

Preparation of the Tissues: Tissue was taken from 1-wk-old White Leghorn chicks and adult male Wistar rats. Pieces from the upper part of the duodenum and distal colon were fixed by immersion in 1% glutaraldehyde in 0.15 M NaCl–0.01 M phosphate buffer (PBS, pH 7.4) for 2 h at room temperature. Afterwards, the tissue pieces were rinsed several times in PBS. Free aldehyde groups were blocked by incubation in 0.5 M NH₄Cl in PBS for 1 h at room temperature. Embedding at low temperature in the hydrophilic resin Lowicryl K4M was performed by stepwise lowering of the temperature down to −35°C during ethanol dehydration. Infiltration with the resin and UV-light polymerization (for 24 h) was done at −35°C as previously described in detail (6, 52). UV-light polymerization was continued for 48 h at room temperature. Thin sections from Lowicryl K4M embedded tissue were mounted on nickel grids having a Parlodion-carbon film and stored at room temperature.

Preparation of Colloidal Gold and H. pomatia Lectin-Gold (HPL-Gold) Complexes: Particles of colloidal gold with a mean diameter of 14 nm were prepared by reducing a boiling solution of aqueous tetrachloroauric acid (0.01%; 100 ml) with trisodium citrate (1%; 4 ml) (14). The pH of the sol was adjusted to 7.4 with 0.2 N K₂CO₃. Details for the preparation of the HPL-gold complex have been reported previously (47, 48, 50). Briefly, 325 μg H. pomatia lectin were dissolved in a small volume of distilled water and 50 ml colloidal gold was added. After 1–2 min, 1 ml 1% aqueous polyethylene glycol (20,000 mol wt) was added. Purification and concentration of this crude HPL-gold complex was done by ultracentrifugation at 60,000 g for 45 min, which resulted in the formation of a dark red sediment in the bottom of the centrifuge tube. The waterclear supernatant was carefully aspirated and discarded, whereas the sedimented HPL-gold complexes were resuspended in PBS (containing 0.01% NaN₃) and stored at 4°C.

Cytochemical Staining Procedure: The incubation of thin sections attached to the nickel grids was done according to published protocols (46, 48). None of the so-called etching procedures was performed before labeling. Nickel grids with the attached thin sections were floated on a drop of PBS for 5 min at room temperature and then transferred to a drop (10–15 μl) of HPL-gold complex. The HPL-gold complex was used at concentrations between 3 to 7.5 μg/ml and the incubation in a moist chamber lasted for 30 min at room temperature. After two rinses for 2 min each with PBS and a short rinse with distilled water, sections were dried and counterstained with 2% aqueous uranyl acetate (7 min) and Millonigs lead acetate (45 s) and examined.

Cytochemical Controls: The specificity of the staining was con-

trolled by addition of GalNAc varying from 3 to 100 mM (final concentration) to the HPL-gold complex 30 min before incubation of the thin sections. Since it was observed that in certain cells, HPL interacts weakly with polymeric N-acetyl-D-glucosamine (20), the HPL-gold complex was preincubated with 3–100 mM N-acetyl-D-glucosamine, and further sugars such as N-acetylneuraminic acid, D-glucose, α-methyl-D-mannopyranoside, L-fucose and β-D-galactose. Another type of control consisted in the incubation of the sections first with an excess of native HPL (50 μg/ml) and second with the HPL-gold complex each for 30 min.

RESULTS

When thin sections from glutaraldehyde-fixed and low temperature Lowicryl K4M embedded duodenum and colon were incubated with HPL-gold complexes an intense labeling with gold particles appeared over several subcellular structures of goblet cells (Fig. 1). Qualitatively this labeling pattern was similar in duodenum and colon. The low temperature embedding conditions with the use of the hydrophilic resin Lowicryl K4M preserved the fine structural details so that the cellular sites of gold-particle labeling could be clearly defined. The labeling over the cisternal space of the rough endoplasmic reticulum and nuclear envelope (Figs. 1–2 and 4–10) ac-
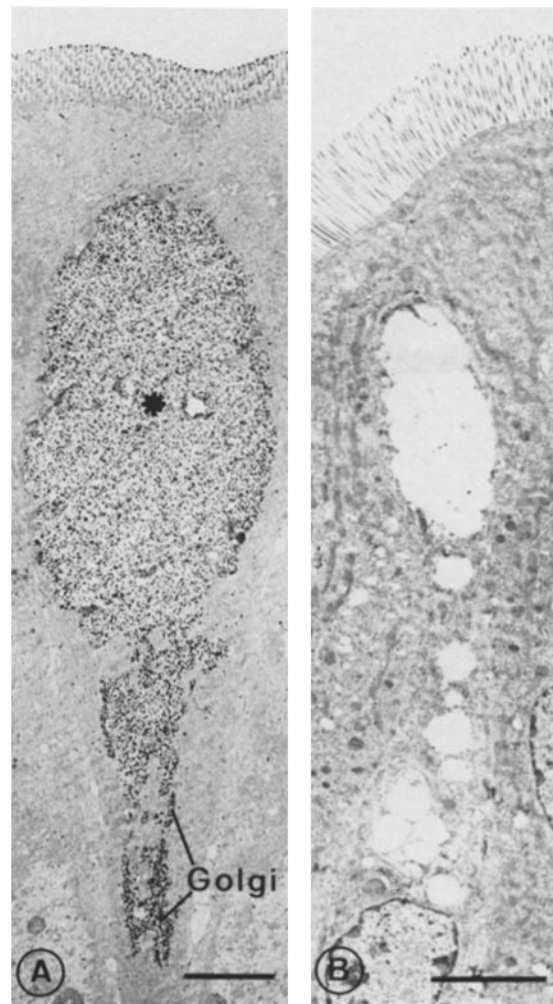

FIGURE 1 These low power micrographs from Lowicryl K4M thin sections of chick duodenum show a part of a goblet cell. A dense labeling with HPL-gold complexes is seen over Golgi apparatus and the mucin (asterisks) in the apical cell region (A). This labeling is abolished when a HPL-gold preincubation with 3 mM GalNAc was used for incubation of thin sections (B). Bar, 0.1 μm. (A) × 1,200; (B) × 1,400.
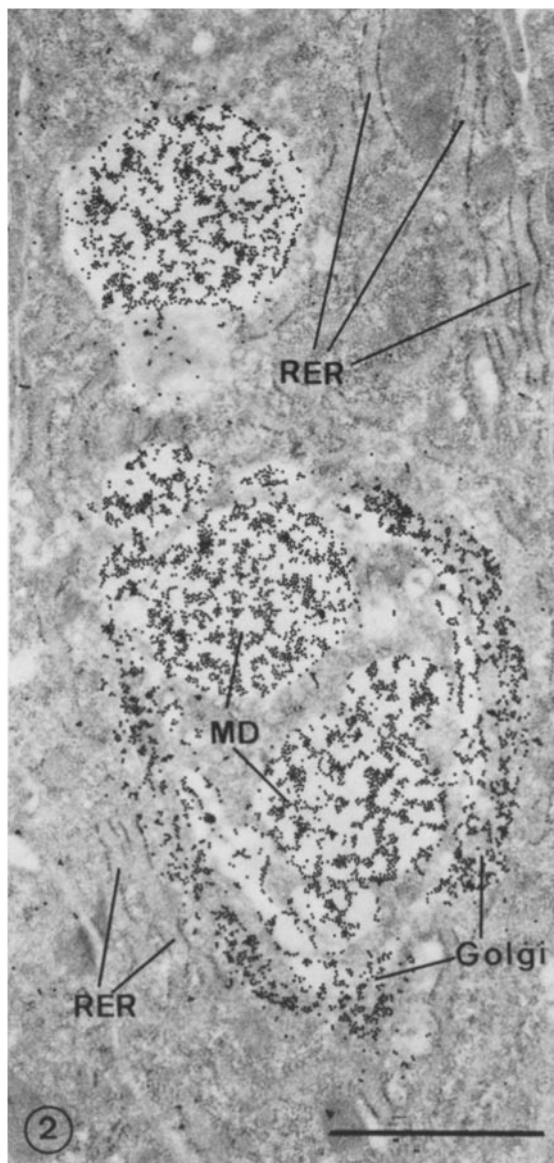
FIGURE 2 Lowicryl K4M thin section. Chick duodenal goblet cell labeled with HPL-gold. The luminal space of the rough endoplasmic reticulum (RER) is free of gold particle label which is in contrast to intensely labeled cytoplasmic regions containing elements of the Golgi apparatus and mucin droplets (MD). Bar, 0.2 μm. × 5,500.

counted for 0.37% of the labeling intensity estimated over the labeled *cis* Golgi cisternae. Labeling over the nucleus and mitochondria corresponded to 3.2% and 2.6%, respectively, of the *cis* Golgi labeling intensity. Over the cytoplasmic matrix between the profiles of the rough endoplasmic reticulum we consistently observed a weak labeling. It could be abolished in the cytochemical controls (see Materials and Methods section) which was in contrast to the labeling over the nuclear interior that remained under such conditions. Transitional elements of the rough endoplasmic reticulum juxtaposed to the *cis* side of the Golgi apparatus were not labeled. The Golgi apparatus of the duodenal and colonic goblet cells exhibited the typical organization (13, 39). It formed a stack of curved, superimposed cisternae; usually seven to ten in duodenal and ten to fourteen in colonic goblet cells. The first cisterna at the *cis* side often appeared interrupted. This structure was intensely labeled with gold particles and this high degree of labeling continued over the succeeding Golgi cisternae (Figs.

3–10). Therefore, in duodenal goblet cells about three to five *cis* Golgi cisternae were heavily labeled. However, the following three to four cisternae towards the *trans* side of the Golgi apparatus were not stained or showed a degree of labeling at least 15 times lower than the positive *cis*-cisternae. A further abrupt change occurred over the last cisterna at the *trans* side of the Golgi apparatus. This dilated cisterna was again labeled with gold particles along the inner membrane aspect and over the lumen as forming and mature mucin droplets. In cross-sections through the Golgi apparatus the slightly dilated extremities of the cisternae sometimes were labeled throughout the stack (Figs. 3 B and 4). Small vesicles at the *trans* side and the extremities of the Golgi stack which resembled coated vesicles were usually not labeled (Figs. 4–6). Principally the same observations were made for colonic goblet cells. The labeling with HPL-gold complexes started abruptly and intensely over the first *cis* Golgi cisterna and extended over the following four to six cisternae (Figs. 7–10). Over the next three to five cisternae toward the *trans* side the labeling decreased abruptly and was almost completely absent. The label occurred again over one or two of the most *trans* Golgi cisternae. Forming and mature mucin droplets were also labeled.

The observed labeling pattern was not seen when thin sections were first labeled with the native HPL followed by the HPL-gold complex (not shown). The specific inhibitory sugar, GalNAc, when added to the HPL-gold complex in a concentration of 3 mM and higher prevented the labeling almost completely (Fig. 1). All the other sugars tested did not impair the staining with HPL-gold complexes (not shown). This observation was especially important with *N*-acetyl-D-glucosamine since it has been shown that HPL interacts in vitro with teichoic acids (20). However, in these studies α-linked *N*-acetyl-D-glucosamine was found to be a considerably poorer inhibitor than α-linked GalNAc and, in addition, had to be present in multivalent form on a macromolecule to bring about precipitation (21).

DISCUSSION

Mucins synthesized in large amounts by intestinal goblet cells contain oligosaccharide chains linked by a ser(thr)-GalNAc bound to the polypeptide (7, 8, 61). The oligosaccharide chains are heterogeneous with respect to sugar composition and molecular size and many of them express blood group activities A or H (8, 26, 61). Such O-glycosidically linked glycoproteins differ from the other types of glycoproteins by their content of GalNAc residues either constantly present as the linkage sugar to the polypeptide or in addition found at the nonreducing terminus of blood group A active oligosaccharides. In the present study the subcellular distribution of terminal GalNAc residues in intestinal goblet cells was investigated using the *H. pomatia* lectin-gold complex. The utility of this lectin as a probe for terminal GalNAc moieties in biopolymers and cells has been noted already in biochemical and recent cytochemical studies (20, 21, 48, 53, 69, 70) and was confirmed in the present investigation. An important feature of the lectin is that it does not show heterogeneity of its sugar combining site and that the size of this site may not be larger than that of an α-linked GalNAc residue (20). This is exceptional, since most lectins have a combining site that fits more complex structures (16, 45). Equally important in explaining the present cytochemical findings is the fact that the *H. pomatia* lectin interacts not only with GalNAc residues
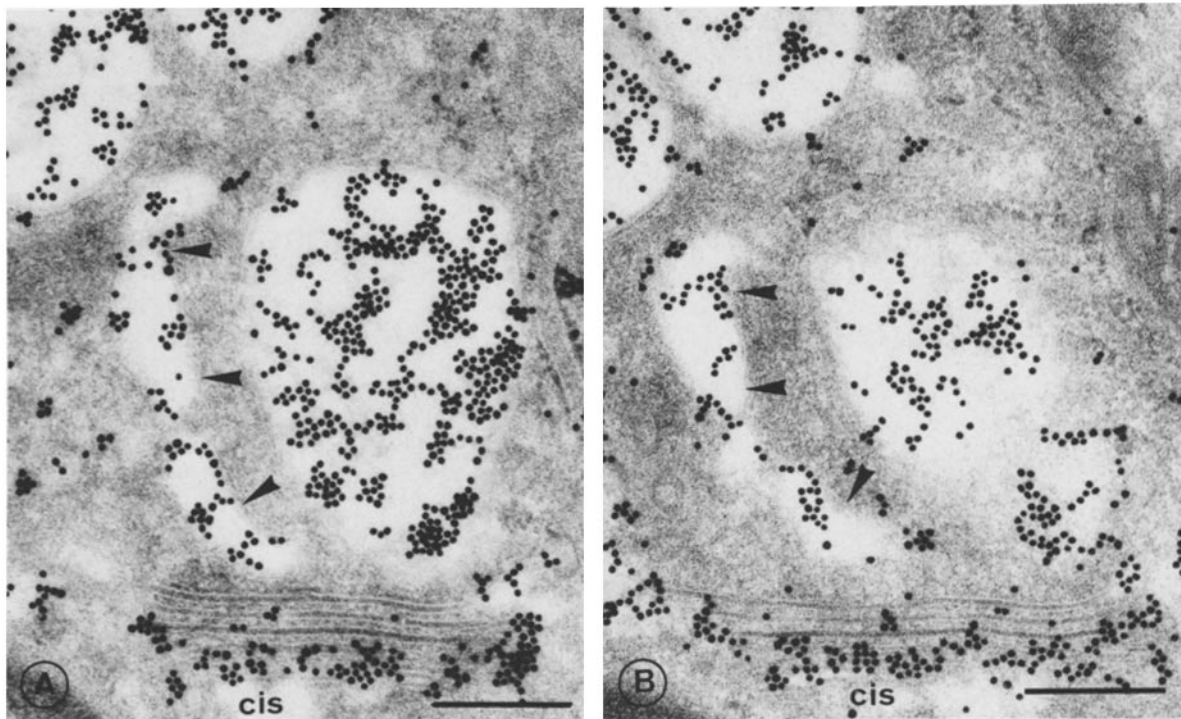
FIGURE 3 Lowicryl K4M serial thin sections of chick duodenal goblet cells. Labeling with HPL-gold complexes results in intense staining over several *cis* Golgi cisternae, whereas subsequent central cisternae are not labeled (A) or show greatly diminished labeling (B). The serial thin sections show that the labeled structure at the *trans* side of the Golgi stack (arrowheads) is an elongated saccular element with regional enlargements of its lumen and which is therefore assumed to represent the last *trans* Golgi cisterna. Bar, 1 μm. × 18,000.

located at the nonreducing terminus of O-glycosidically linked oligosaccharides but also with a single such sugar residue linked to protein (69), a structure characteristic of the initial O-glycosylation step. Concerning the topology of O-glycosylation, the two main observations made in the present investigation are the absence of cytochemically detectable terminal GalNAc residues in the luminal space of the rough endoplasmic reticulum and their presence in two discrete regions of the Golgi apparatus. These cytochemical observations seem to indicate that O-glycosylation does not occur in the rough endoplasmic reticulum. Absence of HPL-gold labeling over the rough endoplasmic reticulum was also observed for rat liver, kidney, and pancreas, both in exocrine and endocrine cells (unpublished observations). The sparse labeling over the cytoplasmic matrix could account for the presence of incompletely extracted UDP-GalNAc, since the synthesis of most sugar nucleotides takes place in this cellular compartment (9). This confirms and extends biochemical studies suggesting different subcellular sites for O- and N-glycosylation. Measurement of N-acetylgalactosaminyl: polypeptide transferase activity in subcellular fractions of hen oviduct (23), rat intestinal mucosa (33), and developing rat brain (34) showed presence of this enzyme in a smooth membrane fraction. Another line of evidence indicating that O-glycosylation takes place in smooth membrane fractions comes from recent work on viral glycoprotein maturation (29, 40, 60). In mammary gland explants, cycloheximide treatment inhibited immediately N-glycosylation, presumably due to lack of nascent polypeptide acceptors, whereas O-glycosylation continued for 30 min, a time period necessary for the movement of possible acceptor polypeptides from the rough endoplasmic reticulum

to the Golgi apparatus (71). The present data point clearly to the Golgi apparatus as the subcellular compartment where O-glycosylation events first occur. It could not be decided whether this glycosylation takes place on glycoproteins or glycolipids either representing membrane constituents or secretory components. The significance of the regional distribution of the cytochemically detectable terminal GalNAc residues in the Golgi apparatus is not clear at present. Among several possible explanations one could assume that all different O-glycosylation steps occur in the *cis* Golgi cisternae but the cytochemical approach used was not sensitive enough to resolve them spatially. Mature O-glycoproteins then could move into the last *trans* Golgi cisterna(e) via the extremities of the Golgi stack or could be transported at a different rate from *cis* to *trans* Golgi cisternae. These possibilities as well as the contrary assumption of a backflow from *trans* to *cis* Golgi cisternae seem to be a rather unlikely. An explanation that appears more likely is that the labeled *cis* Golgi cisternae represent a compartment in which the core O-glycosylation takes place and the *H. pomatia* lectin can recognise and bind to such GalNAc residues situated temporarily in terminal positions. As soon as further glycosylation steps with the addition of galactose, N-acetyl-D-glucosamine, fucose, and sialic acid occur, the initially terminal GalNAc residue of the core linkage becomes internal and is not longer reactive with the *H. pomatia* lectin. In the case of oligosaccharide chains exhibiting blood group A activity, the chains are terminated by a further GalNAc residue, whose addition is controlled by blood group A gene that codes for the blood group A UDP-GalNAc: fucose α 1→2 gal α 1→3-N-acetyl-D-galactosaminyltransferase. Such oligosaccharide chains have been de-
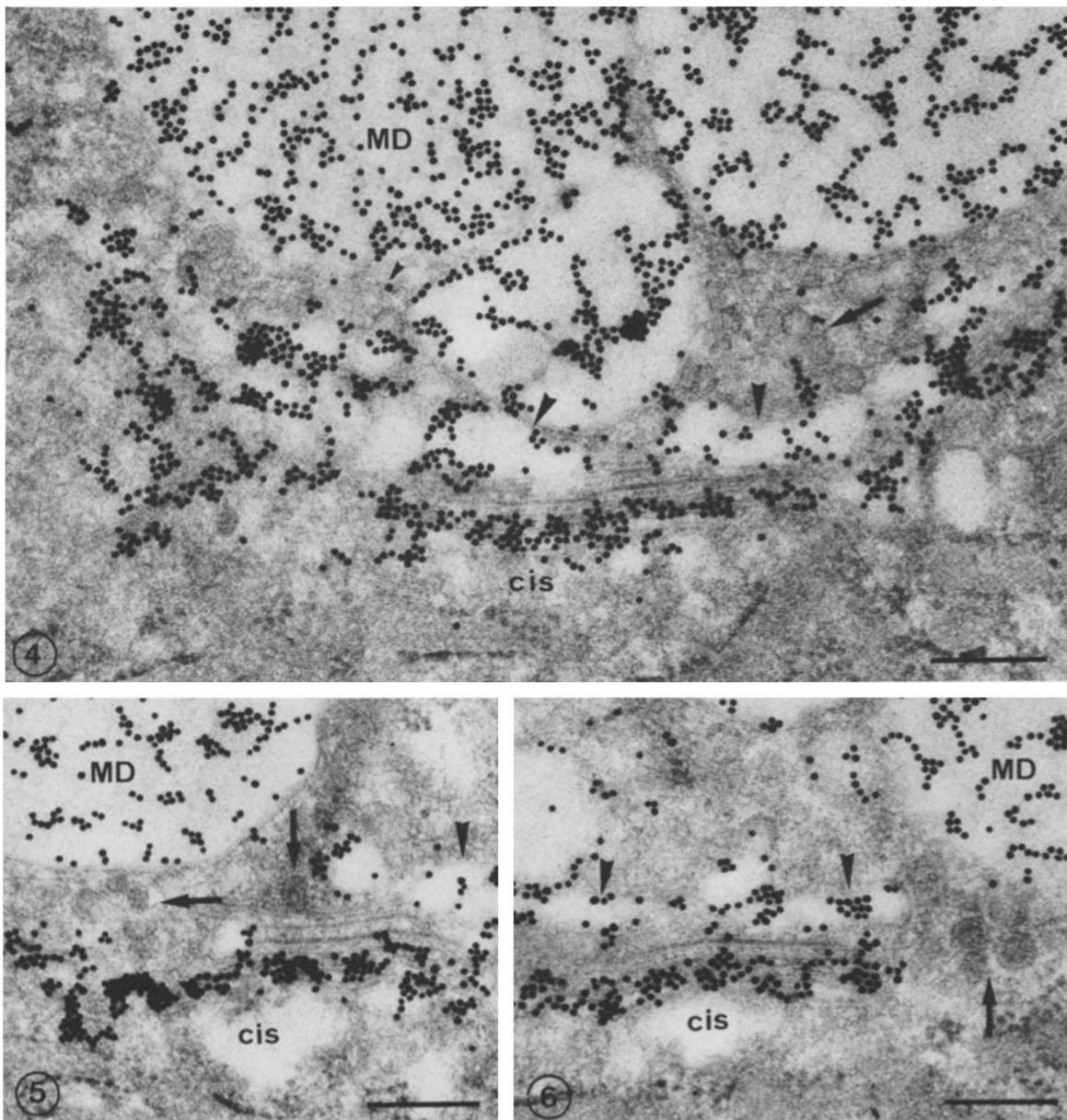
Figures 4–6 Lowicryl K4M thin sections. Rat duodenal goblet cells. HPL-gold labeling. In all cases an intense gold particle labeling is present over several cisternae at the *cis* side of the Golgi apparatus and over the last *trans* Golgi cisterna (arrowheads) that is dilated. A few cisternae in the central region of the stack are unlabeled (Figs. 4 and 5) or show a low degree of labeling (Fig. 6). In Fig. 4 an example is given for the diffuse labeling over the extremities of a Golgi apparatus. Small vesicles (arrows) at the extremities or the *trans* side of the Golgi apparatus appear not labeled. MD-mucin droplets. (Fig. 4) Bar, 1 μm. × 16,000. (Figs. 5 and 6) × 17,000.

tected in rat intestinal mucins (7, 8, 61) and were reactive with *H. pomatia* lectin (8). It is tempting to speculate that this terminal glycosylation step takes place in the last *trans* Golgi cisterna(e) of goblet cells, although it must be stressed that further investigations especially on the immunolocalization of the two different *N*-acetyl-D-galactosaminyltransferases are needed to support such an hypothesis. Altogether this would imply that oligosaccharide chain elongation occurs sequentially and in different Golgi apparatus regions as the glycoproteins move from the *cis* side toward the *trans* side. There are biochemical data that the synthesis of O-linked oligosaccharides occurs by sequential glycosylation and that the sequence of glycosylation steps is dictated by the substrate specificity of the glycosyltransferase. The investigations by Schachter et al.

(59) and Beyer et al. (4) have demonstrated that the preferred order of glycosylation steps after the formation of the ser/(thr)-GalNAc linkage is galactose, sialic acid, fucose, and GalNAc in the case of blood group A type oligosaccharides. Recent cytochemical investigations in fact have shown that galactose residues in goblet cells (49) and in viral glycoproteins transported in cultured baby hamster kidney cells (17) first occur in a few *trans* Golgi cisternae. This distribution of galactose residues principally corresponds with immunocytochemically localized galactosyltransferase in HeLa cells (51) and intestinal goblet cells (54). Furthermore, fucose residues as detected with *Lotus tetragonolobus* lectin-gold complexes were found in *trans* Golgi cisternae of goblet cells (49). Data about the localization of fucosyltransferases as well as sialyl-
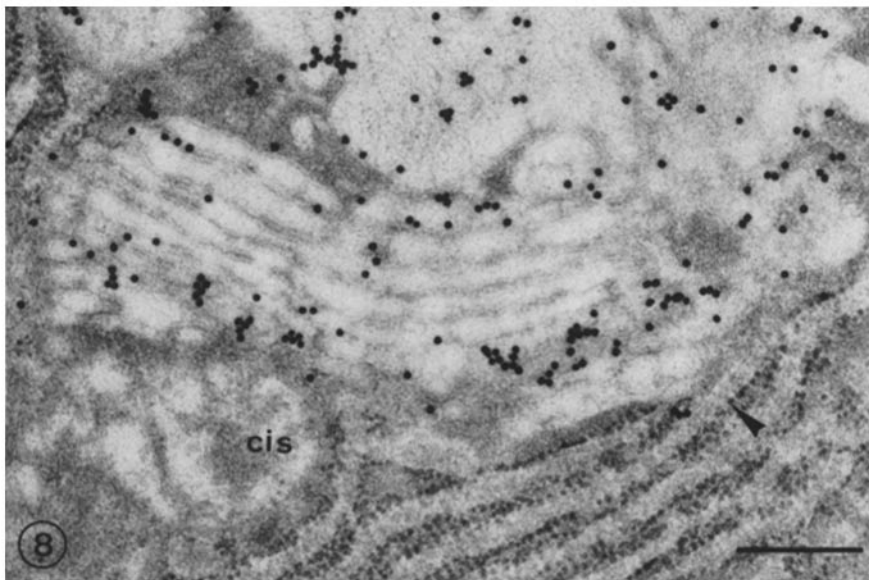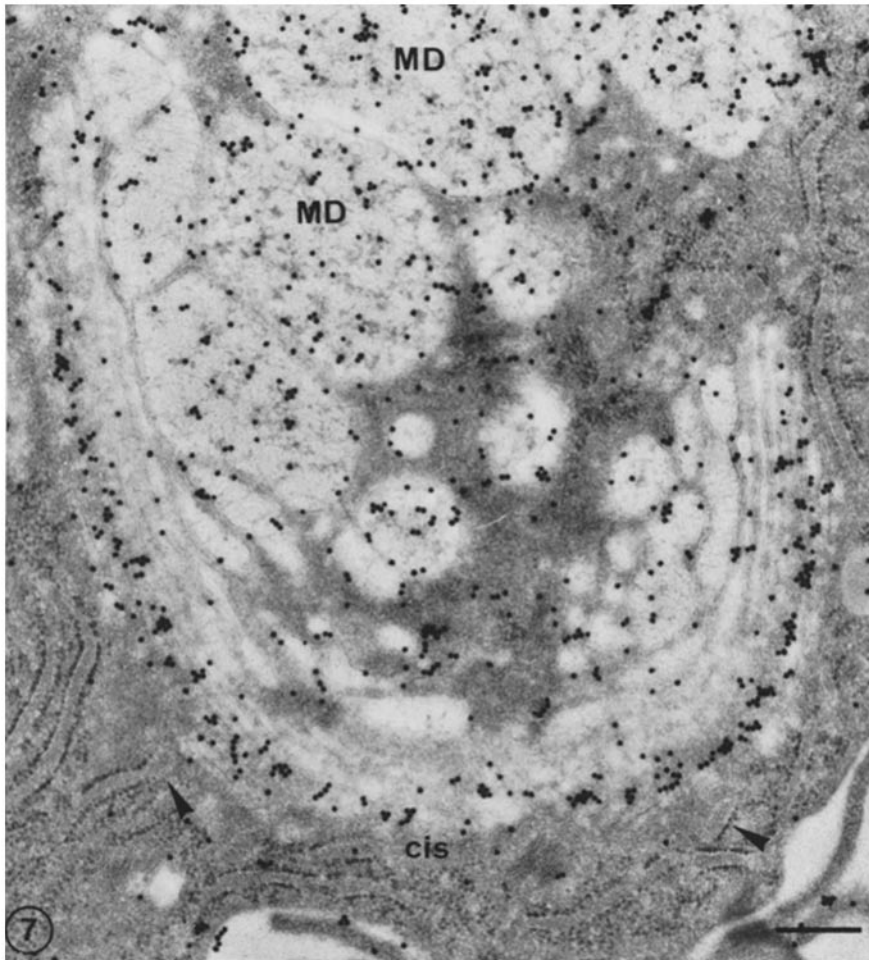
FIGURES 7 and 8   Lowicryl K4M thin sections. Rat colonic goblet cells. Label with HPL-gold complexes is absent over the luminal space of the rough endoplasmic reticulum and transitional elements of the rough endoplasmic reticulum (arrowheads). Gold particle label is distributed over *cis* Golgi cisternae and almost absent over central Golgi cisternae. Dilated cisternae at the *trans* side of the Golgi apparatus and mucin droplets (*MD*) in different stages of formation are labeled. (Fig. 7) Bar, 1 μm. × 11,000. (Fig. 8) × 17,000.

transferases and sialic acid residues are still lacking but it seems that at least in some cell types sialic acid is incorporated into glycoproteins at the *trans* side of the Golgi apparatus as shown by autoradiography (3). Thus, the current hypothesis that the various enzymes involved in glycoprotein biosynthesis have a sequential spatial arrangement in the Golgi apparatus is supported by experimental data (10, 11, 15, 43, 51).

The present study, as a first attempt to elucidate cytochemically the topology of O-glycosylation, has shown the importance of the Golgi apparatus. Further studies are aimed at a better understanding of the compartmentalization of O-glycosylation steps in the Golgi apparatus.
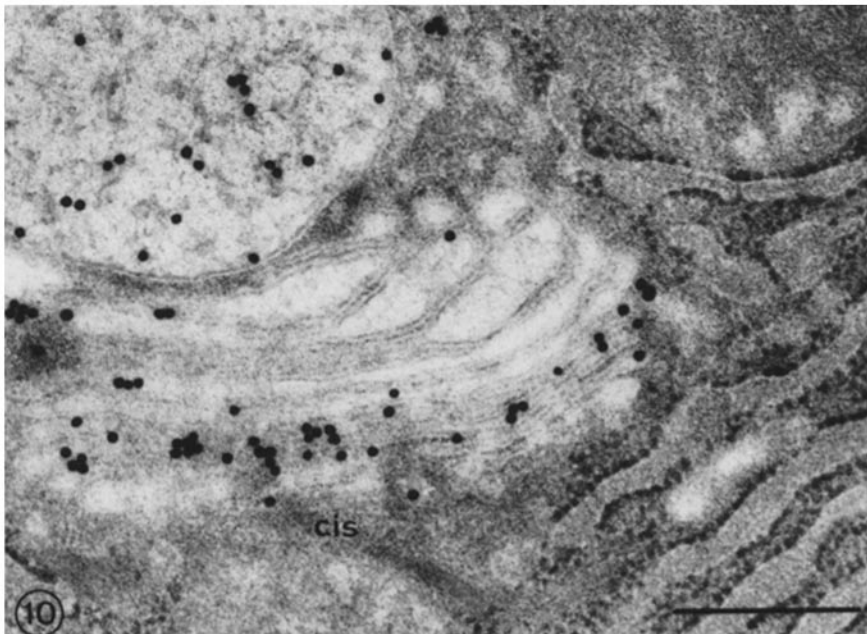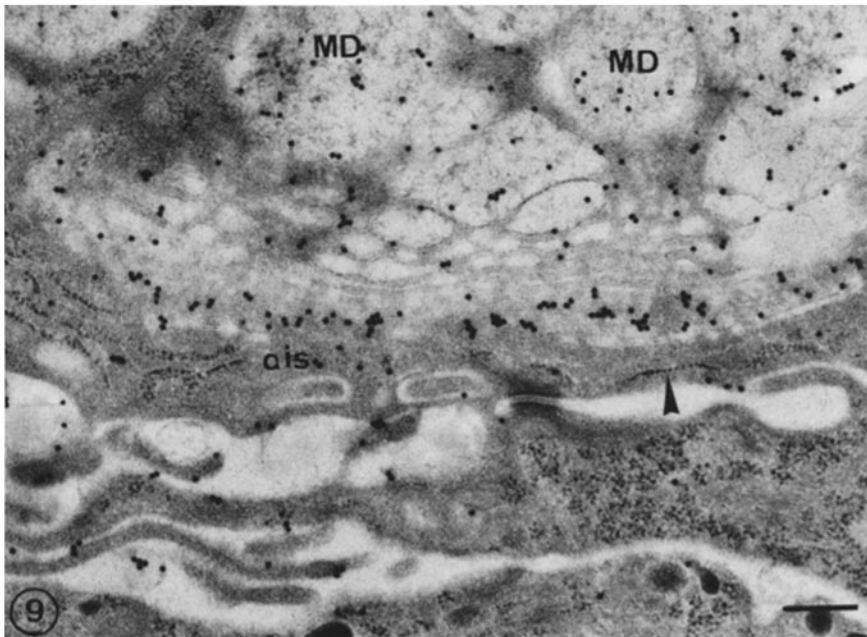
FIGURES 9 and 10 Same material and labeling conditions as in Figs. 7 and 8. The absence of labeling over the rough endoplasmic reticulum is evident. A labeling over cis and trans Golgi cisternae as well as forming mucin droplets (MD) can be appreciated whereas cisternae in the central region of the stack appear free of label. (Arrowhead) Transitional element of the rough endoplasmic reticulum. (Fig. 9) Bar, 1 μm. × 9,000. (Fig. 10) × 24,000.

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