

Lectin-binding Sites as Markers of Golgi Subcompartments: Proximal-to-Distal Maturation of Oligosaccharides

ALAN M. TARTAKOFF and PIERRE VASSALLI

Department of Pathology, University of Geneva, Faculty of Medicine, 1211 Geneva 4, Switzerland

ABSTRACT We investigated the subcellular sites of glycoprotein oligosaccharide maturation by using lectin conjugates to stain lightly-fixed, saponin-permeabilized myeloma cells. At the electron microscopic level, concanavalin A-peroxidase stains the cisternal space of the nuclear envelope, the rough endoplasmic reticulum, and cisternae along the proximal face of the Golgi stack. Conversely, wheat germ agglutinin-peroxidase stains cisternae along the distal face of the Golgi stack, associated vesicles, and the cell surface. These observations confirm the existence of two qualitatively distinct Golgi subcompartments, show that the lectin conjugates can be employed as relatively proximal or distal Golgi markers under conditions of excellent ultrastructural preservation, suggest that the asymmetric distribution of qualitatively distinct oligosaccharides is a property of underlying cellular components and not simply of the principal secretory product, and suggest that the oligosaccharide structure recognized by wheat germ agglutinin is attained during transport from the proximal toward the distal face of the Golgi stack.

The Golgi complex is composed of a set of stacked, closely apposed cisternae and associated vesicles. It is unclear how this structure mediates transport from the rough endoplasmic reticulum (RER) toward the cell surface; however, secretory products are found within each of the cisternae and are suspected to pass successively from one to the next. A limited number of histochemical markers of the relatively proximal (i.e., near the RER) and relatively distal Golgi cisternae have been identified, yet it is not known to what extent these markers may correspond to components which are of functional significance for transport (24, 26, 27).¹

The rat myeloma cells used in this study are highly specialized toward the secretion of a single glycoprotein, immunoglobulin M (IgM).² Judging from structural studies of one human and one murine IgM myeloma protein (1, 5, 9), the heavy chain of the rat myeloma protein would be expected to bear five N-linked oligosaccharides. These oligosaccharides should therefore make a major contribution to the protein-linked carbohydrate along the secretory path. Since it is known that most such N-linked oligosaccharides undergo a sequence of processing events during their intracellular trans-

port (13, 19, 28) and since it is likely that membrane glycoproteins of the cell are processed in similar fashion (13), judicious choice of lectin conjugates for ultrastructural staining of the myeloma should make it possible to detect where selected processing events occur. Furthermore, the detection of qualitatively distinct oligosaccharides should begin the description of Golgi markers of functional significance.

Electron microscopic adaptations of the periodic acid Schiff method have previously shown that a proximal-to-distal increasing gradient of stainable carbohydrate exists across the Golgi complex of many cells (21). Moreover, a limited light microscopic histochemical literature indicates that certain lectins (e.g., wheat germ agglutinin [WGA]) have a preferential affinity for the Golgi complex of fibroblasts while others (e.g., concanavalin A [Con A]) have an affinity for the basophilic part of their cytoplasm, which is known to include the RER (30). Both biochemical and microscopic studies have documented the affinity of Con A for rough microsomes of the liver and myeloma cells (10, 22).

MATERIALS AND METHODS

Cells: The rat IgM myeloma (IR 202) was obtained from Dr. H. Bazin, (University of Louvain, Bruxelles, Belgium), and maintained in ascitic form in pristane-primed LOU rats (see reference 2). The mouse myeloma mutant Sp2/0-Ag 14 synthesizing neither heavy nor light chains was maintained in tissue culture (25).

Conjugates: Lectins (Con A, WGA, soybean agglutinin, ulex lectin, ricin 1) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) or

¹ The classical markers are as follows: a readily osmicated material in proximal cisternae, nicotinamide adenine dinucleotide phosphatase in medial cisternae of selected cells, and nucleoside diphosphatase in distal cisternae.

² *Abbreviations used in this paper:* Con A, concanavalin A; Ig, immunoglobulin; RER, rough endoplasmic reticulum; WGA, wheat germ agglutinin.

Vector Laboratories (Burlingame, CA). They were coupled (a) to tetramethylrhodamine isothiocyanate (BDH Chemicals, Poole, England) (20 $\mu\text{g}/\text{mg}$ protein), at pH 9.5, and 50 mM NaHCO_3 for 1 h, 24°C, followed by extensive dialysis against PBS (10 mM, pH 7.3, Na phosphate, 150 mM NaCl), or (b) to peroxidase (Sigma Chemical Co., St. Louis, MO; Type II) according to the procedure of Nakane and Kawaoi (16), using the proportions recommended for Ig, followed by extensive dialysis against PBS. In all cases, coupling was performed in the presence of 0.1 M of the appropriate sugar (α -methyl mannoside for Con A, *N*-acetyl glucosamine for WGA). The conjugates were stored at 4°C.

Light and Electron Microscopy: Cell suspensions were cytocentrifuged, dried, postfixed 30 min at 4°C in 2% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M cacodylate-HCl, pH 7.3, quenched with 50 mM NH_4Cl in cacodylate-HCl, and stained for 30 min with fluorescent conjugates diluted to $\sim 20 \mu\text{g}/\text{ml}$ in 1% albumin-PBS. The presence of detergents did not influence the staining. For electron microscopy, cells were fixed in suspension, quenched, and incubated 1 h at 24°C with lectin conjugates in the presence of 0.1 mg/ml saponin (BDH Chemicals) and 1 mg/ml bovine serum albumin in cacodylate-HCl buffer. (0.1 mg/ml for Con A-peroxidase or 5–10 $\mu\text{g}/\text{ml}$ for WGA-peroxidase). They were then washed in the presence of saponin, refixed with glutaraldehyde, incubated with peroxidase substrates and fixed in OsO_4 as in Louvard et al. (15). Unstained sections were examined with a Philips EM 300. For light microscopic evaluation of the specificity of WGA staining, cytocentrifuged cells were fixed as indicated above and treated overnight at pH 5 in 0.1 M Na acetate, 40 mM CaCl_2 , in the absence or presence of 50 $\mu\text{g}/\text{ml}$ Cl. perfringens neuraminidase (Sigma Chemical Co., Lot 101F 8061) at 24°C. For detection of nucleoside diphosphatase, uridine 5'-diphosphate was used as substrate (7).

RESULTS

Immunofluorescence

A number of lectins of different oligosaccharide specificity have been conjugated to fluorescein or rhodamine and used to stain cytocentrifuged, air-dried, lightly-fixed IgM myeloma cells. Con A and WGA conjugates gave striking staining patterns—Con A stains the entire cell cytoplasm and WGA stains a single major perinuclear locus, a limited number of tiny spots elsewhere in the cytoplasm, and the cell surface. The staining by Con A and WGA was completely blocked by the inclusion of 0.1 M α -methyl mannoside or 0.1 M *N*-acetyl glucosamine, respectively. The staining with WGA, whose active site is known to accommodate either *N*-acetyl glucosamine or *N*-acetyl neuraminic acid (4), was also eliminated by treatment of the fixed cells with neuraminidase.

The staining of a non-Ig-synthesizing mutant was similar to that of the secreting cells.

Electron Microscopy

The Golgi complex of the myeloma cells is conspicuous and composed of stacks of three to five closely apposed

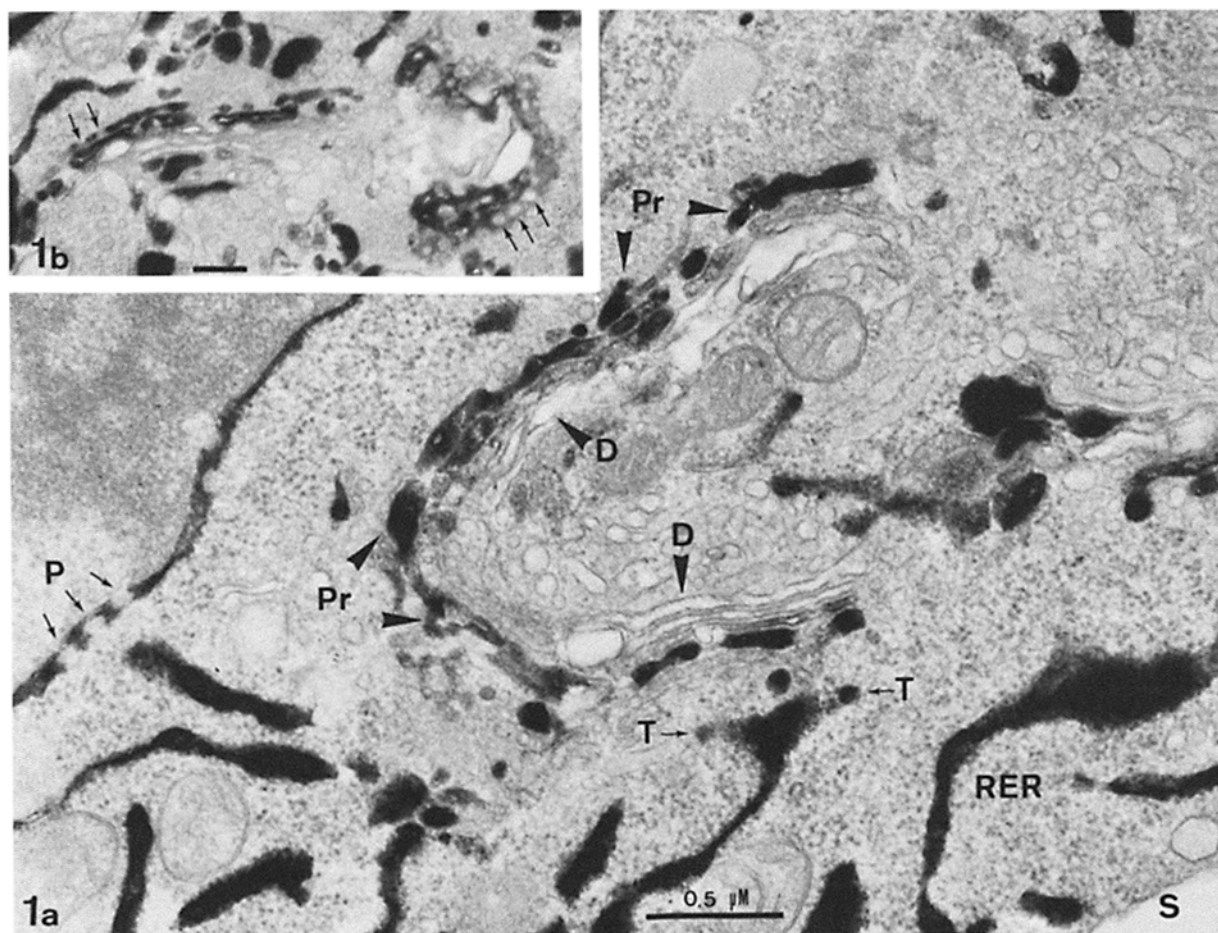


FIGURE 1 (a) Thin section of a cell stained with Con A-peroxidase. Note the intense staining of the nuclear envelope (except for the pores), the RER, and cisternae along the proximal face of the Golgi complex. A couple of possible transitional elements of the RER and small smooth-surfaced vesicles are also stained. (b) A second example of the selective staining of the proximal face of the Golgi stack which indicates its highly fenestrated structure both in cross section and in face view. P, nuclear pore; Pr, proximal cisternae; D, distal cisternae; T, possible transitional elements; and S, cell surface. Bar, 0.5 μM . (a) $\times 12,000$. (b) $\times 34,000$.

cisternae, associated tubules and vesicles. It is not grossly differentiated in such a fashion as to make its relatively proximal and distal faces uniformly distinguishable, however in appropriate sections one face does lie near the transitional elements of the RER and the other face near a population of vesicles somewhat larger than those found along the proximal face. These relations are especially visible in certain of the peroxidase-stained preparations, as is the extensive fenestra-

tion of both the most proximal and most distal cisternae (vide infra).

For examination of lectin-binding at the electron microscope level, lightly fixed cell suspensions were incubated with Con A and WGA conjugates in the presence of saponin to permeabilize membranes and further processed as indicated in Materials and Methods.

The Con A conjugate stained the entirety of the cisternal

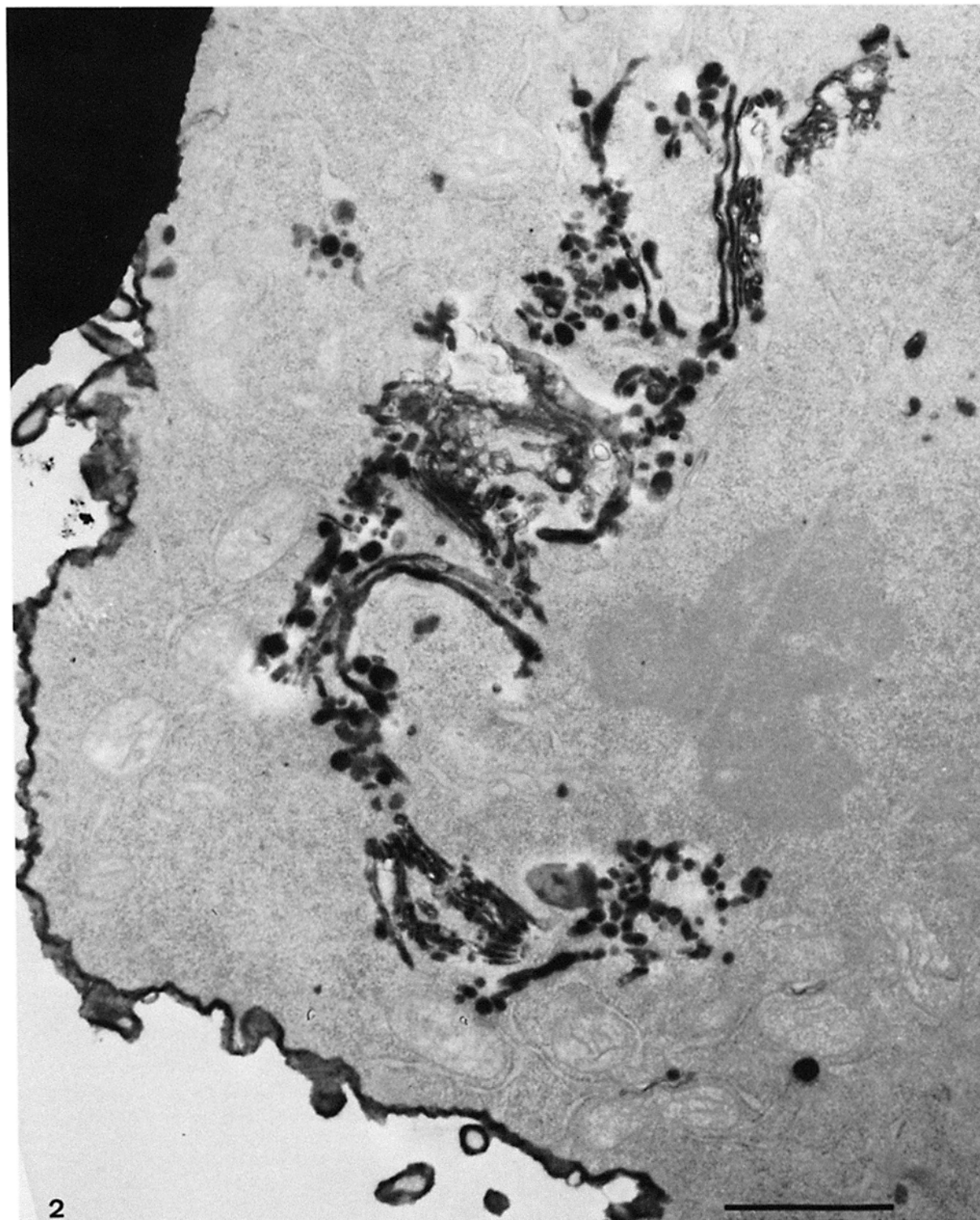
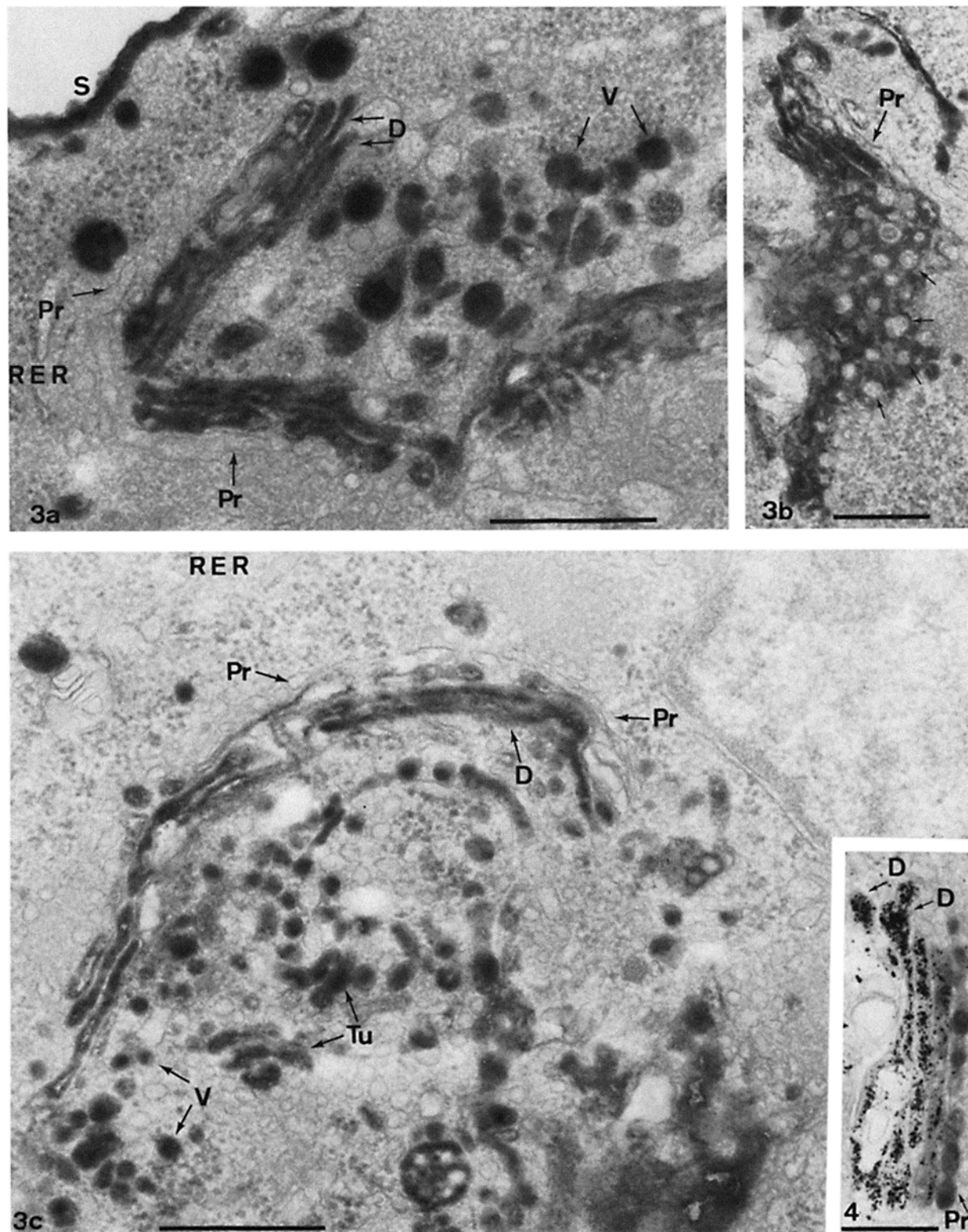


FIGURE 2 Thin section of a cell stained with WGA-peroxidase. In this overview it is dramatically evident that the staining is restricted to the region of the Golgi complex, possibly a few lysosomes, and the cell surface. $\times 30,000$.



FIGURES 3 and 4 Fig. 3: (a-c) Thin sections of cells stained with WGA-peroxidase. Note the selective staining of cisternae along the relatively distal face of the stack of Golgi cisternae, as well as numerous associated vesicles (V) and tubules (Tu). The diameter of such vesicles is somewhat greater than that of the "proximal vesicles" found between the RER and the proximal face of the Golgi complex. (b) A further example of the staining of Golgi cisternae, in this case a tangential view illustrating the highly fenestrated structure. The structure seen within certain fenestrae is thought to result from the inclusion of a partial image of an adjacent tangentially sectioned cisterna. Fig. 4: Thin section of the Golgi complex of a cell doubly stained, first for nucleoside diphosphatase and then with Con A-peroxidase. Note that the two stains are selective for the two faces of the Golgi stack and produce two antiparallel gradients of staining intensity. Fig. 3: (a and c) $\times 54,000$; (b) $\times 32,000$. Fig. 4: $\times 32,000$.

space of the RER and the nuclear envelope, with the exception of nuclear pores (Fig. 1). The cell surface was not stained. This latter observation is consistent with the poor Con A agglutinability of these myeloma cells (unpublished observation).

In the Golgi region, the Con A conjugate stains the cisternal space of cisternae along one face of the Golgi stack. In well oriented sections, the positive cisternae appear to be those closest to the RER, and in fact a limited number of putative transitional elements or small "transit" or "primary" vesicles are also stained. Tangential sections of the proximal face of the stack of Golgi cisternae reveal its highly fenestrated structure (Fig. 1, *a* and *b*).

The WGA conjugate gives a largely reciprocal staining pattern (Figs. 2 and 3). The nuclear envelope, RER, and proximal face of the Golgi stack are unstained, while the more distally disposed Golgi cisternae and associated vesicles, multivesicular bodies, and the cell surface are intensely stained.³ In fortunate sections, the stained face of the Golgi stack can be seen to be adjacent to an anastomosing structure, which is recognizable as GERL and in fact is positive for the WGA conjugate and acid phosphatase activity (unpublished observation). Tangential views of the distal face of the Golgi stack again reveal a highly fenestrated structure (Fig. 3*b*).

The relative polarity of staining observed with the two conjugates has been further established by making use of a classical marker of the distal face of the Golgi stack—nucleoside diphosphatase activity (7, 17, 26). For example, cells can be sequentially processed for uridine diphosphatase and Con A-peroxidase staining. Such experiments clearly prove that these two reagents are preferentially staining opposite faces of the Golgi stack (Fig. 4).

Staining of Sp 2/0 cells, which fail to synthesize Ig, gives results very similar to those obtained with the IgM myeloma.

DISCUSSION

The ability of the Golgi complex to mature the oligosaccharides of glycoproteins and glycolipids by trimming and elongation reactions has been established by an impressive range of autoradiographic, enzymologic and cell fractionation studies (13, 24, 26, 27). Most of these data pertain to the Golgi complex as a whole. The present study seeks to distinguish between two possibilities: that mature oligosaccharides bearing terminal sialic acid are characteristic of the entirety of the Golgi complex; or that the mature structure is characteristic of only a portion of the whole, possibly as a reflection of local addition of the terminal sugars.

We employed a cell type highly specialized toward the biosynthesis and export of IgM, which bears multiple asparagine-linked oligosaccharides, as well as a mutant that fails to synthesize Ig. Both cells were stained with lectin conjugates of defined specificity to localize intracellular oligosaccharides.

Judging from the data presented, the saponin permeabilization procedure employed is adequate to allow the macromolecular conjugates to penetrate all membranes of the RER and Golgi cisternae. Nevertheless, a considerable number of proximal vesicles (between the RER and Golgi cisternae) are not stained. The permeabilization of such vesicles cannot be

directly evaluated. They may simply lack or contain limited amounts of reactive oligosaccharides. Despite the known affinity of saponins for cholesterol-containing membranes, in the present cytochemical context the presence of cholesterol is known not to be a prerequisite for permeabilization (18).

The two lectins used are well characterized from the point of view of their oligosaccharide and glycoprotein specificity. Con A is known to bind both immature and mature (especially biantennary) N-linked oligosaccharides, but to have a considerably higher affinity for those immature structures that terminate in glucosyl, mannosyl, or mannosyl and *N*-acetyl glucosaminyl residues (12). The lack of Con A-peroxidase staining of distal cisternae and the cell surface (where complex oligosaccharides are known to be present) is presumably a reflection of this differential affinity, possibly augmented after fixation. WGA owes its principal glycoprotein-binding properties to the presence of clustered terminal *N*-acetyl neuraminic acid residues (4) and hence can be used to localize completed N- or O-linked oligosaccharide chains or gangliosides. The lack of reaction observed after neuraminidase in this study is consistent with these considerations.

The principal implication of the present investigation is therefore that the stack of Golgi cisternae is composed of at least two subcompartments that can be marked or labeled with distinct lectin conjugates: a proximal one rich in immature oligosaccharides (which can be detected with Con A conjugates) and a distal one containing mature oligosaccharides (detected with the WGA-conjugate). The site of transition between the two extremes, roughly one third to one half the way across, is comparable to that suggested for the incorporation of galactose, as judged by immunocytochemical localization of β (1-4) galactosyl transferase in HeLa cells (23), autoradiography of [³H]galactose-labeled mucin-secreting cells of the stomach (14), and thin section ricin (RCA 120) staining of incomplete oligosaccharides bearing terminal galactose of control and Semliki Forest virus infected baby hamster kidney cells (8). To date, no experimental studies have succeeded in separating the sites of addition of galactose and sialic acid to N-linked oligosaccharides (6), although there have been suggestions of a delayed addition of fucose (11).

To the extent that the two lectin conjugates are staining two stages of maturation of the same macromolecule—a notion which remains to be proven⁴—the data suggest that the axis of trans-Golgi transport indeed runs from the proximal toward the distal face. In the case of a viral membrane protein (G) there are further data in support of this idea (3).

The fact that the staining of cells which do not synthesize Ig is essentially the same as that of the IgM-secreting cells suggests that intrinsic cellular components (membrane proteins or lipids, lysosomal enzymes) are themselves asymmetrically distributed across the Golgi stack.

Finally, the peroxidase-labeling of the Golgi complex calls attention to the highly fenestrated structure of the most proximal and most distal cisternae. Such fenestration has been observed in histochemical preparations of other cells (16, 19). Its functional significance is unknown, but it is striking that

³ When cells are treated with monensin to dilate Golgi cisternae (26, 27), staining with either lectin conjugate stains about half the dilated elements, as expected if the effect of monensin is "segmental," i.e., does not cause intermixing of Golgi subcompartments.

⁴ Smooth microsomal fractions of the IgM myeloma have been run on SDS gels that were blotted onto nitrocellulose paper (29) and the blots then stained with the conjugates used for electron microscopy. By far the most abundant protein stained with Con A-horseradish peroxidase is the Ig heavy chain, WGA-horseradish peroxidase stains a range of proteins, including species that co-migrate with heavy chains.

it is characteristic of just those portions of the Golgi stack that are thought to participate in vesicle fusion or generation.

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