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Immunocytochemical localization of α-D-mannosidase II in the Golgi apparatus of rat liver

(glycoprotein-processing enzymes/staphylococcal protein A-peroxidase cytochemistry/endoplasmic reticulum/lysosomes/GERL)

PHYLLIS M. NOVIKOFF*, D. R. P. TULSIANI†, OSCAR TOUSTER†, ANA YAM*, AND ALEX B. NOVIKOFF*

*Department of Pathology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461; and †Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235

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ABSTRACT **Mannosidase II is involved in the trimming of α-1,6-mannosyl residues during the biosynthesis of glycoproteins containing N-linked oligosaccharides of the complex type. A highly specific polyclonal antibody (IgG) was isolated from rabbits immunized with a homogeneous preparation of mannosidase II prepared from rat liver. With this antibody, light and electron microscopic immunocytochemical studies on rat liver reveal that essentially all mannosidase II in hepatocytes is localized in the Golgi apparatus, the only other site with reaction product being the endoplasmic reticulum. The indirect immunocytochemical method used in this study involved three major steps: exposure of aldehyde-fixed tissue to immune and nonimmune IgG, treatment with staphylococcal protein A labeled with horseradish peroxidase, and incubation in diaminobenzidine to reveal sites of peroxidase activity. The procedures described overcome major problems in immunocytochemistry, allowing preservation of antigenic sites and maintaining adequate ultrastructural integrity. The *in situ* localization of other carbohydrate-processing enzymes, involved in either trimming or attachment of sugar residues, should be possible with this procedure. Because biosynthetic precursors of the processing enzymes may be revealed by an immunocytochemical approach, it is potentially significant that mannosidase II reaction product is present in areas of the endoplasmic reticulum as well as in the Golgi apparatus.**

The Golgi apparatus is involved in the post-translational modification of glycoproteins. This includes proteolytic cleavages as well as glycosylation reactions (1). Secretory and membrane proteins containing N-linked oligosaccharides are synthesized on the endoplasmic reticulum (ER) as precursors whose common oligosaccharide moiety is modified during subsequent processing events. α-1,2-Mannosyl residues are removed by mannosidases IA and IB, and α-1,3- and α-1,6-mannosyl residues are removed from $GlcNAcMan_5(GlcNAc)_2$ species by mannosidase II to produce $GlcNAcMan_3(GlcNAc)_2$ species, which are then converted to complex oligosaccharides by the addition of various sugars (2). Evidence for the Golgi localization of the mannosyl trimming reactions consists of: (*i*) the finding of mannosidases in purified Golgi membrane preparations (2–5); and (*ii*) pulse–chase experiments on glycoprotein biosynthesis (6).

The *in situ* visualization of these enzymes would provide direct confirmation of their intracellular localization and perhaps do so in a more precise manner than possible by biochemical methods. The possibility of cross-contamination of isolated organelle fractions can rarely be excluded unequivocally, particularly contamination by as-yet-undefined cellular elements. Reliable ultrastructural enzyme cytochemical methods for demonstrating α-D-mannosidases and other glycoprotein processing enzymes have not been developed to date.

Rat liver mannosidase II has been isolated in homogeneous form and shown to be different from other liver mannosidases (7). With the availability of highly specific polyclonal antibody to mannosidase II, we chose an indirect immunocytochemical method, staphylococcal protein A-peroxidase (8), to detect the antigenic sites of this enzyme in liver. Protein A interacts with the Fc portion of IgG molecules and can be labeled with horseradish peroxidase, which, in turn, can be cytochemically visualized by reaction with 3,3′-diaminobenzidine (DAB). The distribution of the resulting reaction product is valid at both the light and electron microscope levels.

This report presents light and electron microscopic evidence that mannosidase II is present in the Golgi apparatus in hepatocytes from rat liver. The enzyme appears to be distributed uniformly in the elements of the Golgi apparatus. It is not present in other organelles except in portions of the ER, which may reflect crossreactivity with mannosidase II precursors.

MATERIALS AND METHODS

Materials. Immunodiffusion plates (pattern C) were obtained from Diagnostics, Division of Travenol Laboratories (Deerfield, IL). Homogeneous preparations of Golgi mannosidase II were obtained from 0.4 M NaCl-washed rat liver Golgi membranes, as described previously (7), except that the enzyme from the hydroxylapatite column was eluted with 250 ml of linear potassium phosphate buffer gradient (10–500 mM, pH 7.2), containing 0.1% Triton X-100. The enzymatically active fractions were concentrated to small volumes (0.5–1 ml) by using an Amicon concentrator equipped with a PM-30 membrane. The homogeneity of the enzyme was established on the basis of its specific activity and behavior in polyacrylamide gel electrophoresis (7).

Production of Antiserum. Purified Golgi mannosidase II (70–80 μg of enzyme protein) in 1 ml of 100 mM potassium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.5% Triton X-100, was emulsified with an equal volume of Freund's complete adjuvant. Male New Zealand White rabbits (1 kg) were injected intramuscularly in the thigh and subcutaneously in the back. Immunization was repeated every week for 4 weeks. The animals were bled by ear vein 8 days after the fourth injection. The blood, allowed to clot at room temperature, was centrifuged at 1,600 × *g* for 30 min to obtain antiserum.

Purification of Anti-Mannosidase II IgG. The gamma globulin (IgG fraction) was purified from the antiserum as described (9). In brief, the antiserum was precipitated four times with $(NH_4)_2SO_4$ at 45% saturation, the precipitate being dissolved each time in 10 mM potassium phosphate buffer, pH 8.0. The final precipitate was dissolved in 10–20 ml of the above buffer,

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Abbreviations: DAB, 3,3′-diaminobenzidine; ER, endoplasmic reticulum; HRP, horseradish peroxidase.

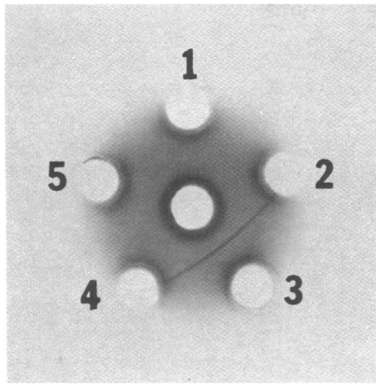

FIG. 1. Immunological characterization of rat liver Golgi mannosidase II by the Ouchterlony double-diffusion technique. The center well contained IgG to Golgi mannosidase II. Peripheral wells are: 1, rat liver Golgi mannosidase IA; 2, rat liver Golgi mannosidase 1B; 3, rat liver Golgi mannosidase II; 4, rat liver lysosomal mannosidase; and 5, rat epididymal mannosidase. The plate was stained for protein, as described (12).

dialyzed extensively against the same buffer, and centrifuged at 35,000 × *g* for 30 min. The clear supernatant was applied to a DE-52 DEAE-cellulose (Whatman) column (1.7 × 34 cm) equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The protein peak (IgG) eluted from the column at about 50 mM phosphate when elution was carried out with 500 ml of a linear phosphate gradient (10–300 mM, pH 8.0). After it was concentrated to a small volume, the IgG was examined by the Ouchterlony double diffusion technique (10) for crossreactivity with rat liver Golgi α-D-mannosidases IA and IB (2), rat liver lysosomal mannosidase (11), and rat epididymal mannosidase (unpublished data). No crossreactivity was observed with these mannosidases (Fig. 1) (12) or with rat liver cytosolic α-D-mannosidase (13) (result not shown).

Morphological Procedures. Untreated Sprague–Dawley male rats (350–400 g) (Marland Farms, Hewitt, NJ) were anesthetized by ether and the livers were fixed either by immersion or by perfusion in the following fixatives at 4°C: a, 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4/7.5% sucrose (P_i/sucrose); b, 4% paraformaldehyde in P_i/sucrose/0.05 M L-lysine/0.01 M sodium periodate (14); and c, 4% paraformaldehyde/0.05% glutaraldehyde/P_i/sucrose (15). Perfusion fixation was employed, as described elsewhere (15). Total fixation time was 4 hr each in a and b and 2 hr in c, with gentle shaking of tissues.

After an overnight rinse in cold P_i/sucrose, nonfrozen sections 30 μm thick were prepared from liver slices from all fixatives with an Oxford Vibratome (Ted Pella, Tustin, CA), using a slow speed and high-amplitude setting, and placed in cold 7.5% sucrose. Sections prepared from tissue fixed in mixture c were treated for 30 min in cold 0.05 M lysine (16) in P_i/sucrose prior to exposure to immunoreagents.

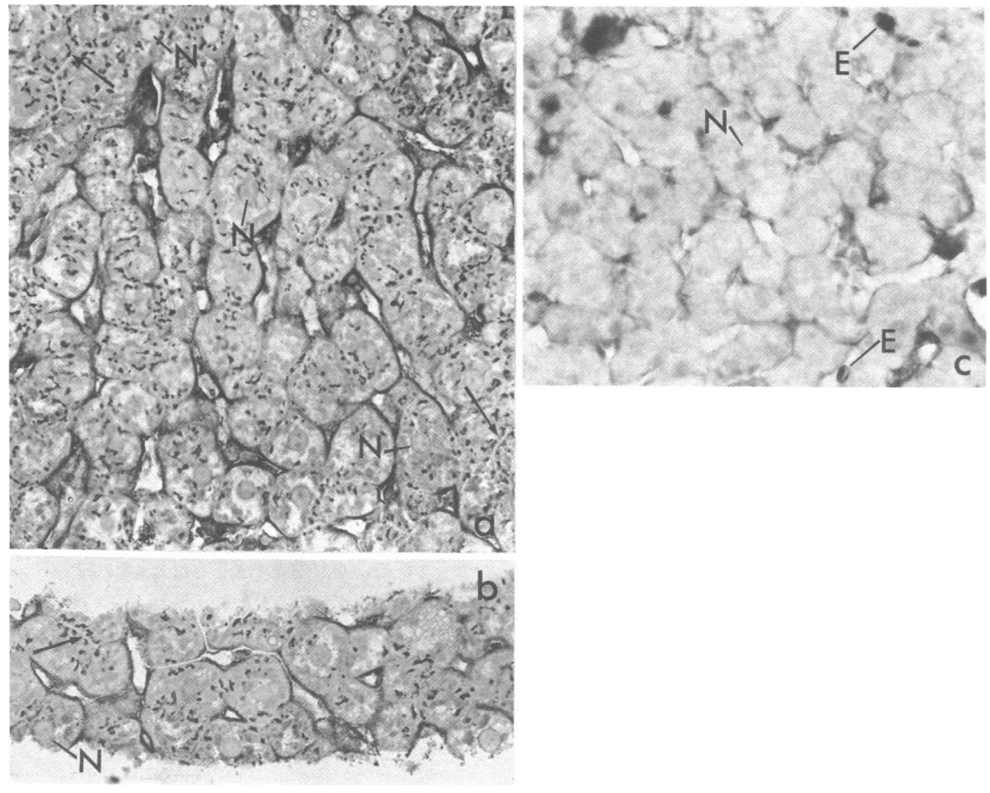

FIG. 2. (*a*) Three- to 4-μm Epon section of rat liver (fixative b). The sections in *a* and *b* were exposed to IgG against rat liver α-D-mannosidase II for 20 hr, counterstained lightly with toluidine blue. Portions of the Golgi apparatus are widespread in the hepatocytes, appearing as darkly stained structures. Some Golgi apparatus are near nuclei, indicated by N; others are near bile canaliculi, indicated by arrows. In 3-μm Vibratome sections, the apparently continuous nature of the Golgi apparatus can be seen more clearly. The stain at the sinusoids is nonspecific, possibly resulting from adsorption of hemoglobin. (×320.) (*b*) Three- to 4-μm Epon section of rat liver (fixative a), exposed to mannosidase II and counterstained with toluidine blue. A section cut through the depth of the Vibratome section is shown. This shows that the diffusion of immunoreagents is not limiting; the Golgi apparatus is visualized in all hepatocytes. The bile canaliculi (arrow) show no reaction product. (×320.) (*c*) Vibratome section (*ca.* 20 μm) of rat liver (fixative a). This section was exposed to nonimmune IgG for 40 hr. Note the absence of reaction product from the cytoplasm of the hepatocytes. Neither the Golgi apparatus nor other organelles are stained. Labeled are the nucleus (N) and erythrocytes (E). As in *a* the sinusoidal staining is evident. (×320.)

Individual Vibratome sections (approximately 2 mm square) were exposed to 0.2 ml of rabbit mannosidase II antibody (0.22 mg of IgG per ml) at 4°C for 18–40 hr, with intermittent mild agitation. After several rinses in cold P_i/sucrose the sections were treated with protein A coupled to horseradish peroxidase (HRP) (0.1 mg/ml) (E. Y. Laboratories, San Mateo, CA) for 1 hr at room temperature, in the dark. After several rinses in P_i/sucrose and in 7.5% sucrose, the sections were incubated in DAB medium (17) at pH 7.6, containing 7.5% sucrose, at room temperature for 10 min. The following controls were performed: (*i*) exposure of sections to nonimmune or normal rabbit antibody (final concentration 0.22 mg of IgG per ml) (Cappel Laboratories, Cochranville, PA), treatment with protein A-HRP and incubation in DAB, as described above; (*ii*) treatment with protein A-HRP and incubation in DAB, as above, but without prior exposure to immune or nonimmune antibody; (*iii*) treatment with unlabeled protein A and incubation in DAB, as above; and (*iv*) incubation in DAB, as above, but no treatment with antibody, protein A-HRP, or protein A.

Vibratome sections treated in the above procedures were examined by light microscopy after mounting in glycerogel and processed for electron microscopy after additional fixation in cold 2.5% (vol/vol) glutaraldehyde/P_i/sucrose for 30 min before treatment with osmium tetroxide. The electron microscope procedures followed were as described elsewhere (15). Epon sections were examined with and without *en bloc* uranyl acetate stain (18, 19) and with and without lead stain (20), using a Philips EM-300.

RESULTS

Light Microscopic Observations. Intense staining for mannosidase II is dramatically revealed in light microscope preparation of 3- to 4-μm Epon sections (Fig. 2 *a* and *b*). All the hepatocytes are positive for mannosidase II localization. By light microscopy, mannosidase II distribution appears to be exclusively localized to the Golgi apparatus. The widespread nature of the Golgi apparatus is evident. Portions of the apparatus are seen near the bile canaliculus, near the nucleus, and throughout the hepatocyte cytoplasm. The staining of the sinusoidal areas is nonspecific, most probably due to diffusion of hemoglobin or leakage of endogenous peroxidase from disrupted Kupffer cells that occurred during the preparative procedures. The bile canaliculi are devoid of reaction product. All controls showed complete absence of reaction product in the cytoplasm of all hepatocytes. Fig. 2*c* illustrates a control incubated with nonimmune rabbit IgG. When thickness of sections (20 μm as against 4 μm) is considered, the sinusoidal nonspecific staining is less intense than in Fig. 2 *a* and *b*.

Fig. 2*b* shows that all immunoreagents penetrate through the depth of the section (two to three hepatocytes). All hepatocytes show immunoreactive Golgi apparatus (Fig. 2 *a* and *b*).

Electron Microscope Observations. Figs. 3, 4, and 5*a* show Golgi-GERL regions in hepatocytes. Reaction product appears to be present in all the elements of the Golgi stack. Because the Golgi apparatus is an extensive organelle that undulates and twists through the cytoplasm, perpendicular cuts that reveal all the elements from the *cis* to the *trans* aspect are not regularly encountered. Figs. 3 and 4 show portions of three or four distinct elements that are parallel to each other and separated by a relatively small distance. The reaction product appears to be localized to the membrane rather than within the cisternae of the elements (see *Discussion*). GERL and its derivatives (21–23) are consistently negative for mannosidase II (Figs. 3*a* and 4). Coated regions are seen attached to areas of GERL and lipoprotein-like particles are present within the cisternae of GERL (Figs. 3*a* and 4). Fig. 3*b* shows that residual body type lysosomes and another type of lysosome, autophagic vacuoles, are also devoid of reaction product.

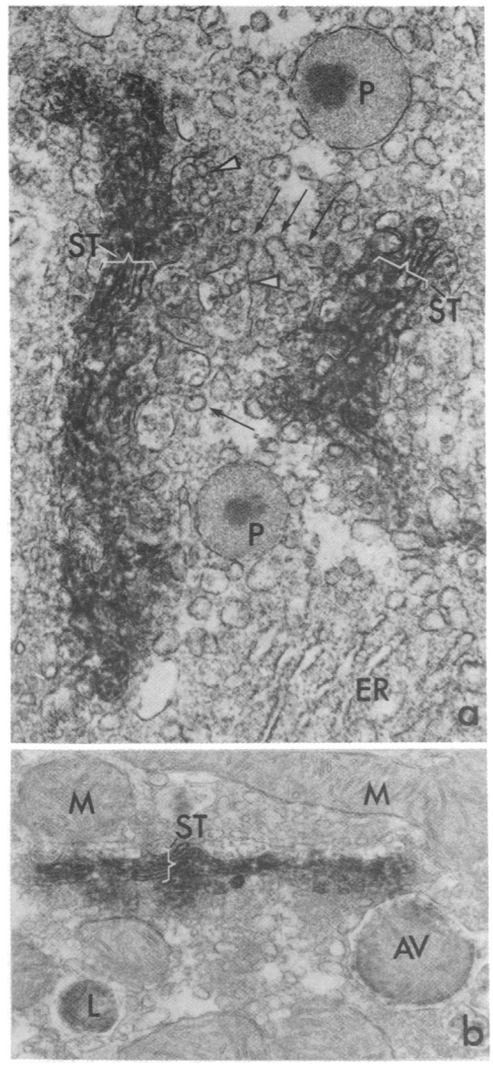

FIG. 3. The elements of the Golgi stack are seen most clearly at ST, where they are sectioned transversely; elsewhere they are cut tangentially. All elements appear to have reaction product. No reaction product is seen elsewhere. In *a*, negative structures include ER, peroxisomes (P), and GERL. The coated areas of GERL are indicated by arrows, and its lipoprotein-like particles by arrowheads. In *b* the micrograph includes lysosomes. Two types are seen: residual bodies (L) and autophagic vacuoles (AV) without reaction product. Also without reaction product are mitochondria (M). (Section *a* is fixed with fixative b, and treated with uranyl acetate and stained with lead ions; ×32,300. Section *b* is fixed with fixative a, treated with uranyl acetate, and not stained with lead ions; ×19,500.)

Mitochondria and peroxisomes are always devoid of reaction product. Fig. 5 *a* and *b* shows electron-dense reaction product associated with the membranes of the ER. Fig. 5 *c* and *d* illustrates the absence of reaction product from the Golgi apparatus and the ER in sections exposed to nonimmune IgG.

Reaction product is observed associated with the ER even in sections exposed to immune IgG that were neither stained *en bloc* with uranyl acetate nor stained with lead ions. Reaction product is absent from the ribosomes attached to the ER in sections exposed to both immune and nonimmune IgG.

All three fixatives used in this study yielded adequate but not optimal preservation. However, each fixation showed unequivocal localization of mannosidase II to the Golgi apparatus.

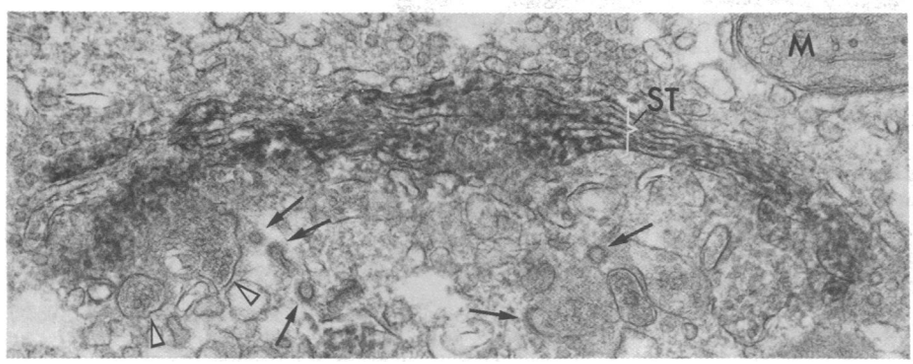

FIG. 4. All elements of the Golgi stack (ST) have reaction product. No reaction product is seen elsewhere. This includes GERL with its coated regions (arrows) and its lipoprotein-like particles (arrowheads) and the mitochondria (M). (Section of material fixed in fixative a, treated with uranyl acetate, and stained with lead ions; ×33,800.)

When low concentrations of glutaraldehyde were used (as in fixative procedure c), variability in the intensity of the reaction product was observed at both light and electron microscopic levels. At the electron microscope level, not only was there variability in the number of elements that were reactive but also there was a nonuniform distribution throughout the individual elements. It should be stressed that reaction product does not fill in the spaces between successive elements and that there are no continuities between elements to be seen in unincubated tissue. This speaks against the possibility that oxidized DAB has diffused from one Golgi element to the other (cf. ref. 24). Improvements in presently available procedures are required that will keep antigenic sites in place and reactive and will improve ultrastructural preservation.

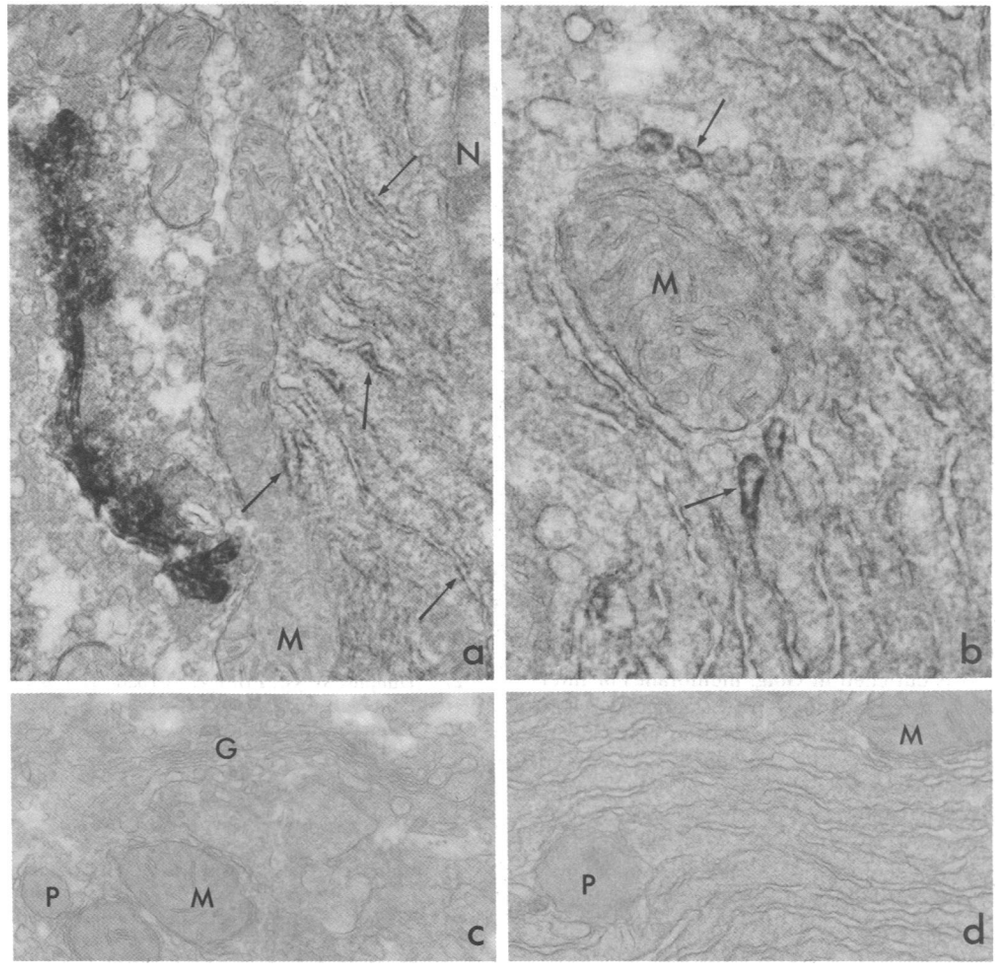

FIG. 5. (*a*) Reaction product is seen in the Golgi stack but is so sectioned that the individual elements are difficult to discern. Dilatations of the elements are evident at the bottom. Note areas of reaction product in the ER (arrows). The mitochondria (M) and nucleus (N) show no reaction product. (Fixation in fixative a; section treated in uranyl acetate and not stained with lead ions; ×24,000.) (*b*) Reaction product is seen in areas of the endoplasmic reticulum (arrows). Note that the ER is sectioned lengthwise and transversely. The mitochondrion (M) has no reaction product. (Fixation in fixative b; section treated in uranyl acetate and not stained with lead ions; ×41,300.) (*c*) Control section exposed to nonimmune IgG. Note the Golgi elements (G) are totally negative. Included in the section are mitochondria (M) and peroxisomes (P), also negative. (Fixation in fixative b; section treated in uranyl acetate and not stained with lead ions; ×21,000.) (*d*) Control, as in *c*. The parallel array of ER has no reaction product. Also seen are a peroxisome (P) and a mitochondrion (M), both negative. (Fixation in fixative b; section treated in uranyl acetate and not stained with lead ions; ×26,300.)

DISCUSSION

We report the demonstration of the *in situ* localization of the carbohydrate-trimming enzyme mannosidase II to the Golgi apparatus of rat hepatocytes. In contrast, preliminary studies on the immunocytochemical localization of a lysosomal α-D-mannosidase purified from rat epididymis show this enzyme to be present in lysosomes, but to be absent from the Golgi apparatus (unpublished data). Biochemical studies on Golgi-enriched fractions isolated from rat liver indicate that mannosidase II is membrane bound (6). In our electron micrographs, reaction product appears to be associated with the membranes of the elements rather than the cisternae. However, its localization to the Golgi membrane must be interpreted with caution because of the possibility of diffusion artifacts with DAB cytochemistry (24). Of particular interest is the reaction product associated with the ER. Until we can rule out an adsorption artifact, its interpretation as either an early cellular site in the biosynthesis of mannosidase II or, less likely, as a crossreaction with an antigenic site of an-as-yet-uncharacterized ER mannosidase (25) remains uncertain. However, it may be mentioned that in HeLa cells mannosidase II precursor has in fact been found to react with mannosidase II antibody (unpublished results). *In situ* procedures of the type reported in the present paper, in contrast to methods employing isolation of fractions [e.g., the study of galactosyltransferase by Bretz, *et al.* (26)], can offer a more precise localization to specific Golgi elements and regions within the elements.

The subfractionation of the Golgi apparatus has been undertaken in several laboratories in an attempt to localize specific processing reactions of glycoprotein biosynthesis in particular regions of the apparatus. For example, galactosyltransferase was reported to be enriched in a fraction mainly representing *trans* Golgi elements, whereas α-1,2-mannosidase activity was reported to have a predominantly *cis* localization (27, 28). The densities of the subfractions studied apparently did not permit their clear separation. We have utilized a variety of methods (28–30) in attempts to separate Golgi into subfractions differing substantially in their mannosidase II activity (unpublished experiments). The lack of success of these attempts might be explained by the fact that all Golgi membranes are revealed, by immunocytochemistry, to contain immunoreactive mannosidase II. However, it is possible that biosynthetically mature, enzymatically active mannosidase II is present only in limited regions of the Golgi. A very recent study (31) involving sucrose gradient centrifugation for the fractionation of membranes from a mouse lymphoma cell line indicated that mannosidase II is enriched in Golgi membranes of intermediate density as compared with the fractions enriched in early or late enzymes of the glycoprotein biosynthesis pathway.

Our procedure visualized all of the rat hepatocyte Golgi apparatus. The widely used cytochemical procedure for staining the Golgi apparatus is thiamin pyrophosphatase or nucleoside diphosphatase activity (32). This shows less of the Golgi apparatus than does our present method, as can be seen in figure 6 of ref. 33. Only the *trans* element responds to the thiamin pyrophosphatase procedure (34). Another difference between the present immunocytochemical method and the thiamin pyrophosphatase procedure is that the former method does not stain the bile canaliculi. The classical metal preparations also appear, by light microscopy, to show all of the Golgi apparatus (figures 13 and 14 of ref. 35). In a recently published paper (36) showing frozen sections of rat liver treated with antisera raised against Golgi membranes, only very small portions of the Golgi apparatus were revealed. The thiamin pyrophosphatase method stains the Golgi apparatus of all tissues and cells thus far studied (37). Preliminary studies on the epithelial absorptive cells, the crypt cells of the intestine, and the neurons in the cerebellum indicate that the same is true with this mannosidase II immunocytochemical procedure.

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