Retention of Membrane Proteins by the Endoplasmic Reticulum

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ABSTRACT We have used a monoclonal antibody specific for a hydrocarbon-induced cytochrome P450 to localize, by electron microscopy, the epitope-specific cytochrome P450. The cytochrome was found in the rough and smooth endoplasmic reticulum (ER) and the nuclear envelope of hepatocytes. Significant quantities of cytochrome P450 were not found in Golgi stacks. We also could not find any evidence of Golgi-associated processing of the Asn-linked oligosaccharide chains of two well-characterized ER membrane glycoprotein enzymes (glucosidase II and hexose-6-phosphate dehydrogenase), or of the oligosaccharides attached to the bulk of the glycoproteins of the ER membrane. We conclude that these ER membrane proteins are efficiently retained during a process of highly selective export from this organelle.

Evidence from subcellular fractionation (1–5; and reviewed in reference 6) suggests that membrane proteins (enzyme markers) that are most concentrated in the endoplasmic reticulum (ER)[1] membranes are also found at high concentrations in the Golgi complex, a highly compartmentalized organelle (6–12).

To examine this issue further, we have determined the intracellular localization of a major ER membrane protein, cytochrome P450 (13), by electron microscope immunocytochemistry. We have also studied the structures of the oligosaccharide chains of two particular ER membrane glycoproteins (glucosidase II [14–16] and hexose-6-phosphate dehydrogenase [H6PDH; 17]) as well as those of a broad spectrum of membrane glycoproteins prepared from ER fractions of rat liver to seek evidence of Golgi-associated oligosaccharide processing of these glycoproteins.

MATERIALS AND METHODS

Cytochrome P450 Experiments

ANIMALS: Wistar rats (Simonson Labs) were either starved for 24 h or fed ad libitum (as indicated) before they were killed. Induction of 3-methylcholanthrene (3-MC)–cytochrome P450 was achieved by intraperitoneal injection of 10 mg 3-MC (Eastman Kodak Co., Rochester, NY) per 340 gm body weight. 3-MC was dissolved at 50°C in corn oil at 10 mg/ml concentration. Control rats were mock-induced by injection of an equivalent amount of corn oil. Livers were excised 1 d after a single injection.

ANTIBODIES: Monoclonal IgG antibodies to a 3-MC–induced form of cytochrome P450 (MC-P450 1-7-1) and to a phenobarbital-induced cytochrome P450 (PB-P450 2-66-3) have been described (18, 19). Both are of the IgG_1 subtype and react with different forms of P450. Affinity-purified rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA), rhodamine-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA), and protein A (Pharmacia Fine Chemicals, Piscataway, NJ) were purchased. Protein A/gold conjugates of ~5-, 8-, or 12-nm diam were prepared as described (20).

TISSUE PREPARATION: Rat liver was fixed by portal vein perfusion with a gradient of 2–8% paraformaldehyde (in phosphate-buffered saline [PBS]) as described (21). 1-mm^3 blocks of liver were stored in 8% paraformaldehyde in PBS. A Golgi-enriched fraction of rat liver was prepared according to Bergeron et al. (22) and fixed in formaldehyde (21).

IMMUNOCYTOCHEMISTRY: Semithin (200 nm) and ulthrathin (50–100 nm) cryosectioning (for light microscopy and electron microscopy, respectively) of liver and liver Golgi-enriched fractions, as well as the subsequent immunoincubation procedures, were done according to Brands et al. (21). The anti-MC-P450 monoclonals were used at 50 μg/ml. Since the mouse monoclonals do not bind protein A/gold conjugates, we applied rabbit anti-mouse IgG as an intermediate antibody (as described in reference 21) to localize the cytochrome P450 subtypes studied. For reference to standard morphology, some tissue blocks were Epon-embedded (Polysciences, Inc., Warrington, PA) after osmium postfixation and subsequent dehydration, and stained with uranyl acetate after sectioning.

H6PDH and Glucosidase II

PROTEINS: H6PDH and glucosidase II were purified as previously described (16, 17).

ENDOGLYCOSIDASE H DIGESTION OF H6PDH: Digestion of H6PDH with endoglycosidase H (Endo H) was essentially as described (16). 2 μg of H6PDH preparation (20 μl) was mixed with an equal volume of 0.1 M Tris-

[1] *Abbreviations used in this paper:* Con A, concanavalin A; H6PDH, hexose-6-phosphate dehydrogenase; 3-MC, 3-methylcholanthrene; RCA I, ricin agglutinin I; RM_2, rough microsome fraction.

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HCl buffer (pH 6.8) containing 30 mM dithiothreitol and 2% SDS, and heated at 100°C for 5 min. Then 20 μl of 0.3 M sodium citrate buffer (pH 5.5) containing 1 mg/ml SDS, either in the presence or absence of Endo H (6 ng protein, 2 × 10^4 IU), was added, and the mixture was incubated for 16 h at 37°C. The digest was subjected to SDS PAGE, and the gel was analyzed for protein, concanavalin A (Con A) binding, or ricin binding as will be described below.

LECTIN BINDING: ^{125}I-Con A was used in conjunction with SDS PAGE to determine if a polypeptide has a Con A–binding oligosaccharide. Paper blots of the SDS gels were incubated with ^{125}I-Con A as before (16). Ricin agglutinin I (RCA I) binding was studied by essentially the same procedure (16) except that an anti–RCA I IgG and ^{125}I-protein A were used for detection. The diazonium paper transfer of the gel (~100 cm^2) was thoroughly washed with 0.25% gelatin solution containing 0.1 M Tris-HCl buffer (pH 9.0) and then with a detergent solution (0.15 M NaCl, 50 mM Tris-HCl [pH 7.4], 0.25% gelatin, and 0.05% Nonidet P-40) to inactivate any remaining diazonium groups. The inactivated paper was incubated for 1 h at room temperature with 1.5 ml of the same detergent solution now containing RCA I (20 μg/ml). Then, the paper was incubated (in 1.5 ml vol) for 1 h each at room temperature successively with mucin (1 mg/ml), anti–RCA I IgG (1.5 μg/ml; kindly provided by Vector Laboratories, Inc.), and ^{125}I-protein A (80 ng protein, 7 × 10^5 cpm).

PERIODATE OXIDATION: Sialic acid is specifically oxidized by sodium metaperiodate under mild conditions (23). The aldehyde group formed by the oxidation can be reduced to the corresponding radioactive alcohol group by $NaB[^3H]_4$. A glucosidase II preparation (100 μg/ml; 0.5 ml) was extensively dialyzed against 0.1 M acetate buffer (pH 5.6) containing 0.15 M NaCl, was mixed with 0.05 ml of 60 mM $NaIO_4$ (final concentration 5 mM), and incubated for 10 min on ice. The reaction was then quenched by adding an excess of ethylene glycol (20 μl). The whole mixture was then thoroughly dialyzed at 4°C against 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl to remove the remaining periodate. Then 0.6 mCi of carrier-free $NaB[^3H]_4$ (100 mCi/ml dissolved in 0.01 M NaOH) was added to the oxidized sample, and the mixture was incubated for ~1 h at room temperature. The sample obtained as above was precipitated with 6% trichloroacetic acid, using 0.2 mg of cytochrome *c* as a carrier protein. The protein precipitate was neutralized and subjected to SDS PAGE. After staining with Coomassie Blue, the gel was treated with ENHANCE (New England Nuclear, Boston, MA) and autoradiographed.

Analysis of Endo H–Sensitive Glycopeptides of Rough ER Membranes

Microsomes were extracted with 0.05% deoxycholate to remove soluble content proteins as described (25). Lyophilized membrane pellets (6–10 mg protein) were rehydrated in 0.5 ml of 0.1 M Tris-HCl, pH 8.0, 1 mM NaN_3, and 40 μl of 20 mg/ml pronase (CB grade, Calbiochem-Behring Corp., La Jolla, CA) was added. The pronase had been dissolved in this same buffer, but also containing 10 mM $CaCl_2$, and was incubated for 1.5 h at 37°C before use. Digestion was for 36 h at 50°C, with additional 25-μl portions of pronase being added after 12 and 24 h of incubation (26). The resulting glycopeptides were lyophilized, redissolved in water, and desalted on a Sephadex G-10 column. Glycopeptides were then reduced with 0.1 M $NaBH_4$ –0.15 M NaOH overnight at room temperature (to react any groups available before the Endo H cleavage), and then the reaction was stopped by the addition of glacial acetic acid, and the water evaporated under a stream of nitrogen. The glycopeptides were then treated with endoglycosidase H as described (26), and the liberated oligosaccharides were isolated by passing samples over columns of Sephadex G-10 and AG-1 X-2 (formate form, Bio-Rad Laboratories, Richmond, CA) using water as eluant. The resulting Endo H–sensitive oligosaccharide preparation was then reduced with 0.5 mCi $NaB[^3H]_4$ in 0.5 ml of 0.1 M NaOH for 24 h at room temperature. The reactions were desalted on AG-1 X-2 (H^+ form), and boric acid was removed by repeated evaporation from methanol, all as described (27). Contaminating, free radioactivity was removed by descending paper chromatography in ethyl acetate/acetic acid/formic acid/water (18:3:1:4) overnight (28). Material that ran in the first 2.5 cm of the chromatogram was cut out, eluted with water, and lyophilized. Samples were analyzed (26) by chromatography on 1 × 120-cm columns of Biogel P-4 (400 minus) or high pressure liquid chromatography on Lichrosorb Si-60 in the presence of 1,4-diaminobutane (29). Reduced oligosaccharides were treated with jack bean alpha-mannosidase (Sigma Chemical Co., St. Louis, MO) and the products analyzed on Biogel P-4 as previously described. Mild acid hydrolysis and chromatography on QAE Sephadex were performed according to Varki and Kornfeld (30).

RESULTS

Subcellular Localization of Cytochrome P450

After induction by substrates, certain forms of cytochrome P450 become major components of the ER membrane (13). The 3-MC–induced forms of cytochrome P450 (MC-P450) are virtually absent before induction, but are abundant after induction (31). We have used a monoclonal antibody (18, 31) together with electron microscope immunocytochemistry to localize the 3-MC–induced cytochrome P450 in rat liver hepatocytes. For this purpose, frozen sections of livers from control and from 3-MC–induced rats were prepared and were incubated with an anti–cytochrome P450 monoclonal antibody, and then rabbit anti–mouse antibody, and finally either rhodamine-conjugated goat anti–rabbit IgG (for immunofluorescence) or a protein A–gold conjugate (for electron microscopy).

The specificity of the monoclonal antibody for the induced form of MC-P450 in the context of the cytochemical procedures used here was demonstrated by comparing the level of immunofluorescent staining of 3-MC–induced (Fig. 1 *B*) and uninduced (Fig. 1 *C*) livers. Note that the section in Fig. 1 *C* (control) was photographed with 18 times the exposure used for Fig. 1 *B* (induced).

The distribution of cytochrome P450 can be explored at much higher resolution by electron microscopy. No qualitative changes occur as a result of 3-MC treatment, either in the structure of the Golgi complex or in the proportion or distribution of ER membranes (data not shown). Figs. 2 and 3 show that the 3-MC–induced cytochrome P450 is (as expected) found in both the rough and smooth ER membranes. The outer nuclear envelope (Fig. 2 *A*) also stains, consistent with the findings of Matsura et al. (42). Lysosomes, peroxisomes, and the nucleus do not stain. Glycogen granules, when present in livers from fed rats, also do not stain (Fig. 3 *A*). Despite some biochemical evidence suggesting a mitochondrial localization (43), mitochondria do not stain. The specificity of the immunocytochemical procedure for MC-P450 could be further demonstrated in two different ways. First, a control monoclonal antibody (also IgG_1) specific for a different kind of P450 induced by phenobarbital (19, 31) did not stain sections of 3-MC–induced livers (Fig. 2 *D*) but did stain sections of livers from phenobarbital-induced rats (not shown). Second, hepatocytes from control rats (not induced by 3-MC) did not stain with the anti–MC-P450 monoclonal antibody (Fig. 2 *B*). Together, these controls establish that the gold particles represent sites at which 3-MC–induced forms of cytochrome P450 are present.

Despite the ease with which cytochrome P450 can be demonstrated in the surrounding ER membranes, we could not detect this protein in the Golgi stack. Fig. 2 *C* illustrates a typical Golgi area in which the surrounding ER is clearly labeled for cytochrome MC-P450, but in which the Golgi stack itself has few, if any, gold particles above the background.

We have also examined isolated Golgi stacks in a Golgi-rich fraction (22) from which most of the ER membranes that surround the Golgi in the cell have been removed by fractionation. Staining of these isolated Golgi membranes for MC-P450 was also insignificant; microsomal membranes, obtained from the same homogenate, were heavily stained for P450 (data not shown).

In summary, the 3-MC–induced form of cytochrome P450 is a major component of the ER membrane, and we have been unable to detect significant quantities of this protein in Golgi stacks.

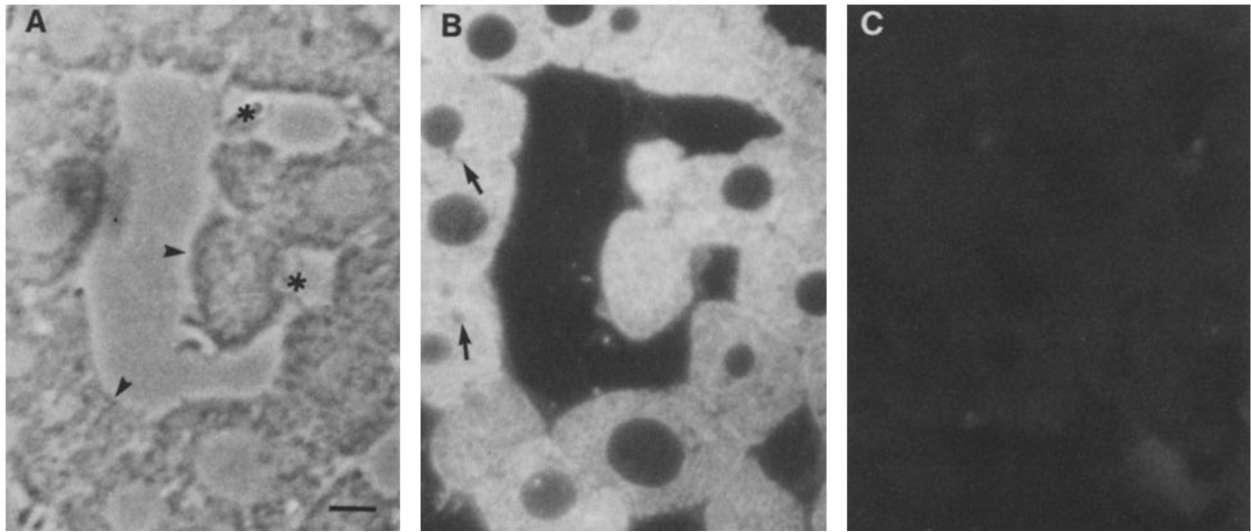


FIGURE 1 Specificity of monoclonal antibody to MC-P450 demonstrated by immunofluorescence (*A* and *B*); sections from an induced (fed) rat. (*A*) Phase contrast, (*B*) immunofluorescence (10-s exposure). (*C*) Section from an uninduced (control, fed) rat, stained in parallel for immunofluorescence (3-min exposure). Fluorescence is limited to the cytoplasm of hepatocytes. Endothelial cells (arrowheads) lining the sinusoids and Kuppfer cells (*) do not label. Labeling of Golgi regions (arrows) does not occur. Albumin is concentrated in the Golgi complex in these same areas (21). Bar, 10 μm. × 600.

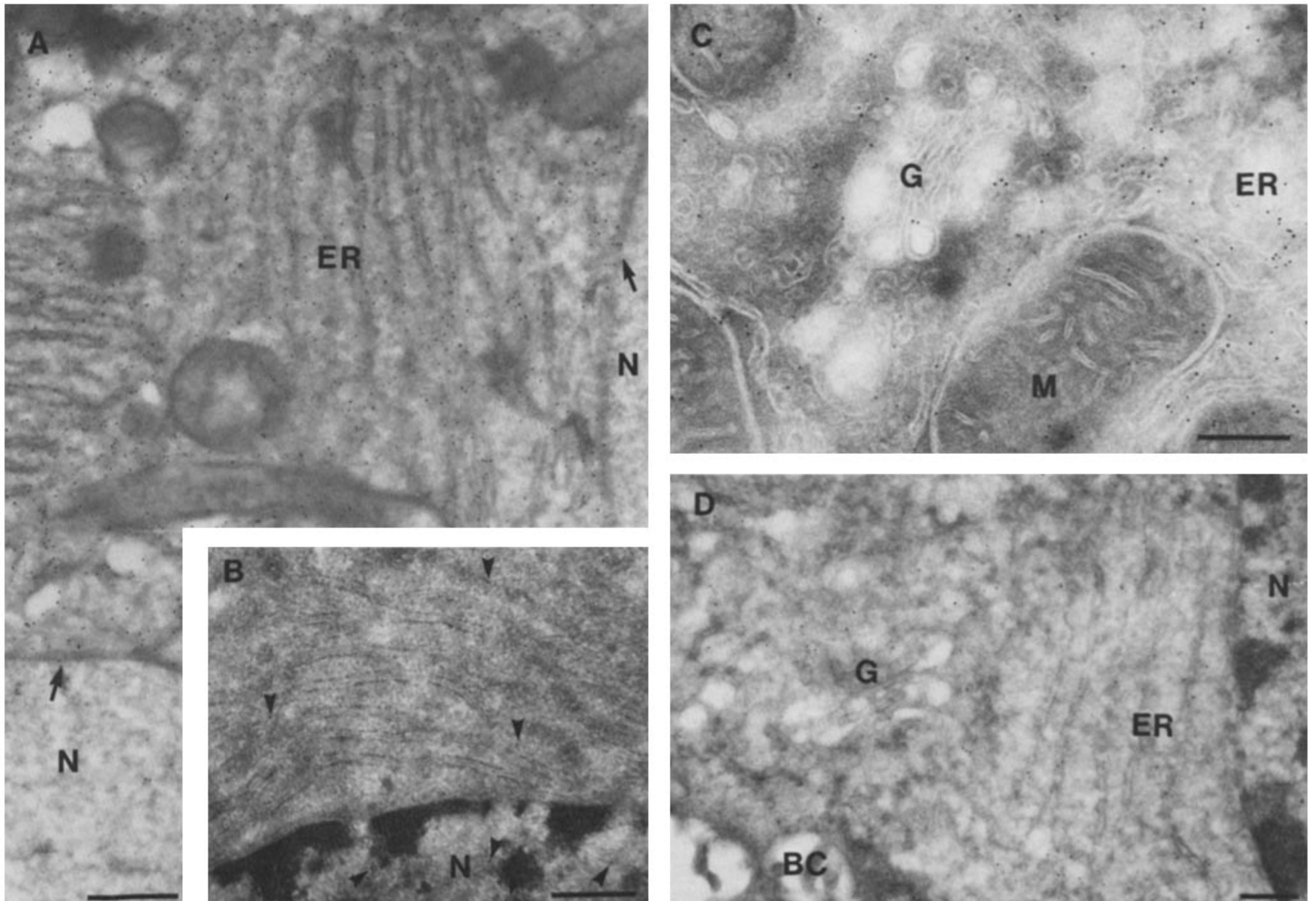


FIGURE 2 Electron microscopic immunolocalization of 3-MC–P450 in hepatocytes using 8-nm protein A/gold conjugates as the electron dense marker. (*A*) Label is specific for ER including the nuclear envelope (arrows). (*B*) Hepatocyte from an uninduced rat. The ER does not label for 3-MC P450 with the 3-MC P450 antibody (the few gold particles present are indicated by arrowheads). (*C*) Hepatocyte from induced rat. The ER surrounding a Golgi area is labeled, however, the Golgi complex itself is not labeled. Here, 12-nm gold conjugates were used. (*D*) Control in which a monoclonal antibody to a phenobarbital-induced form of cytochrome P450 was used to stain sections of MC-induced liver. Label over ER was at background levels as judged by the similar densities over nuclei (*N*) and mitochondria (not shown). 8-nm gold. (*A*) Bar, 0.5 μm. × 25,000. (*B*) Bar, 0.5 μm. × 22,000. (*C*) Bar, 0.25 μm. × 50,000. (*D*) Bar, 0.25 μm. × 33,000. The rats used for this figure had been starved before they were killed.

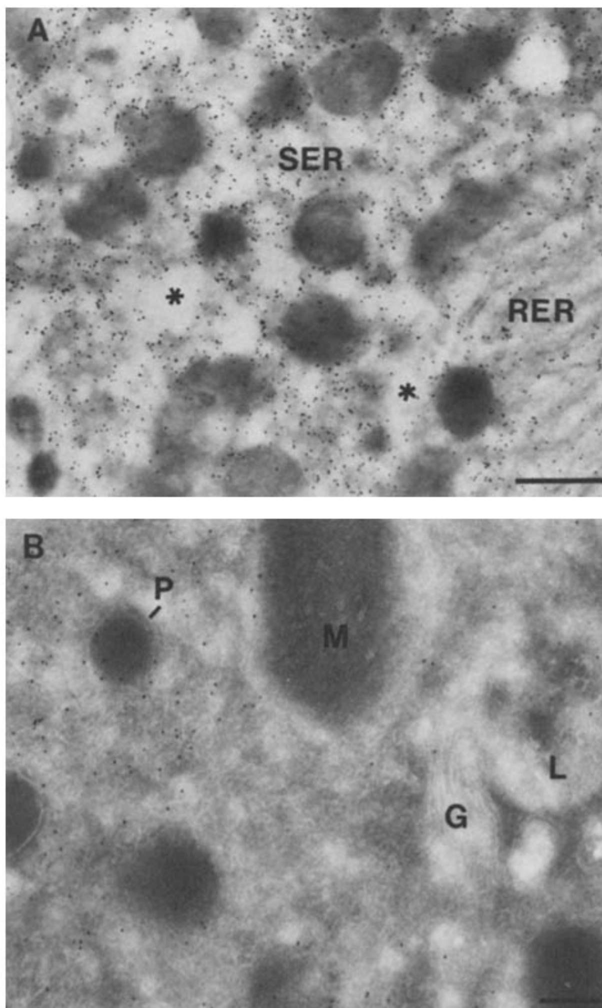


FIGURE 3 (*A*) Liver from fed rat, to retain glycogen granules. Note the density of 3-MC P450 label in smooth ER, which is now spaced out by glycogen filled areas (*). Rough ER is not affected. 9-nm gold conjugate. MC-450 labeled with 12-nm gold in livers from starved rats. Golgi (*G*) stacks are not labeled, whereas surrounding ER is labeled. Negligible label is seen over mitochondria (*M*), lysosomes (*L*), and peroxisomes (*P*). (*A*) Bar, 0.5 μm. × 22,000. (*B*) Bar, 0.25 μm. × 32,000.

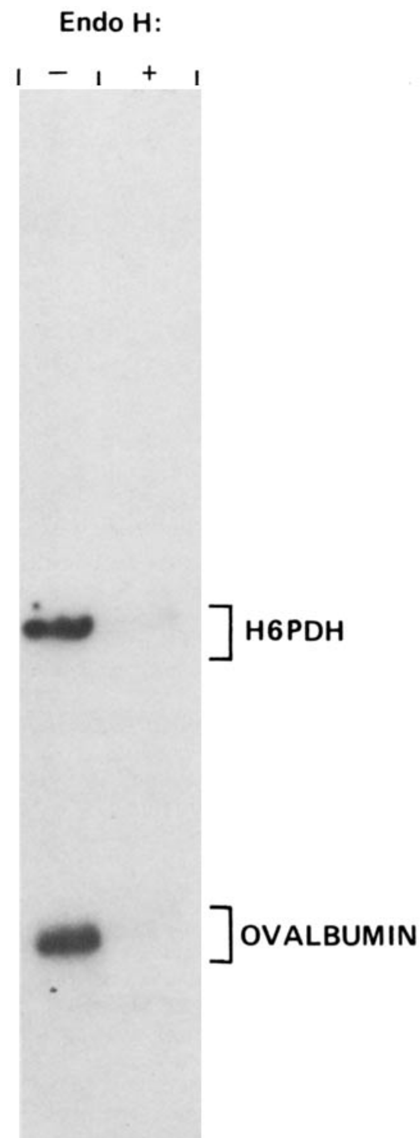


FIGURE 4 H6PDH has Endo H–sensitive Con A–binding sites. A mixture containing H6PDH (0.5 μg) and ovalbumin (2 μg) (as an internal standard) was incubated for 16 h at 37°C with or without Endo H, and then subjected to SDS PAGE. A paper transfer was incubated with ^{125}I-Con A to localize polypeptides with Man-containing oligosaccharide chains.

The Oligosaccharide Chains of H6PDH and of Glucosidase II

We have examined the oligosaccharide chains present on two glycoprotein enzymes of the ER membrane to see whether any evidence could be obtained of passage into (or back from) the Golgi. H6PDH was purified to homogeneity from rat liver microsomes (17). The polypeptide chain (108 kD) has previously been suggested to be a glycoprotein because it stained with the PAS reagent. Glucosidase II is an enzyme that acts in the ER to remove the inner two (1,3-linked) glucose residues from oligosaccharide chains soon after their transfer to Asn residues of nascent glycoproteins. Glucosidase II is itself a glycoprotein (14–16) as its polypeptide chain is sensitive to Endo H (15, 16). Glucosidase II has also been reported to contain sialic acid, on the basis of the effect of neuraminidase on its isoelectric point (14).

Fig. 4 confirms that H6PDH is glycoprotein, and shows that it contains Endo H–sensitive oligosaccharide chains. For this experiment, untreated and Endo H-digested H6PDH were electrophoresed on an SDS gel and then transferred to diazonium paper. The paper blot of the SDS gel was then incubated with ^{125}I-labeled Con A to reveal the location of any Con A-binding polypeptides. The intact enzyme, retaining its Endo H–sensitive oligosaccharides, bound radioactive Con A. The binding sites for Con A were largely lost upon removal of the Endo H–sensitive oligosaccharide chains. This suggests an absence of complex-type Asn-linked oligosaccharide chains.

We have previously reported that glucosidase II is Endo H–sensitive, and that all of the Con A binding sites are also lost after an Endo H digestion (16). Fig. 5*a* tests whether or not any RCA I (a lectin specific for Gal attached to N-linked chains) binding sites may be present on the glucosidase II polypeptide chains. For this purpose, an SDS gel of glucosi-

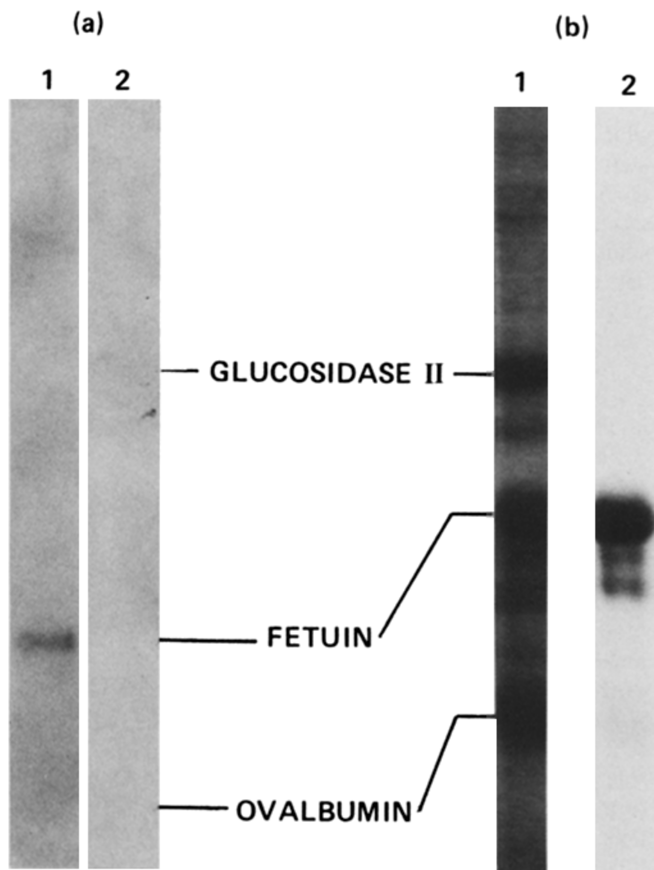


FIGURE 5 Glucosidase II appears to lack N-linked galactose as well as sialic acid residues. (a) A glucosidase II preparation (13.4 μg) was electrophoresed on an SDS gel (in lane *2*), and the paper blot of the gel was analyzed for RCA I binding polypeptides (lane *2*). Ovalbumin (0.5 μg) and fetuin (0.5 μg) were electrophoresed together (in lane *1*) to validate the technique as negative and positive controls, respectively. Note that fetuin (lane *1*) binds RCA, but ovalbumin (lane *1*) and glucosidase II (lane *2*) do not. The position of these proteins was established from a stained replica (not shown). (*b*) A mixture of glucosidase II (50 μg), fetuin (50 μg), and ovalbumin (50 μg) was treated with sodium metaperiodate and then with $NaB[^3H]_4$, to label sialic acids. The mixture was electrophoresed on an SDS gel. One lane was stained with Coomassie Blue for protein (lane *1*). An identical lane was autoradiographed with fluorographic enhancement (lane *2*) to locate the sialic acid–containing species. Note that fetuin (a positive control) was labeled, whereas ovalbumin (negative control) and glucosidase II were not labeled.

dase II (lane *2*) was transferred to paper, and the paper blot was probed with ^{125}I-labeled RCA I. No binding to the glucosidase II polypeptide chain could be detected. Positive and negative controls were included in this experiment to validate the technique. Fetuin, a glycoprotein that contains galactose, did bind RCA I (lane *1*). Ovalbumin, lacking Gal residues, did not bind any RCA I (lane *1*).

To test whether or not sialic acid residues might be present in glucosidase II, we have taken advantage of the finding (23) that sialic acid (whether attached to N-linked or O-linked chains) can be specifically oxidized by sodium metaperiodate under mild conditions. The aldehyde group formed by the oxidation can be reduced to the corresponding alcohol by ^{3}H-labeled borohydride, thus incorporating ^{3}H into the glycoprotein. The results are shown in Fig. 5 *B*. Lane *1* was stained for protein. An identical lane (lane *2*) was autoradiographed. No incorporation of radioactivity into glucosidase II occurred as a result of this procedure (lane *2*). As positive and negative controls we included fetuin in the same reaction. Fetuin, which contains terminal sialic acid residues, incorporated ^{3}H to a high level (lane *2*). Ovalbumin was also included in the reaction mixture as a negative control. Ovalbumin, which lacks sialic acid, did not incorporate any ^{3}H (lane *2*).

Together, these results establish that glucosidase II and hexose-6-phosphate dehydrogenase, both integral proteins of the ER membrane, contain high Man-type Asn-linked oligosaccharide chains. The findings strongly suggest, though do not prove, that these polypeptides also lack complex-type asparagine-linked oligosaccharides and any sialic acid (in the case of glucosidase II).

Bulk Glycoproteins of the ER Membrane

To help establish whether or not the bulk of the ER membrane glycoproteins also lack Endo H–resistant oligosaccharide chains, and to examine the structure of these chains in greater detail, we have prepared a highly purified rough microsome fraction (RM_2) from rat liver, using established procedures (24). The purity was confirmed by electron microscopy (not shown). The integral membrane glycoproteins were separated from the soluble, content glycoproteins of the RM_2 fraction using a published procedure (25). Briefly, the microsomes were permeabilized with 0.05% deoxycholate to release the content glycoproteins while retaining most of the integral membrane glycoproteins in a sedimentable form. The pattern of polypeptides present in stained SDS gels of this membrane fraction (data not shown) agreed closely with that reported (25).

Fig. 6 shows that the glycoproteins of the ER membrane can bind Con A, but lose their ability to do so after a digestion with Endo H. For this purpose, the membrane fraction (i.e., the 0.05% deoxycholate pellet) was further extracted with 2% deoxycholate, and the supernatant was chromatographed on Con A–Sepharose to separate a Con A–binding fraction from a nonbinding fraction (as a control). Samples of these fractions were then incubated with or without Endo H and then electrophoresed on an SDS gel. The Con A–binding polypeptides were localized in this gel after an in situ staining procedure (25). As expected ^{125}I-Con A was bound to polypeptide chains present in the Con A–binding fraction derived from the RM_2 membrane (lane *1*, 120 μg loaded). As a control for the specificity of the binding, ^{125}I-Con A did not bind to any of the polypeptides that had flowed through the Con A–Sepharose column (lane *6*, 100 μg loaded). Little if any Con A binding was lost during a mock digestion of the Con A–binding fraction without Endo H (lane *3*, 25 μg loaded). However, when the same amount of the Con A–binding fraction (25 μg) was digested with Endo H, virtually all of the binding of ^{125}I-Con A was lost (lanes *4* and *5*). This implied that most if not all of the Con A–binding sites in the major ER membrane glycoproteins consist of Endo H–sensitive high-mannose oligosaccharide chains. This also argued against the presence of complex (Golgi-derived) chains in the bulk of ER membrane proteins, consistent with the conclusions of Rodriguez-Boulan et al. (24).

The apparent absence of Endo H–resistant chains attached to principal ER membrane proteins would suggest that these proteins, as a group, do not travel as far as the *medial* Golgi cisternae, in which GlcNAc residues are added (11) (confer-

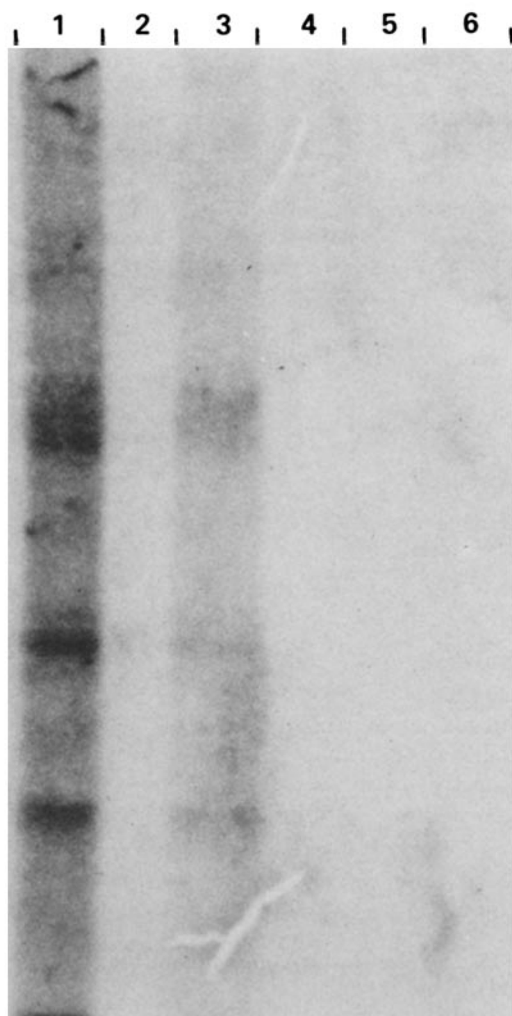


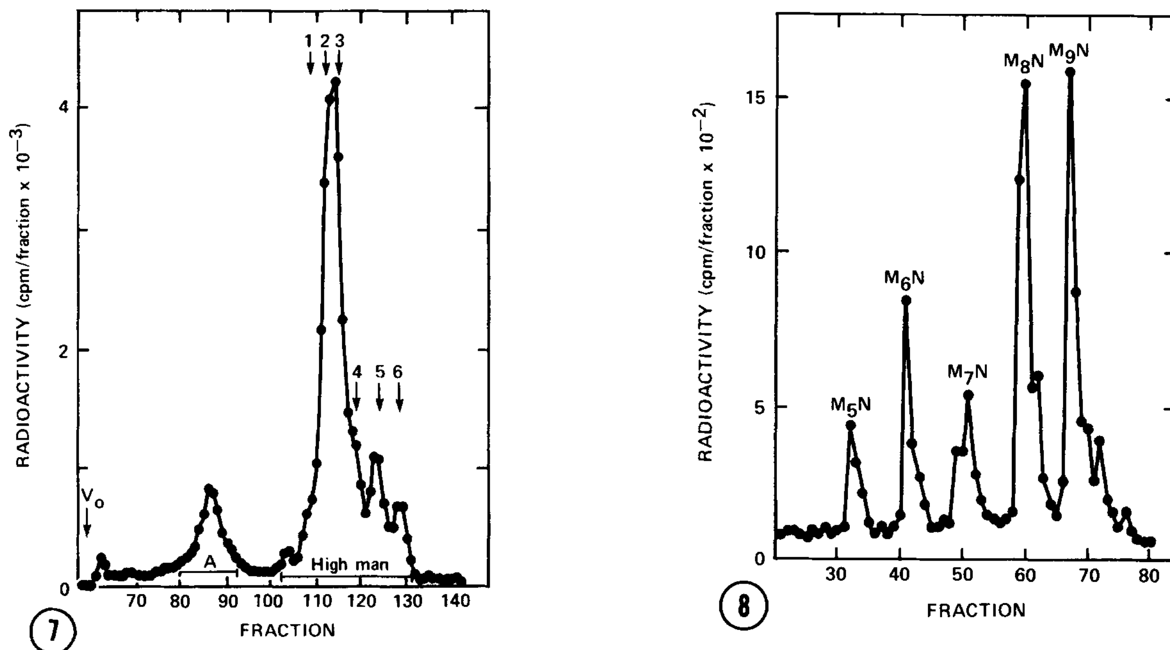
FIGURE 6 Binding of ^{125}I-Con A to proteins from the ER membrane before and after digestion with Endo H. The glycoprotein fraction and the nonglycosylated protein fractions of the membranes RM_2 were prepared by chromatography on Con A-Sepharose, exactly as described (25). Samples of these fractions, in some cases digested with Endo H as described (16), were electrophoresed on an SDS polyacrylamide gel. The gel was incubated with ^{125}I-Con A, washed exhaustively, dried, and autoradiographed as described (25). Shown is the autoradiograph. Lane *1*, 120 μg of the glycoprotein fraction from RM_2 membranes. No Endo H treatment. Lane *2*, empty. Lane *3*, 25 μg of RM_2 membrane glycoprotein fraction, mock-digested without Endo H for 16 h at 37°C. Lane *4*, same as lane *3*, except 0.01 U/ml of Endo H was added. Lane *5*, same as lane *3*, except 0.02 U/ml Endo H was added. Lane *6*, 100 μg of the nonglycosylated fraction (the flow-thru fraction of the Con A-Sepharose column) from the RM_2 membrane fraction, without an Endo H treatment. Parallel experiments (not shown) in which gels of Endo H-treated and untreated protein fractions were stained ruled out the possibility that the loss of Con A binding by the RM_2 membrane glycoprotein fraction was due to proteolysis accompanying the Endo H digestion.

ring resistance to Endo H). Still, they might have had their high-mannose chains trimmed in the Golgi. Therefore, we have determined the size distribution of the oligosaccharide chains released by Endo H from rough ER membrane glycoproteins. From this size distribution, the extent to which Man residues have been removed by the earlier-acting Golgi mannosidase I can be assessed (8, 32). The Endo H-sensitive oligosaccharide chains were released and then labeled at their reducing ends with [^{3}H]$NaBH_4$ (27). Fig. 7 shows the profile of oligosaccharides analyzed by chromatography on Biogel P-4. 80% of the radioactivity appeared in the fraction in which reduced high mannose oligosaccharides elute, with the major peak eluting between the $Man_9GlcNAc_{OT}$ and Man_8-$GlcNAc_{OT}$ markers (the subscript OT denotes that the oligosaccharide is labeled with tritium at the reducing end). In addition, 13% of the radioactivity eluted in a peak midway between the void volume and the reduced high mannose oligosaccharides (peak *A*). When control samples were treated identically, except for the omission of the digestion with Endo H, radioactivity was only found in the void volume fractions. The reduced, high-mannose oligosaccharides and peak A were pooled separately, lyophilized, and analyzed further by high performance liquid chromatography.

High performance liquid chromatography offers a better resolution of individual species in the high mannose fraction (Fig. 8). Material chromatographing as $Man_8GlcNAc_{OT}$ and $Man_9GlcNAc_{OT}$ were the major peaks, each comprising ~25% of the total. In addition, more minor peaks corresponding to $Man_{5-7}GlcNAc_{OT}$ and $GlcMan_9GlcNAc_{OT}$ were seen, each making up ~10% of the total. To confirm the identification of the peaks corresponding to $Man_{5-9}GlcNAc_{OT}$, each peak was treated exhaustively with jack bean alpha-mannosidase and then analyzed on a Biogel P-4 column. In each case, the sole product migrated with $ManGlcNAc_{OT}$ (not shown), indicating that the identification of the oligosaccharides had been made correctly, since each contained exclusively alpha-linked Man residues attached to a Man-β-$GlcNAc_{OT}$. It is interesting to note that several of the peaks seen on high performance liquid chromatography particularly Man_7-$GlcNAc_{OT}$ and $Man_8GlcNAc_{OT}$, appear to be heterogeneous, suggesting that several isomers of these high-mannose oligosaccharides are found on rough ER glycoproteins.

The size distributions of the Endo H-sensitive chains are essentially those expected from the actions of the ER-associated mannosidase (32). A repeated exposure to Golgi mannosidase I would have been expected to result in the eventual production of $Man_5GlcNAc_{OT}$ as the major species.

To determine if the oligosaccharides in peak A were negatively charged, they were characterized by chromatography on QAE-Sephadex (30). Material eluting at 5 mM NaCl accounted for 40% of the total radioactivity of peak A while the balance eluted at 50 mM NaCl (not shown). To test for the presence of sialic acid or phosphodiester residues on these oligosaccharides, each of the two QAE fractions from peak A was treated with mild acid (30). Sialic acid residues should be removed by this treatment, resulting in reduced charge and elution at a lower NaCl concentration. Phosphodiesters should be cleaved yielding phosphomonoesters with increased charge, resulting in elution at high NaCl concentrations. Phosphomonoesters should be unaffected. The elution position of neither QAE pool was affected by the mild acid treatment, suggesting that the oligosaccharides in peak A do not contain sialic acid, -P-GlcNAc, or -P-glucose diesters. Because of the small amount of material available, peak A material could not be further characterized. Thus, the significance of peak A is presently unclear. It is negatively charged and derived from high-Man Asn-linked oligosaccharide chains. The negative charge is not due to sialic acid, or a phosphodiester, but could conceivably be either a phosphomonoester or a sulfate.



FIGURES 7 and 8 Analysis of Endo H-sensitive oligosaccharides of RM_2. The microsomes were extracted with 0.05% deoxycholate to release soluble content (25) and pelleted. The Endo H-sensitive oligosaccharides of RM_2 membranes were labeled by reduction with $[^3H]NaBH_4$ and analyzed by column chromatography (Fig. 7) as described under Materials and Methods. The void volume (V_o) and the elution positions of authentic reduced high mannose oligosaccharide are shown: *1*, $GlcMan_9GlcNAc$; *2*, $Man_9GlcNAc$; *3*, $Man_8GlcNAc$; *4*, $Man_7GlcNAc$; *5*, $Man_6GlcNAc$; *6*, $Man_5GlcNAc$. The regions marked with horizontal bars were pooled for further analysis by high performance liquid chromatography in Fig. 8 (29). Fractions of 1 ml were collected.

DISCUSSION

The evidence from subcellular fractionation (1–5) that ER membrane proteins are also found at high concentrations in Golgi membranes led to the proposal (6) that the ER marker proteins putatively present in the Golgi escape from the ER as a result of a low level of errors made during the process of a selective but imperfect export from ER. The fact that these ER marker proteins are not present in the plasma membrane, a major target for export from the Golgi apparatus, gave rise to the idea (6) that the escaped ER membrane proteins are eventually removed from the Golgi and returned to the ER. The stack would then act as a multistage filter, improving the overall fidelity of the process of protein transport from the ER to other organelles.

This distillation hypothesis (6) makes several predictions. First, the major components of the ER membrane should be readily demonstrable in the Golgi stack by immunocytochemical methods. In particular, the concentration of ER marker proteins should be greatest at the *cis* (entry face) and least at the *trans* (exit face). Second, a typical ER membrane protein would escape into the Golgi and then be retrieved many times during its lifespan of a few days. Thus, oligosaccharide chains attached to ER membrane proteins should, in many cases, bear the characteristic imprints of the actions of Golgi-localized mannosidases and glycosyltransferases (7, 8). These include the removal of Man residues (most likely in the *cis* or *medial* Golgi [9, 10]), the addition of GlcNAc (in *medial* Golgi [11]), and of Gal and sialic acid (in *trans* Golgi [12]). Even ER glycoproteins that are relatively poor substrates for these enzymes would be expected to eventually be processed in the Golgi, due to a repeated exposure.

A third prediction is that proteins in the ER membrane should be in a dynamic equilibrium with the same molecules in the Golgi. Unfortunately, pulse-chase experiments in which the ER marker proteins would be labeled biosynthetically in the ER are likely to be insensitive indicators of potential ER–Golgi traffic. Since the total amount of ER membrane in a cell is typically in great excess of the total amount of Golgi membrane, most of the labeled ER protein will be in the ER membranes at any given time with very little in the Golgi membranes, whether or not there is a traffic of ER markers between these two organelles.

We have been unable to confirm the first two basic predictions of the distillation hypothesis for the ER proteins that we have studied. Using sensitive electron microscopic immunocytochemical methods, we have been unable to demonstrate the presence of significant quantities of the 3-MC–induced form of cytochrome P450 in the Golgi stack, even though this component is abundant in the ER membrane. C. DeLemos and D. Sabatini have reached a similar conclusion for phenobarbital-induced cytochrome P450 (personal communication). Also, Lucocq et al. (34) have reported similar findings in the case of glucosidase II. Our findings with cytochrome P450 are consistent with ferritin–antibody studies of isolated Golgi fractions (33) but inconsistent with conclusions that can be drawn from subcellular fractionation experiments (1–5). The latter studies have suggested that ER membrane proteins are also present as bona fide components in Golgi membranes, at concentrations perhaps as much as one half of that in the ER membrane. It seems likely that artifacts of contamination by ER membranes, although apparently ruled out in these studies, nonetheless account for the presence of most of the cytochrome P450 and other ER markers in the Golgi-rich fractions. A combined electron microscope and subcellular fractionation study by Ito and Palade (5) showed that membranes containing the bulk of the cytochrome P450

reductase (an ER membrane-bound enzyme) in a Golgi fraction were absorbed in an immunospecific fashion to beads coated with anti-reductase antibody. Among the absorbed membranes were Golgi cisternae and very low density lipoprotein-containing, Golgi-associated vesicles. Evidently, these Golgi membranes contain some of the P450 reductase (and, presumably, other ER membrane proteins). However, it is important to note that the presence of only a few molecules of cytochrome P450 reductase in each cisterna would be sufficient to account for their absorption to the beads.

Our studies of the oligosaccharide chains attached to two individual ER membrane proteins, as well as those attached to the bulk population, also support the view that ER membrane proteins are efficiently retained within this organelle. We have been unable to detect evidence of passage into *medial* or *trans* cisternae of the Golgi stack, as would be indicated by the presence of GlcNAc and Gal or sialic acid, respectively, on ER glycoproteins. This is in keeping with the study of Rodriguez-Boulan et al. (24), who could not detect binding sites for either wheat germ, ricin, or soybean agglutinins in rough microsomes. They concluded that microsomal membrane glycoproteins have incomplete carbohydrate chains that lack the characteristic terminal trisaccharides (GlcNAc-Gal-sialic acid) present in many glycoproteins that are transported through the Golgi body.

We have confirmed and also extended their work by examining the number of Man units present in the Asn-linked oligosaccharide chains of ER membrane glycoproteins. This analysis makes it seem unlikely that the bulk of the ER membrane protein even enters the Golgi cisternae. We found that the predominant oligosaccharide chains are those with either eight or nine Man units, with smaller amounts of Man_5, Man_6, and Man_7. This pattern would be predicted from the spectrum of products of the ER-localized mannosidase described by Bischoff and Kornfeld (32). A similar product distribution is found when proteins that are normally transported rapidly out of the ER are artificially retained there (35–38). Had the Golgi-localized mannosidase I acted upon these ER glycoproteins, the number of Man units could have been reduced to as few as five. Certainly, some of the ER membrane glycoproteins could pass into the Golgi and retain their Man units because their oligosaccharide chain happens to be relatively inaccessible to the action of Golgi mannosidase I. However, it seems unlikely that this would be the case for the bulk of ER membrane polypeptides, especially with many repeated exposures as the distillation hypothesis must propose (6). An analysis of the Endo H-sensitive oligosaccharides of ribophorin (39) has shown that they, too, consist mostly of Man_9 and Man_8 chains. These observations may not be pertinent to the issue of ER-Golgi traffic, however, since ribophorins may not be diffusible in the plane of the ER; rather, they may be fixed in rough ER regions. Hydroxymethylglutaryl-CoA reductase, a transmembrane glycoprotein of the ER, has an oligosaccharide chain of which Man_6 is the principal species (40).

In conclusion, the bulk of proteins of the ER membrane appear to be very efficiently retained in the face of a massive and continuous export of newly made secretory, cell surface, and lysosomal proteins, even though there is no evidence (apart from that of cell fractionation) in keeping with the basic predictions of the distillation hypothesis (6) for the ER membrane proteins that have been studied to date. Because these findings are intrinsically negative, it is formally possible though unlikely that this hypothesis is correct although difficult to confirm, or that it holds for a subset of ER proteins that have not yet been studied. A similar conclusion has been reached by Green and co-workers (41). The notion that is most fundamental to the distillation hypothesis, that the Golgi stack exists to carry out sorting operations in a multistage process, could nonetheless still be correct. Indeed, it is increasingly clear that the Golgi stack consists of sequential compartments as originally proposed (6) and thus can carry out multistage operations. However, it is most unlikely that the bulk of ER membrane proteins are the substrates of such a sorting cascade.

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