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) 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 (3-MC-P450) was achieved by intraperitoneal injection of 10 mg 3-MC (Eastman Kodak Co., Rochester, NY) per 340 g body weight. 3-MC was dissolved at 50°C in corn oil at 0.1 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, subtype and react with different forms of P450. Affinity-purified rabbit antiserum to 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 ultrathin (50-100 nm) cryosectioning (for light microscopy and electron microscopy, respectively) of liver and liver Golgi-enriched fractions, as well as the subsequent immunocytochemical 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 (Polytechnics, Inc., Warrington, PA) after osmium postfixation and subsequent dehydratation, 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-
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 NaN3, 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 0.05% of 60 mM NaCl (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 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 pronase. Then 0.6 M cI of carrier-free NaB\(^{35}\)H\(_4\) (100 µCi/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.

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 microscopy 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. 1B) and uninduced (Fig. 1C) livers. Note that the section in Fig. 1C (control) was photographed with 18 times the exposure used for Fig. 1B (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. 2A) 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. 3A). 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. 2D) but did stain sections of livers from phenobarbital-induced rats (not shown).

Despite some biochemical evidence suggesting a mitochondrial localization, this is a major component of the ER membrane, and we have been unable to detect significant quantities of this protein in Golgi stacks.

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. 2C 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.

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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 Kupffer 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. X 22,000. (B) Bar, 0.25 μm. X 32,000.

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 125I-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. 5a 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-
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 Con A-binding sites in the major ER membrane glycoproteins consist of Endo H-sensitive high-mannose oligosaccharide chains. This also argues 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-
with Endo H as described (16), were electrophoresed on an SDS polyacrylamide gel. The gel was incubated with 125I-Con A, washed exhaustively, dried, and autoradiographed as described (25). Shown is the autoradiograph. Lane 1, 120 μg of the glycoprotein fraction from RM2 membranes. No Endo H treatment. Lane 2, empty. Lane 3, 25 μg of RM2 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 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 RM2 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 RM2 membrane glycoprotein fraction was due to proteolysis accompanying the Endo H digestion.

FIGURE 6 Binding of 125I-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 RM2 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 125I-Con A, washed exhaustively, dried, and autoradiographed as described (25). Shown is the autoradiograph. Lane 1, 120 μg of the glycoprotein fraction from RM2 membranes. No Endo H treatment. Lane 2, empty. Lane 3, 25 μg of RM2 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 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 RM2 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 RM2 membrane glycoprotein fraction was due to proteolysis accompanying the Endo H digestion.

High performance liquid chromatography offers a better resolution of individual species in the high mannose fraction (Fig. 8). Material chromatographing as ManαGlcNAcOT and ManαGlcNAcOT were the major peaks, each comprising ∼25% of the total. In addition, more minor peaks corresponding to Manα,6-GlcNAcOT and GlcManαGlcNAcOT were seen, each making up ∼10% of the total. To confirm the identification of the peaks corresponding to Manα,6-GlcNAcOT, 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 ManαGlcNAcOT (not shown), indicating that the identification of the oligosaccharides had been made correctly, since each contained exclusively alpha-linked Man residues attached to a Manα,6GlcNAcOT. It is interesting to note that several of the peaks seen on high performance liquid chromatography particularly Manα-GlcNAcOT and ManαGlcNAcOT, 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 ManαGlcNAcOT 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 were 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. Phosphodiesterases 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.
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

![Figures 7 and 8](image-url)
repeated exposures as the distillation hypothesis must propose.

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-NeuAc) 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$_6$, Man$_7$, and Man$_8$. 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$_9$ 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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