Biosynthesis and Processing of Ribophorins in the Endoplasmic Reticulum

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ABSTRACT Ribophorins are two transmembrane glycoproteins characteristic of the rough endoplasmic reticulum, which are thought to be involved in the binding of ribosomes. Their biosynthesis was studied in vivo using lines of cultured rat hepatocytes (clone 9) and pituitary cells (GH 3.1) and in cell-free synthesis experiments. In vitro translation of mRNA extracted from free and bound polysomes of clone 9 cells demonstrated that ribophorins are made exclusively on bound polysomes. The primary translation products of ribophorin messengers obtained from cultured hepatocytes or from regenerating livers co-migrated with the respective mature proteins, but had slightly higher apparent molecular weights (2,000) than the unglycosylated forms immunoprecipitated from cells treated with tunicamycin. This indicates that ribophorins, in contrast to all other endoplasmic reticulum membrane proteins previously studied, contain transient amino-terminal insertion signals which are removed co-translationally. Kinetic and pulse-chase experiments with [^{35}S]methionine and [^{3}H]mannose demonstrated that ribophorins are not subjected to electrophoretically detectable posttranslational modifications, such as proteolytic cleavage or trimming and terminal glycosylation of oligosaccharide side chain(s). Direct analysis of the oligosaccharides of ribophorin I showed that they do not contain the terminal sugars characteristic of complex oligosaccharides and that they range in composition from $Man_8GlcNAc$ to $Man_5GlcNAc$. These findings, as well as the observation that the mature proteins are sensitive to endoglycosidase H and insensitive to endoglycosidase D, are consistent with the notion that the biosynthetic pathway of the ribophorins does not require a stage of passage through the Golgi apparatus.

It is now well established that many proteins destined for different subcellular compartments share a common site of synthesis in ribosomes bound to membranes of the endoplasmic reticulum (ER)[1] and are initially incorporated into this organelle (49). However, nothing is known about the discriminating mechanisms that effect the sorting of these polypeptides and determine their different fates, or of the features of the polypeptides that serve as sorting signals in this process.

It is clear that the pathways followed by secretory, lysosomal, and membrane proteins of different plasma membrane domains do not diverge before the polypeptides enter the Golgi apparatus, since proteins of these different classes may be processed by the same Golgi enzymes (12, 33), and some have been found in the same Golgi cisternae (44, 53). On the other hand, it has not yet been determined if the biosynthesis of membrane polypeptides characteristic of the ER requires their transfer to the Golgi apparatus and if this organelle plays a role in ensuring the segregation of certain proteins within the ER. Significant amounts of several ER proteins have been detected in fractions of Golgi membranes (5, 17–19, 20, 22, 23), and it has been proposed (47) that an important function of the *cis* portion of the Golgi apparatus is the refinement of a crude export from the ER, from which membrane proteins to be segregated in the ER must be continuously removed and returned to this organelle. If this is the case, it may be expected that some ER membrane polypeptides are subjected

[1] *Abbreviations used in this paper: cl-9, clone 9; ER, endoplasmic reticulum.*

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to posttranslational modifications carried out by Golgi enzymes, such as those that affect oligosaccharide chains in glycoproteins. Of the many ER proteins characterized to date, however, only the ribophorins, which are restricted to the rough ER and appear to play a role in ribosome binding (26–28), and HMG-CoA reductase, an enzyme of cholesterol biosynthesis (31), have been found to bear covalently bound oligosaccharides, and therefore may serve as natural probes to study this question.

The biosynthesis of ribophorins is also of interest because of the role that these proteins appear to play in the binding of ribosomes to the ER membranes and hence the co-translational incorporation of all proteins into the ER (49). Although ribophorins have been best characterized in rat liver microsomes, from where they can be recovered in association with the ribosomes after other membrane polypeptides are dissolved by non-ionic detergents (28), they appear to be universal components of rough ER membranes in all cell types, and basic structural features of both ribophorins seem to have been conserved during evolution (34). If ribophorins are indeed necessary for the incorporation of proteins into the ER, their synthesis may be part of the regulatory mechanism that controls the development of the rough ER and the extent to which synthesis of ER membrane proteins can take place.

In this paper we demonstrate that the ribophorins themselves are products of bound polysomes which contain transient amino-terminal insertion signals and acquire asparagine-linked oligosaccharides during their co-translational insertion into ER membranes. Because all posttranslational changes that affect the ribophorins can be carried out by enzymatic activities found in the ER, the biosynthesis of these polypeptides does not require their transfer to the Golgi apparatus.

MATERIALS AND METHODS

Guanidine thiocyanate was from Fluka (Hauppauge, NY), [^{35}S]methionine (specific activity 1,000 Ci/mmole), 2[^{3}H]mannose (25 Ci/mmole), and [^{125}I]Na (100 Ci/mmole) from New England Nuclear (Boston, MA), Trasylol from Mobay Chemical Corp. (New York, NY), oligo-dT cellulose from Collaborative Research, Inc. (Lexington, MA), and tunicamycin from Calbiochem-Behring Corp. (La Jolla, CA). RPMI 1640, spinner minimal essential medium, horse serum, and fetal calf serum were from Grand Island Biological Co. (Grand Island, NY). Wheat germ was a gift of The General Mills Corp. (Minneapolis, MN). Most of the reagents were purchased from Sigma Chemical Co. (St. Louis, MO), or from Fisher Scientific Co. (Pittsburgh, PA). Sprague-Dawley female rats (150–200 g) were obtained from Taconic Farms, (Germantown, NY). When necessary, partial hepatectomies were performed as described by Higgins and Anderson (16).

Preparation of Antiserum: Antibodies against rat liver ribophorins I and II were produced and characterized as previously described (34).

Cell Culture: A rat hepatocyte line (clone-9; Cl-9) was kindly provided by Dr. M. Kaighn of The Pasadena Foundation for Medical Research (Pasadena, CA). Cells were cultured at 37°C in an air/5% CO_2 atmosphere with RPMI 1640 medium supplemented with 10% horse serum and antibiotics. A rat pituitary cell line (GH 3.1) was obtained from Dr. Carter Bancroft (Memorial Sloan Kettering Cancer Center, NY) and cultured as above in spinner minimal essential medium supplemented with 2.5% fetal calf serum/15% horse serum and antibiotics.

Labelling and Preparation of Cell Extracts: Hepatocyte monolayers were grown in 35-mm dishes to a density of approximately 5×10^6 cells per dish. Before labeling, the cultures were rinsed with RPMI free of methionine and incubated in this medium for 1 h to allow for the depletion of the intracellular methionine pool. Cells were pulse-labeled for 1 h in methionine-free medium supplemented with 10% dialyzed horse serum and [^{35}S]methionine (100 μCi/ml).

In experiments employing tunicamycin (2 μg/ml), confluent cultures were first incubated with the drug for 1 h in complete medium and then for 1 h with methionine-free medium before adding methionine (100 μCi/ml) and continuing incubation in the presence of tunicamycin.

For mannose labeling, hepatocytes (2×10^7 cells) were incubated for the indicated times in RPMI-10% horse serum supplemented with 2[^{3}H]D-mannose at a concentration of 1 mCi/ml (24.3 Ci/mmol).

After labeling, monolayers were washed twice with Moscona's PBS (36) and cells were scraped off the dish into an SDS solution (20 mM Tris-HCl, 7.4, 2% SDS) and sonicated 10–30 s in a Heat Systems-Ultrasonics Instrument with a microtip (Heat Systems-Ultrasonics, Inc. Plainview, NY). The suspension was boiled for 2 min and centrifuged (2 min at 15,000 *g*) in an Eppendorf 5412 to remove unsolubilized material. The supernatant was diluted fourfold with 50 mM Tris-HCl 7.4, 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 0.02% NaN_3, and 100 U/ml Trasylol (Solution A).

Immuneprecipitation: Cell extracts were incubated with IgG fractions (300 μg/ml) at room temperature for 1 h and at 4°C for 14 h before addition of protein-A-sepharose CL-4B beads. After incubation for 3 h at room temperature, the beads were sedimented, washed three times with Solution A containing 0.2% SDS, and boiled for 2 min in a solution of 10% SDS, 1 M dithiothreitol, 2 mM EDTA.

RNA Extraction: Clone 9 hepatocytes or GH 3.1 pituitary cells were homogenized in a solution containing 5 M guanidine thiocyanate plus 100 mM mercaptoethanol at pH 5.0 (58). RNA was separated by centrifugation through 0.2 vol of 5.7 M $CsCl_2$ as described by Liu et al. (32). Similarly, total RNA was prepared from control rats starved for 18 h or from rats sacrificed 15–24 h after partial hepatectomy (16).

Free and membrane-bound polysomes were prepared from cl-9 hepatocytes by a modification of the procedure of Ramsey and Steele (43). Polysome pellets were rinsed with sterile water and the RNA was extracted with 5 M guanidine thiocyanate as described above. Poly A^+ mRNA was purified by chromatography on oligo-dT cellulose (2). Preparations of messenger RNA from the pituitary cell line GH 3.1 were fractionated on a methyl mercury gel (40) to enrich for ribophorin encoding molecules.

Cell-free Protein Synthesis: Wheat germ extracts (35) containing 12.5 μCi of [^{35}S]methionine and 0.03 A_{260} U of mRNA in 0.025 ml were incubated at 25°C for 90 min. Dog pancreas microsomal membranes, prepared as described by Shields and Blobel (52), were added to a final concentration of 2 A_{260} U/ml. Samples from translation mixtures (0.2 ml) were boiled in 2% SDS, centrifuged (2 min at 15,000 *g*), and diluted fourfold with solution A. Immuneprecipitation was carried out as described above.

Treatment with Endo H- and Endo D-N-acetyl-glucosaminidases: Detergent extracts of rat liver rough microsomes were iodinated by the chloramine T procedure as previously described (34). After immuneprecipitation of the ribophorins, the beads were boiled in 2% SDS and centrifuged. Supernatants diluted with 1 M citrate-phosphate buffer (pH 5.0 or pH 6.4) to a final concentration of 50 mM were incubated with endoglycosidase H (8×10^{-4} U/ml) or with a crude preparation of Endoglycoside D (38) for 1 h at 37°C at pH 5.0 or 6.4, respectively. Samples were then analyzed by PAGE (10%) followed by autoradiography.

Sizing Oligosaccharides: Oligosaccharides labeled with 2[^{3}H]mannose were released from glycopeptides derived from ribophorin I as previously described for Sindbis virus glycoproteins (13). Essentially, the labeled proteins were exhaustively digested with pronase, the samples were heated to inactivate the enzyme, and the oligosaccharides were released with endo-β-*N*-acetyl-glucosaminidase-H. These were separated on a Biogel P4(400 mesh) column (175 × 1.5 cm) and the sizes were determined by co-elution with Sindbis virus marker oligosaccharides labeled with [^{14}C]mannose.

RESULTS

Levels of ribophorin synthesis in normal rat liver are low, and the presence of newly synthesized ribophorins in microsomal membranes obtained after short times of in vivo labeling could not be demonstrated by immuneprecipitation with specific antibodies. In fact, in vitro translation experiments indicated that ribophorin mRNAs account for <0.001% of the [^{35}S]methionine incorporation promoted by total mRNA extracted from resting liver. However, synthesis of ribophorins and the levels of the corresponding mRNAs increased significantly when hepatocyte proliferation was induced by partial hepatectomy, and 15 h after the operation ribophorin synthesis accounted for 0.05% of the total mRNA dependent in vitro incorporation (Fig. 1, compare lanes *a* and *b*).

The initial observations described above led us to examine growing cultures of rat hepatocytes (cl-9 cells) as possible rich

sources of ribophorin-mRNA. After short periods of in vivo labeling with [^{35}S]methionine, newly synthesized ribophorin molecules were easily detected in these cells by immunoprecipitation with specific antibodies. These appeared indistinguishable electrophoretically from those previously characterized in rat liver (Fig. 2, compare lanes *a* and *c* and lanes *b* and *d*) and therefore cl-9 cells appeared appropriate subjects for biosynthesis studies. Moreover, when added to the in vitro translation system, messenger RNA extracted from these cells directed the synthesis of measurable amounts of both ribophorins (see Fig. 1, lane *c* for ribophorin I).

Analysis of the products of in vitro translation of poly A$^+$ mRNAs extracted from free (Fig. 3, lanes *a* and *c*) and membrane-bound polysomes (lanes *b* and *d*) isolated from cl-9 cells showed that both ribophorin mRNAs are found only in polysomes bound to ER membranes and therefore indicated that in intact cells the polypeptides must be co-translationally inserted into the ER membranes. The in vitro synthesized ribophorin polypeptides had the same electrophoretic mobility as the corresponding proteins found in cl-9 cells (Fig. 4, compare lanes *a* and *c* for ribophorin I). Because both ribophorins are glycoproteins (45, 46), the size of the primary translation products were also compared with that of the respective unglycosylated polypeptides synthesized in cells treated with tunicamycin, an inhibitor of N-linked glycosylation (30) (Fig. 4, lane *b*). It was observed that each of the unglycosylated ribophorins synthesized in vivo was ~2,000 mol wt smaller (ribophorin I, M_r = 63,000; ribophorin II, M_r = 61,000, not shown) than the products of in vitro translation. These observations strongly suggest that both ribophorins contain amino-terminal signal sequences which are removed during co-translational insertion of the polypeptides into the ER membrane. Co-translational core glycosylation must also take place, however, so that no net change in the apparent molecular weight of the ribophorins is observed after their insertion into the ER membrane is completed. These presumptions were confirmed when the co-translational insertion of ribophorin I into the ER membrane was carried out in a cell-free translation system supplemented with dog pancreas microsomes (Fig. 5). The presence of membranes during

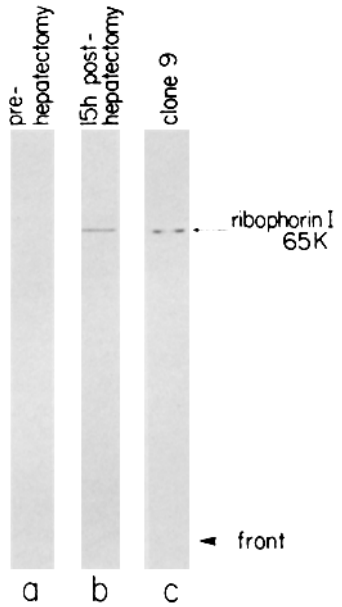

FIGURE 1 Increase in ribophorin I rat liver mRNA after partial hepatectomy. Messenger RNA samples isolated from control rat liver (lane *a*), from liver 15 h after partial hepatectomy (lane *b*), or from cl-9 cells (lane *c*) were used to program wheat germ cell-free translation systems (12 A_{260} of mRNA/ml). After 90 min of incubation at 25°C, aliquots (0.2 ml) containing ~10^7 cpm of incorporated [^{35}S]methionine were used for immunoprecipitation with anti-ribophorin I IgG (300 μg/ml). The immunoprecipitates were analyzed by SDS PAGE (10%) followed by fluorography (72 h).

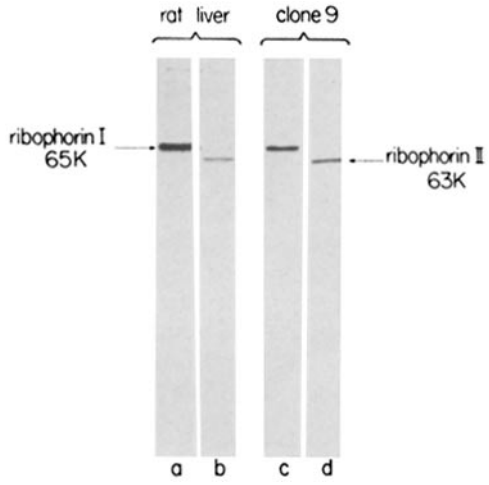

FIGURE 2 Electrophoretic analysis of ribophorins from rat liver and clone 9 hepatocytes. Ribophorins I (lanes *a* and *c*) and II (lane *b* and *d*) were precipitated with the appropriate antibodies from aliquots (containing 5 × 10^3 cpm) of the detergent-insoluble residue obtained from rat liver rough microsomes labeled with ^{125}I (lanes *a* and *b*) or from rat liver cl-9 cells (10^7 cpm) labeled for 24 h with [^{35}S]methionine (lanes *c* and *d*).

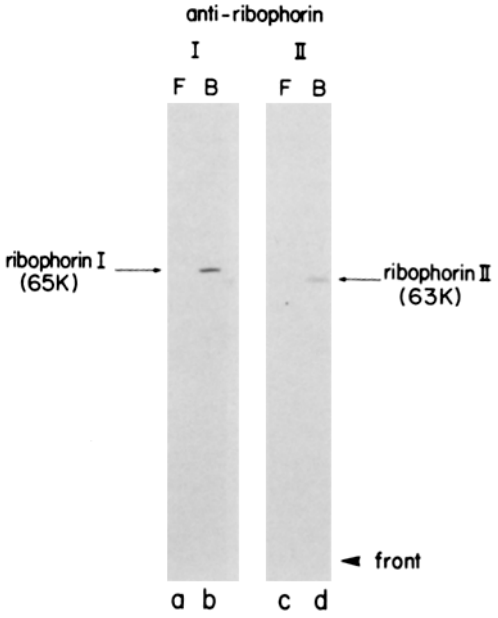

FIGURE 3 Site of synthesis of ribophorins. Messenger RNA isolated from free (lanes *a* and *c*) or membrane-bound polysomes (lanes *b* and *d*) prepared from rat hepatocyte cultures (cl-9) was used to program a wheat germ cell-free translation system (12 A_{260} of mRNA/ml). Samples (0.2 ml) containing ~10^7 cpm of [^{35}S]methionine-incorporated radioactivity were used for immunoprecipitation of in vitro synthesized polypeptides with antibodies against ribophorin I or ribophorin II (300 μg/ml IgG for each). Immunoprecipitates were analyzed by SDS PAGE (10%) followed by fluorography (72 h).

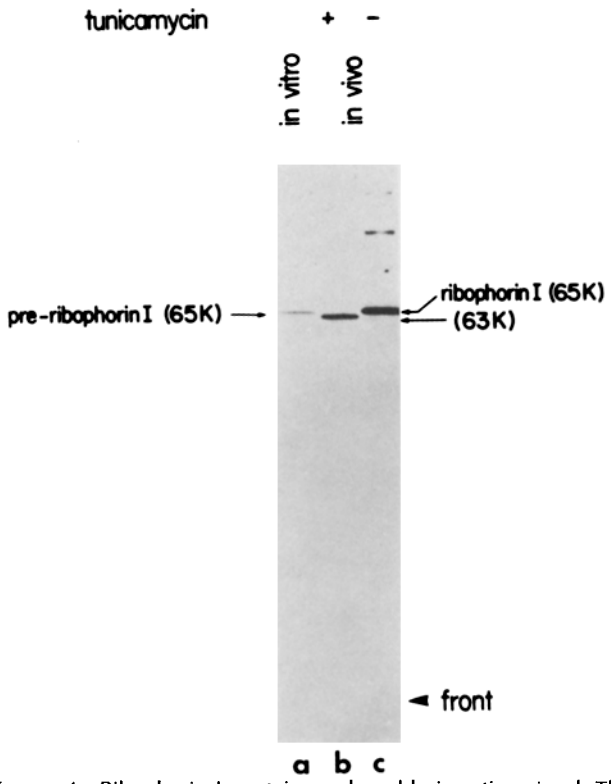

FIGURE 4 Ribophorin I contains a cleavable insertion signal. The relative mobility of the in vitro synthesized ribophorin I (lane *a*, M_r = 65,000) is compared with that of the corresponding polypeptides recovered from cl-9 cultures labeled for 15 min with [^{35}S]methionine either in the presence (lane *b*, M_r = 63,000) or absence of tunicamycin (2 μg/ml) (lane *c*, M_r = 65,000). Samples were immunoprecipitated and analyzed as described in Fig. 1.

translation (Fig. 5, lane *a*), but not their addition after translation was completed (Fig. 5, lane *c*), rendered the product (M_r = 65,000) sensitive to endoglycosidase H treatment, which resulted in a decrease in the apparent molecular weight to M_r = 63,000 (Fig. 5, lane *b*), the size of the polypeptide found in tunicamycin-treated cells (Fig. 4, lane *b*).

After their insertion in the ER, newly synthesized ribophorins did not appear to undergo major posttranslational modifications, since the electrophoretic mobility of the labeled proteins did not change when incubation of cl-9 cells with [^{35}S]methionine was extended for various periods, from 6 min to 15 h (for ribophorin I see Fig. 6). Thus, it appeared unlikely that the asparagine-linked oligosaccharides were modified to the complex forms characteristic of many glycoproteins (21, 25), or that O-linked sugars are added to the polypeptides (9, 24). Indeed, the effect of endoglycosidases on mature ribophorins demonstrated the presence of high mannose oligosaccharide chains in these proteins. ^{125}I-labeled ribophorins were resistant to endoglycosidase D (Fig. 7, lane *b* for ribophorin I) and sensitive to endoglycosidase H treatment (Fig. 8, lanes *b* and *d*). Moreover, treatment with endoglycosidase H released >95% of the [^{3}H]mannose radioactivity in ribophorins obtained from cells labeled for 48 h with [^{3}H]mannose (data not shown). It can therefore be concluded that all mannose-containing oligosaccharides in ribophorin I are of the high mannose type.

The extent to which the high mannose oligosaccharide side chains in ribophorin I undergo removal of peripheral mannose residues was assessed by a kinetic analysis of the distribution of radioactive mannose in ribophorin oligosaccharides obtained from cells labeled with 2[^{3}H]mannose for 1, 7, and 24 h. The oligosaccharides obtained from immunoprecipitated ribophorin I by pronase digestion and endoglycosidase

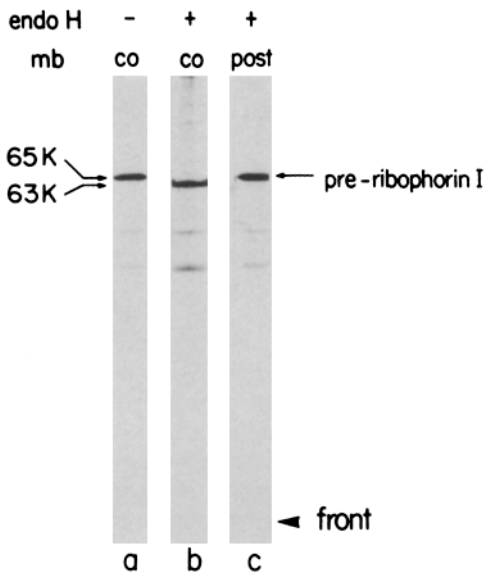

FIGURE 5 Co-translational insertion of ribophorin I into microsomal membranes. Messenger RNA isolated from rat pituitary cultures (GH 3.1) was translated in wheat germ cell-free systems (12 A_{260} of mRNA/ml) supplemented with dog pancreas microsomal membranes (*mb*, 2 A_{260}/ml) (lanes *a* and *b*) or to which membranes were added posttranslationally (lane c). Samples recovered by immunoprecipitation were analyzed directly or after treatment with endoglycosidase H (*endo H*) (8 × 10^{-4} U/ml) for 1 h at 37°C (lanes *b* and *c*).

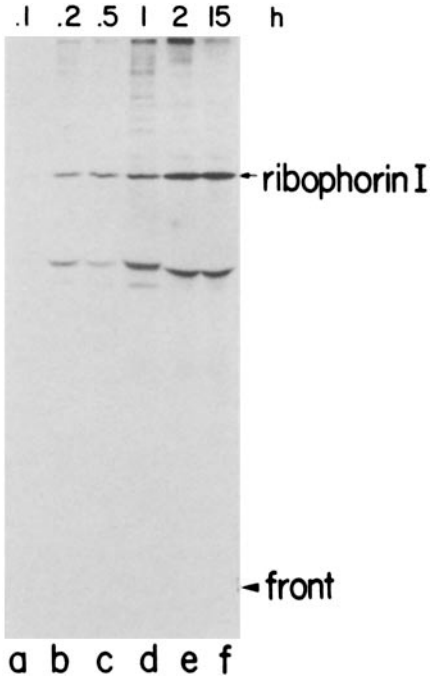

FIGURE 6 In vivo labeling of ribophorin I. Rat hepatocyte cultures (cl-9) were continuously labeled with [^{35}S]methionine (100 μCi/ml). At various times (lanes *a–f*) samples containing ~10^{7} cpm were processed for immunoprecipitation and analyzed electrophoretically.

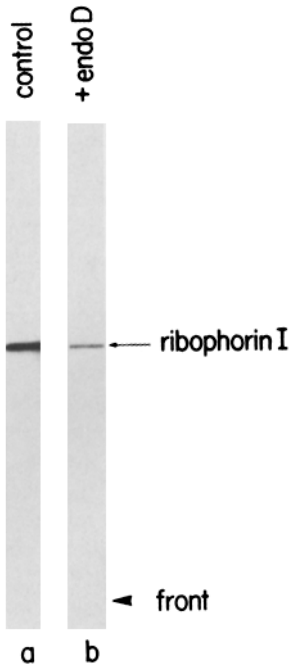

FIGURE 7 Insensitivity of ribophorin I to endoglycosidase D. Immunoprecipitates of ^{125}I-labeled ribophorins were incubated for 60 min at 37°C with (*b*) or without (*a*) a crude preparation of endoglycosidase D (*endo D*) before analysis by PAGE (6–11%) followed by autoradiography (24 h).

H treatment were analyzed by high resolution Biogel P-4 gel filtration chromatography (13). After 1 h of labeling, the major radioactive peaks corresponded to $Man_9GlcNAc$ and $Man_8GlcNAc$, and a small shoulder to $GlcMan_9GlcNAc$ (Fig. 9*C*). After 7 h of labeling (Fig. 9*B*), peaks corresponding to these species were well resolved and $Man_7GlcNAc$, $Man_6GlcNAc$, and $Man_5GlcNAc$ species could also be identified. A steady state seemed to be reached after 24 h (Fig. 9*A*), when the most prominent peaks corresponded to $Man_8GlcNAc$ and $Man_6GlcNAc$, but all species above $Man_5GlcNAc$ were represented.

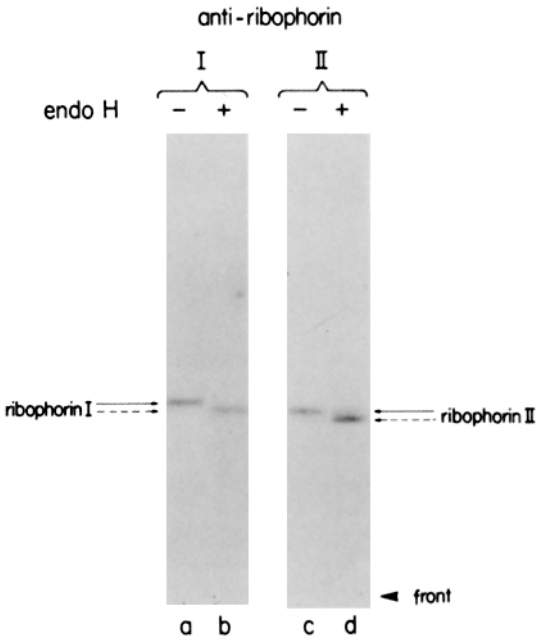

FIGURE 8 Sensitivity of ribophorins I and II to endoglycosidase H. Immunoprecipitates of ^{125}I-labeled ribophorins were incubated for 60 min at 37°C with (*b* and *d*) or without (*a* and *c*) endoglycosidase H (*endo H*, 8×10^{-4} U/ml) and samples were analyzed as described in Fig. 7.

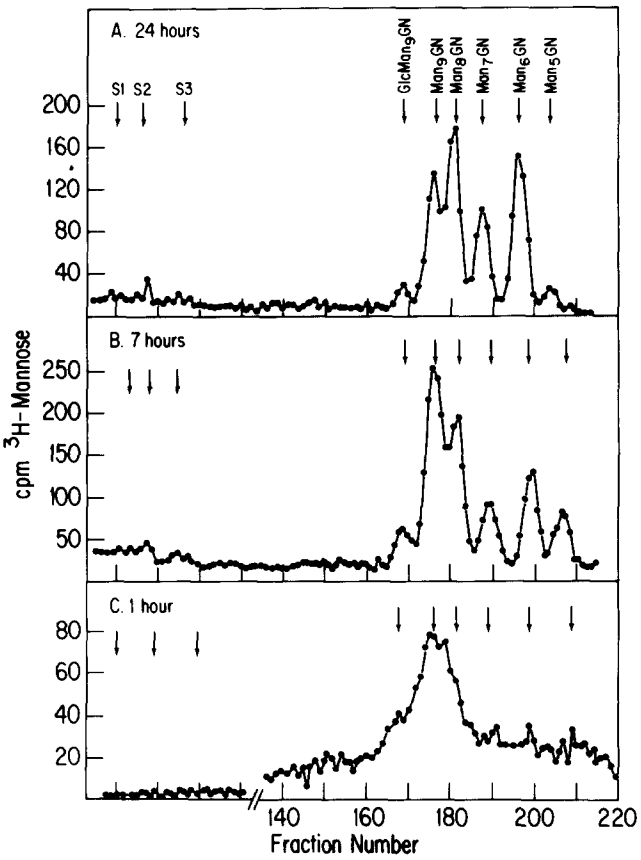

FIGURE 9 Biogel P4 chromatography of oligosaccharides released by endoglycosidase H from ribophorin I. Rat liver hepatocyte cultures (cl-9) were labeled with [^{3}H]mannose for 24 h (*A*) 7 h (*B*), and 1 h (*C*) and processed for immunoprecipitation with anti-ribophorin I antibodies as described in Materials and Methods. [^{14}C]mannose-labeled Sindbis virus glycopeptides were added as markers and the mixture was treated with endoglycosidase H in 0.1 M citrate-phosphate, pH 5.0, for 18 h at 37°C. The digest was boiled for 3 min and applied to a Biogel P4 (400 mesh) column equilibrated and eluted in 0.1 M NH_4HCO_3. The arrows from left to right indicate the peak elution fractions of Sindbis virus S1, S2, S3 glycopeptides, and S4 oligosaccharides.

DISCUSSION

The preceding observations demonstrate that ribophorins are synthesized in membrane-bound ribosomes and that during their co-translational insertion into the ER membrane the nascent polypeptides undergo cleavage of an amino-terminal insertion signal, as well as core glycosylation of asparagine residues. Aside from trimming of the oligosaccharide chains, no major posttranslational modifications such as addition of terminal sugars, O-glycosylation, or further proteolytic cleavage of the polypeptide seemed to affect the ribophorins. A kinetic analysis indicated that after rapid removal of glucose residues, further trimming of mannoses from the core oligosaccharide is a slow process: a steady-state distribution of labeled mannoses in the different stages is attained only after 24 h. Several lines of reasoning indicate that this trimming takes place in the ER itself. If the slow trimming of ribophorin oligosaccharides were carried out by the Golgi mannosidases which process other glycoproteins (55–57), ribophorins would be expected to reside for a rather long period of time in Golgi membranes, where their presence cannot be detected by immunoprecipitation (unpublished observations). The fact that

no ribophorin molecules containing oligosaccharides shorter than $Man_5GlcNAc$ could be detected in cl-9 cells, even after 72 h of labeling (not shown), also suggests that the proteins do not reach the *cis* portion of the Golgi apparatus where the enzyme transferase I, the action of which is required for the subsequent removal of mannoses from $Man_5GlcNAc$, is thought to be located (54).

The existence of mannosidases in the ER was first suggested by the observation that when transport to the Golgi apparatus is blocked, peripheral mannose residues are removed from secretory glycoproteins which accumulate in this organelle (10, 11, 14). The existence of ER mannosidases has recently been demonstrated directly (1, 4, 15), but for most glycoproteins only partial removal of mannose residues seems to take place in the ER. Complete removal of peripheral mannoses from ribophorins could however take place in the ER because these proteins, as permanent residents of the organelle, remain available as substrates for the local α-1,2-mannosidase for long periods of time. A similar argument has been presented (15) correlating the extent of removal of mannoses from IgA in the ER with the slow rate of secretion of this protein.

Clearly, the observations reported in this paper and the recent finding (6, 31) that another ER glycoprotein, HMG-CoA reductase, is also of the high mannose type do not offer support for the notion (47) that sorting of all membrane polypeptides synthesized in the ER occurs in the Golgi apparatus. Instead, it would appear that selective retention in the ER is a more likely sorting mechanism for polypeptides destined to be segregated in this organelle.

It is not at all clear what mechanisms could effect the permanent segregation of certain polypeptides in ER membranes, since no common features have been identified among the ER membrane proteins so far studied. It has been noted that several ER membrane proteins have substantial portions of their polypeptide chains exposed on the cytoplasmic aspect of the membrane, but this feature is not one shared by the ribophorins (28, 29) or by the E_1 glycoprotein of coronavirus, which is thought to trigger budding of the virion into the ER (48). Obviously, glycosylation is not required for retention in the ER since, in contrast to the ribophorins and the recently characterized HMG-CoA reductase (6, 7, 31), all other ER proteins investigated, such as cytochrome P-450 (3) and its reductase, cytochrome b_5 and its reductase, epoxide hydrolase (29, 41), and Ca^{++} ATPase (8, 37) are not glycoproteins. Moreover, retention of the signal for co-translational insertion, which is characteristic of all ER proteins studied except the ribophorins, is also known to take place for several plasma membrane proteins (39, 42, 50, 51). It is perhaps important to note that ribophorins are thought to form an intramembranous meshwork in the rough ER and that the tendency of these proteins to interact with each other may be sufficient to ensure that they are not transported from this organelle to the Golgi apparatus. Thus, oligomeric interaction of at least some other proteins with the ribophorins could also ensure their retention in the ER.

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