Biosynthesis and Processing of Ribophorins in the Endoplasmic Reticulum

MELVIN G. ROSENFELD,* EUGENE E. MARCANTONIO,* JOHN HAKIMI,* VICTORIA M. ORT,* P. H. ATKINSON,* DAVID SABATINI,* and GERT KREIBICH*

*Department of Cell Biology and The Kaplan Cancer Center, New York University, New York 10016; and
*Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, New York 10461

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 [35S]methionine and [3H]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 ManαGlcNAc to Man6GlcNAc. 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) 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
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 which 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

Guandine thiocyanate was from Fluka (Haupeauge, NY), [35S]methionine (specific activity 1,000 Ci/m mole), [3H]mannose (25 Ci/m mole), and [125]Na (100 Ci/m mole) 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 antibiotics.

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. Knecht of The Pasadena Foundation for Medical Research (Pasadena, CA). Cells were cultured at 37°C in an air/5% CO2 atmosphere with RPMI 1640 containing 10% dialyzed horse serum and antibiotics.

Cell-free Protein Synthesis: Wheat germ extracts (35) containing 125 µCi of [35S]methionine and 0.03 A260 U/ml RNA were incubated at 25°C for 90 min. Dog pancreas microsomal membranes, prepared as described by Shiels and Blobel (52), were added to a final concentration of 2 A260 U/ml. Samples from translation mixtures (0.2 ml) were boiled for 2 min in 2% SDS and centrifuged. The supernatants diluted with 1 M citrate-phosphate buffer (pH 5.0 or pH 6.4) to 10 A260 U/ml were added to a final concentration of 120 U/ml, 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 [3H]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-µ-N-acetylgalactosaminidase-H. These were separated on a Biogel PA400 mesh column (175 x 1.5 cm) and the sizes were determined by co-elution with Sindbis virus marker oligosaccharides labeled with [14C]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 [35S]methionine incorporation promoted by total mRNA extracted from resting liver. However, synthesis of ribophorins and the levels of the corresponding mRNA 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 [35S]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 4 Ribophorin I contains a cleavable insertion signal. The relative mobility of the in vitro synthesized ribophorin I (lane a, Mr = 65,000) is compared with that of the corresponding polypeptides recovered from cl-9 cultures labeled for 15 min with [35S]methionine either in the presence (lane b, Mr = 63,000) or absence of tunicamycin (2 μg/ml) (lane c, Mr = 65,000). Samples were immunoprecipitated and analyzed as described in Fig. 1.

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 A260 of mRNA/ml) supplemented with dog pancreas microsomal membranes (mb, 2 A260/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 [35S]methionine (100 μCi/ml). At various times (lanes a–f) samples containing ~10^7 cpm were processed for immunoprecipitation and analyzed electrophoretically.

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 [35S]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. 125I-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 [3H]mannose radioactivity in ribophorins obtained from cells labeled for 48 h with [3H]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[3H]mannose for 1, 7, and 24 h. The oligosaccharides obtained from immunoprecipitated ribophorin I by pronase digestion and endoglycosidase translation (Fig. 5, lane a), but not their addition after translation was completed (Fig. 5, lane c), rendered the product (Mr = 65,000) sensitive to endoglycosidase H treatment, which resulted in a decrease in the apparent molecular weight to Mr = 63,000 (Fig. 5, lane b), the size of the polypeptide found in tunicamycin-treated cells (Fig. 4, lane b).
FIGURE 7 Insensitivity of ribophorin I to endoglycosidase D. Immuneprecipitates of 125I-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 Man9GlcNAc and Man8GlcNAc, and a small shoulder to GlcMan9GlcNAc (Fig. 9 C). After 7 h of labeling (Fig. 9 B), peaks corresponding to these species were well resolved and Man7GlcNAc, Man6GlcNAc, and Man5GlcNAc 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 Man7GlcNAc and Man6GlcNAc, but all species above Man5GlcNAc were represented.

FIGURE 9 Biogel P4 chromatography of oligosaccharides released by endoglycosidase H from ribophorin I. Rat liver hepatocyte cultures (cl-9) were labeled with [3H]mannose for 24 h (A) 7 h (B), and 1 h (C) and processed for immuneprecipitation with anti-ribophorin I antibodies as described in Materials and Methods. [14C]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 NH4HCO3. 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 immuneprecipitation (unpublished observations). The fact that
no ribophorin molecules containing oligosaccharides shorter than Man$_5$GlcNAc 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$_5$GlcNAc, 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 proteins investigated, such as cytochrome P-450 (3) and its chromophore b$_5$ reductase, an integral membrane protein, in rat liver cells. J. Cell Biol. 73:901-915.

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