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Isolation and Characterization of cDNA Clones for Rat Ribophorin I: Complete Coding Sequence and In Vitro Synthesis and Insertion of the Encoded Product into Endoplasmic Reticulum Membranes

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Abstract. Ribophorins I and II are two transmembrane glycoproteins that are characteristic of the rough endoplasmic reticulum and are thought to be part of the apparatus that affects the co-translational translocation of polypeptides synthesized on membrane-bound polysomes. A ribophorin I cDNA clone containing a 0.6-kb insert was isolated from a rat liver lambda gtll cDNA library by immunoscreening with specific antibodies. This cDNA was used to isolate a clone (2.3 kb) from a rat brain lambda gtll cDNA library that contains the entire ribophorin I coding sequence. SP6 RNA transcripts of the insert in this clone directed the in vitro synthesis of a polypeptide of the expected size that was immunoprecipitated with anti-ribophorin I antibodies. When synthesized in the presence of microsomes, this polypeptide, like the translation product of the natural ribophorin I mRNA, underwent membrane insertion, signal cleavage, and co-translational glycosylation. The complete amino acid sequence of the polypeptide encoded in the cDNA insert was derived from the nucleotide sequence and found to contain a segment that corresponds to a partial amino terminal sequence of ribophorin I that was obtained by Edman degradation. This confirmed the identity of the cDNA clone and established that ribophorin I contains 583 amino acids and is synthesized with a cleavable amino terminal insertion signal of 22 residues. Analysis of the amino acid sequence of ribophorin I suggested that the polypeptide has a simple transmembrane disposition with a rather hydrophilic carboxy terminal segment of 150 amino acids exposed on the cytoplasmic face of the membrane, and a luminal domain of 414 amino acids containing three potential N-glycosylation sites. Hybridization measurements using the cloned cDNA as a probe showed that ribophorin I mRNA levels increase fourfold 15 h after partial hepatectomy, in confirmation of measurements made by in vitro translation of liver mRNA. Southern blot analysis of rat genomic DNA suggests that there is a single copy of the ribophorin I gene in the haploid rat genome.

THE rough endoplasmic reticulum (ER)[1] plays a major role in cellular protein synthesis; it contains sites for the binding of ribosomes synthesizing specific classes of proteins, and effects the co-translational translocation of certain nascent polypeptides into the lumen of the organelle and the insertion of others into the ER membrane itself (cf. Sabatini et al., 1982; Walter et al., 1984; Wickner and Lodish, 1985).

Although the details of the insertion and translocation processes are not at all understood, much progress has been made towards elucidating the mechanism of assembly of membrane-bound polysomes (see Walter et al., 1984; Hortsch and Meyer, 1984). It is now clear that a segment of the nascent polypeptide serves as a signal that initiates co-translational insertion (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975*a*). As the signal segment emerges from the large ribosomal subunit during the course of polypeptide chain elongation, it interacts with a macromolecular complex, the signal recognition particle (SRP) (Walter and Blobel, 1981; Walter et al., 1981), which in turn binds to a specific receptor in the ER membrane (Warren and Dobberstein, 1978; Walter and Blobel, 1981; Gilmore et al., 1982) that is also known as the docking protein (Meyer and Dobberstein, 1980; Meyer et al., 1982). During this process, binding of the ribosome to specific receptors in the ER membrane also takes place and conditions are established that in some way enable insertion of the nascent chain into the membrane and translocation to begin. The SRP and its receptor, however, appear to only function during the initial stages of the insertion process since they are present in less than stoichiometric amounts with respect to the number of ribo-

1. *Abbreviations used in this paper*: ER, endoplasmic reticulum; SRP, signal recognition particle.

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somes engaged in translocation (Walter and Blobel, 1980; Gilmore and Blobel, 1983; Gilmore et al., 1982).

Progress is also being made toward the identification and characterization of the ER membrane proteins that are involved in the translocation process. The SRP receptor, or docking protein, has been well characterized (see Walter et al., 1984; Hortsch and Meyer, 1984) and its amino acid sequence deduced (Lauffer et al., 1985) from the nucleotide sequence of a cDNA clone. The membrane-associated signal peptidase (Blobel and Dobberstein, 1975*b*; Jackson and Blobel, 1977) that effects the removal of the signal sequence located at the amino terminus of the nascent chain of many polypeptides synthesized in membrane-bound ribosomes, has been recently purified (Evans et al., 1986) and shown to consist of a complex of at least six relatively small polypeptides. Other membrane-bound enzymes, which act on specific residues of the nascent polypeptides to effect core glycosylation or other side chain modifications, must also be present near the ribosome–membrane junction but have not yet been characterized. Finally, the molecular architecture that integrates the co-translational processing elements, the components of the translocation apparatus, and the ribosome binding site remains to be elucidated.

Several ER membrane proteins have been shown to be specifically restricted to the rough domains of this organelle (Kreibich et al., 1978*a*, *b*; Sharma et al., 1978) and its seems likely that they also participate in some aspects of the membrane insertion and translocation processes. Ribophorins I and II (Kreibich et al., 1978*a*, *b*) are transmembrane glycoproteins that are characteristic of the rough ER of a wide variety of eukaryotic cell types (Marcantonio et al., 1982) and, unlike the SRP receptor, are present in a 1:1 ratio with respect to the number of membrane-bound ribosomes (Marcantonio et al., 1984). The strict segregation to the rough domains of the ER (Kreibich et al., 1978*a*, *b*), their copurification with membrane-bound ribosomes and with the SRP receptor when the membranes of rough microsomes are partially dissolved with neutral detergents (Kreibich et al., 1978*a*, *b*), and the finding that they can be cross-linked to membrane-bound ribosomes by bifunctional reagents (Kreibich et al., 1978*a*) strongly suggest that ribophorins are additional components of the translocation apparatus that may be associated directly or indirectly with the ribosome-binding sites. In addition, ribophorins appear to exist as part of a protein network that may be responsible for the morphological configuration of the rough ER cisternae as extended and flattened sacs, quite distinct from the tortuous tubular membrane system that makes up the smooth ER (Kreibich et al., 1978*a*, *b*). Recent experiments (Hortsch et al., 1986; Todd et al., 1984) indicate that ribophorins alone could not provide a binding site for the ribosomes and that at least one other protein component, highly sensitive to proteases, is required for ribosome binding.

An insight into the role of ribophorins in the organization and function of the rough ER may be gained from the elucidation of the structure and membrane topology of these proteins. In this paper we report the determination of the complete amino acid sequence of ribophorin I, derived from the nucleotide sequence of a cloned cDNA, and present the implications of this sequence for the disposition of this protein in the membrane. Regions of homology between the cytoplasmic domain of ribophorin I and several cytoskeletal proteins were detected. This suggests that an interaction of ribophorin I molecules between themselves or with other cytoskeletal components may contribute to the maintenance of the organization of the rough ER.

Materials and Methods

cDNA Library Construction and Isolation of Clones for Ribophorin I

For the construction of a rat brain cDNA library, total brain poly $(A)^+$ mRNA was prepared (Chirgwin et al., 1979) from 20-d-old rats and 10 μg was used as a template for cDNA synthesis. The first strand was synthesized with M-MLV reverse transcriptase using the protocol provided by the supplier (Bethesda Research Laboratories, Gaithersburg, MD) and second strand synthesis was performed by published procedures (Gubler and Hoffman, 1983). Double-stranded cDNA (2 μg) was treated (20 min, 37°C) with mung bean nuclease (5 U; Pharmacia, Inc., Piscataway, NJ) in 50 mM NaCl, 30 mM Na acetate pH 5.5, 1 mM $ZnCl_2$, and 3% glycerol (100 μl final vol) to produce blunt ends (Gubler, 1987). The double-stranded cDNA was methylated at internal EcoRI sites, and EcoRI linkers were attached in a standard overnight ligation reaction (Maniatis et al., 1982). Redundant linker sequences were excised by digestion with EcoRI, and the double-stranded cDNA was size fractionated on a Sepharose CL-4B (Pharmacia, Inc.) column (10 ml). Double-stranded cDNA larger than 1.5 kb (as assessed by electrophoresis of aliquots of the column fractions in 1.5% agarose gels containing NaOH-EDTA followed by autoradiography) was ligated to lambda gtll arms (Stratagene Cloning Systems, San Diego, CA) and the ligated DNA was packaged into bacteriophage particles using a commercial packaging extract from the same manufacturer to generate a library that contained $\sim 3 \times 10^6$ independent recombinants.

A lambda gtll rat liver cDNA library (Gonzalez et al., 1985), as well as the brain library, were screened using either a ribophorin I antibody or, at subsequent stages, a ^{32}P-labeled ribophorin I cDNA probe. Immunoscreening and plaque hybridization were performed by established procedures (Young and Davis, 1983; Maniatis et al., 1982). The preparation and the purification of goat anti-rat ribophorin I antibodies have been previously described (Marcantonio et al., 1982).

Demonstration of the Presence of Ribophorin I Epitopes Within the Fusion Proteins Encoded by cDNA Clones

A culture of the hfl *Escherichieae coli* strain Y 1089 was lysogenized (Schwarzbauer et al., 1983) with the lambda gtll phage containing the 1A insert and incubated at 42°C for 20 min to induce the prophage. Isopropyl β-D thiogalactoside (10 mM) was then added and the culture was incubated for 2 h at 37°C. The bacteria were recovered by centrifugation, resuspended in gel buffer, and sonicated. The lysate was fractionated by electrophoresis on a 7.5% polyacrylamide gel and the fractionated proteins were transferred to nitrocellulose filters (Millipore/Continental Water Systems, Bedford, MA) that were stained with Ponceau S stain (Sigma Chemical Co., St. Louis, MO). The fusion protein band, as well as a control band of similar intensity, were excised and the two strips of nitrocellulose were incubated overnight at 4°C with polyclonal ribophorin I antibody diluted 1:10. The strips were washed with PBS containing 0.1% Triton X-100 and 0.2% gelatin, and bound antibody was eluted by incubating the strips in 0.2 N glycine-HCl (pH 3.5) for 1 min. The eluates were neutralized and were used to probe Western blots of solubilized rough microsomal proteins. The bound goat antibodies were localized by the peroxidase reaction using anti-goat IgG conjugated to horseradish peroxidase (Towbin et al., 1979).

Hybridization Analysis

The cDNA probes were labeled by nick-translation to a specific activity of $\sim 1 \times 10^8$ cpm/μg using a kit from Bethesda Research Laboratories. For Southern blotting analysis (Southern, 1975) of genomic DNA, high molecular weight DNA was isolated from rat liver (Dillela et al., 1986), cleaved with restriction endonucleases (New England BioLabs, Beverly, MA, or Boehringer Mannheim Biochemicals, Indianapolis, IN), and, after electrophoresis in 0.8% agarose gels, transferred to nitrocellulose sheets (Schleicher & Schuell, Inc., Keene, NH). Hybridization was carried out in 5 × SSC, 5 × Denhardt's solution, and 100 μg/ml of salmon sperm DNA,

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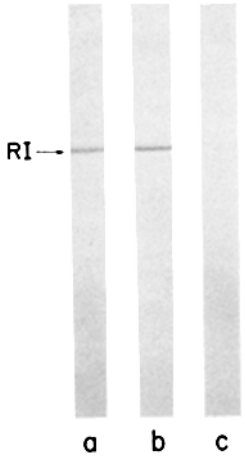

Figure 1. The β-galactosidase fusion protein encoded in clone 1A specifically adsorbs antiribophorin I antibodies. The electrophoretically purified fusion protein produced by *E. coli* Y 1089 lysogenized with clone 1A and a control band from the bacterial lysate were used to adsorb antibodies from an anti-ribophorin I IgG sample. The antibodies adsorbed to the fusion protein (lane *b*) and to the control protein band (lane *c*) were used to probe a Western blot of rat liver microsomal proteins (10 μg) fractionated in a 7.5% polyacrylamide gel. For comparison, an identical sample of rough microsome proteins was probed with the unfractionated anti-ribophorin IgG preparation (lane *a*).

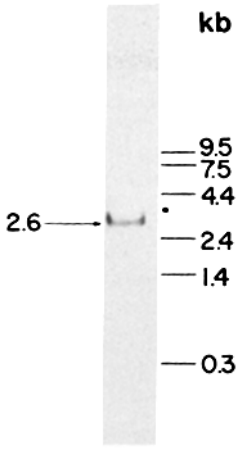

Figure 2. The ribophorin I cDNA insert in clone 1A hybridizes to a 2.6-kb mRNA species. Rat liver poly $(A)^+$ mRNA (5 μg) obtained 15 h after partial hepatectomy was fractionated on a formaldehyde-agarose gel, transferred to a GeneScreen filter, and hybridized to the ^{32}P-labeled insert from clone 1A. The chain lengths of a series of RNA standards (Bethesda Research Laboratories) are indicated.

and after hybridization the filters were washed at 65°C with 0.1 × SSC and 0.1% SDS (Maniatis et al., 1982). For Northern blotting analysis, mRNA was fractionated on 1.8% agarose gels containing formaldehyde and transferred to GeneScreen filters and hybridized to labeled probes as recommended by the filter manufacturer (New England Nuclear, Boston, MA).

For slot blot analysis of rat liver RNA, RNA samples were adjusted to 12 × SSC and 9% formaldehyde, incubated to 65°C for 15 min, and cooled on ice. Samples were applied to nitrocellulose filters as described (White and Bancroft, 1982) using a Schleicher & Schuell, Inc. apparatus. The hybridization procedure was the same used for Northern blotting.

DNA Sequencing

Both the chemical method of Maxam and Gilbert (1980) and the enzymatic dideoxy chain termination method of Sanger et al. (1977) were used. For the first method, the ~600-bp EcoRI fragment (clone 1A; Fig. 4) was 5′ end labeled with ^{32}P using gamma ^{32}P-ATP and polynucleotide kinase (Boehringer Mannheim Biochemicals), and digested with HindIII to generate two fragments of 350 and 270 bp. Alternatively, the cDNA was labeled at the internal HindIII site before cleavage of the recombinant plasmid fragments with the EcoRI. The fragments were separated by electrophoresis, purified on polyacrylamide gels, and subjected to chemical cleavage. For dideoxy sequencing, various fragments generated by restriction digestion of the plasmid pGEM-6-1 were subcloned (Messing et al., 1981) into the M13mp18 and M13mp19 vectors (Pharmacia, Inc.). The sequencing strategy used is shown in Fig. 6. When necessary, specific oligonucleotide primers corresponding to previously determined sequences were synthesized using a DNA synthesizer (model 380A; Applied Biosystems, Inc., Foster City, CA), and purified by chromatography on a Sep-pak column (Waters Associates, Millipore Corp., Milford, MA). Complete sequences of both strands of the cDNAs were determined.

Cell-Free Transcription-Translation and Immunoprecipitation

The cDNA insert was subcloned into the plasmid vector pGEM-1 (to yield pGEM-6-1) and transcribed using a riboprobe kit (Promega Biotec, Madison, WI), essentially as described by Melton et al. (1984) except that Hepes was used as a buffer instead of Tris-HCl in the transcription reaction mixture. The final reaction mixture of 10 μl contained 2 μl of 5 × transcription buffer (100 mM Hepes (pH 7.4), 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 1 μl of 100-mM DTT, 2 μl of 5-mM $m^7G(5')ppp(5')G$ (Pharmacia, Inc.), 1 μl (20 U) RNasin, (Amersham Corp., Arlington Heights, IL), 1 μl (10 U) SP6 polymerase (Promega Biotec, Madison, WI), and 2 μl (2 μg) of plasmid DNA. The mixture was preincubated for 5 min at 40°C and then 1 μl of a 10 mM ribonucleotide triphosphate mixture was added before continuing the incubation for an additional 60 min. Translation of the in vitro–transcribed mRNA was carried out in the wheat germ system as described for the natural mRNA (Rosenfeld et al., 1984) except that 1.0 mM $MgCl_2$ was used instead of 2.4 mM, and each 25-μl incubation mixture contained 1.5 μl of in vitro–transcribed mRNA as template. Dog pancreas microsomal membranes were prepared and used as described by Walter and Blobel (1983). For protease digestion, a mixture of chymotrypsin and trypsin was added to a final concentration of 50 μg/ml of each enzyme and the samples were incubated for 30 min at 0°C. The procedures for immunoprecipitation and endoglycosidase H digestion have been previously described (Rosenfeld et al., 1984).

Computer Analysis of the Amino Acid Sequence of Ribophorin I

The signal cleavage site was predicted using a method designed by von Heijne (1983) based on the frequency distribution of the different amino acids near the cleavage site. Secondary structure analysis was carried out by J. Jensen at Hoffmann-La Roche, Inc. (Nutley, NJ) using the Delphi computer program (written by Morten Kjeldgaad) based on the work of Garnier et al. (1978), Lifson and Sanders (1979), and Levitt (1978).

The protein libraries of the National Biomedical Research Foundation (Washington, DC) and of Hoffmann-La Roche, Inc. were searched for homologous protein sequences using the computer program FASTP (Lipman and Pearson, 1985). The significance of each homology was further tested using the RDF program (Lipman and Pearson, 1985).

Results and Discussion

Isolation of Ribophorin I cDNA Clones and Confirmation of Their Identity

A rat liver cDNA library constructed in the lambda gtll vector was screened using a polyclonal antibody against ribophorin I. One putative ribophorin I clone (clone 1A) was identified amongst 6 × 10^5 recombinant clones that were screened. After plaque purification, the DNA from this clone was analyzed by digestion with EcoRI and found to contain an insert of ~600 bp. The ribophorin identity of this clone was verified by a clonal epitope selection technique (Weinberger et al., 1985) in which the fusion protein encoded in the recombinant phage was shown to specifically adsorb, from a polyclonal anti-ribophorin I antiserum, antibodies that recognized only ribophorin I in Western blots of solubilized rough microsomes (Fig. 1). This provided strong evidence that the cDNA clone did not correspond to a contaminating protein that was present in the ribophorin I preparation used to generate the antiserum used in the library screening. Furthermore, antibodies raised in rabbits against the purified fusion protein bound specifically to ribophorin I in immunoblots of microsomes from rat liver, rat lacrimal gland, and dog pancreas (data not shown), tissues previously shown to contain relatively high levels of ribophorin I (Mar-

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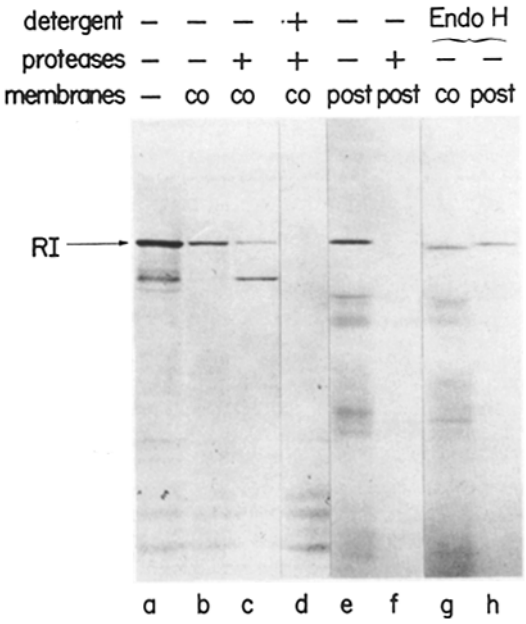

Figure 3. In vitro expression of the cloned ribophorin I cDNA containing the complete coding region and insertion of the encoded polypeptide into membranes. The SP6 polymerase transcripts of plasmid pGEM-6-1 were translated in a wheat germ cell-free system to which dog pancreas microsomes were added before translation was initiated (*co*, lanes *b–d*) or after translation was completed (*post*, lanes *e*, *f*, and *h*). Samples were analyzed by 10% SDS PAGE and autoradiography, either directly (lanes *a*, *b*, and *e*), after treatment with endoglycosidase H (lanes *g* and *h*), or a mixture of chymotrypsin and trypsin (lanes *c*, *d*, and *f*). The arrow indicates the position of ribophorin I.

cantonio et al., 1982). In Northern blot analysis of rat liver poly $(A)^+$ RNA, the insert in clone 1A hybridized to a mRNA of 2.6 kb (Fig. 2), which is approximately the size of the translatable ribophorin I mRNA purified by electrophoresis in methyl mercury-containing agarose gels (not shown). Clone 1A was then used to rescreen 10^6 recombinants of the same library by plaque hybridization. Three additional positive clones were identified but their inserts co-migrated with that in clone 1A. Given the extremely low abundance of ribophorin I mRNA in normal liver (Rosenfeld et al., 1984), the rarity of the ribophorin I clone in the rat liver library was not unexpected.

Clones containing larger inserts of ribophorin I cDNA were obtained, however, by hybridization screening of a lambda gtll cDNA library constructed from brain mRNA isolated from 20-d-old rats. It was expected that higher levels of ribophorin I mRNA would be present in this RNA because at this stage of development neurons and glial cells that contain a relatively well-developed ER are still undergoing rapid differentiation (Colman et al., 1982). Screening of $\sim 1 \times 10^5$ independent clones by hybridization, using the ^{32}P-labeled 600-bp insert as a probe, yielded three positive clones with inserts of ~0.75, 1.3, and 2.3 kb (data not shown). The clone containing the 2.3-kb insert (clone 6-1) was large enough to encode the entire ribophorin polypeptide and was further characterized.

In Vitro Expression of the Cloned Ribophorin cDNA and Insertion of the Encoded Polypeptide into Membranes

The insert in clone 6-1 was subcloned into pGEM-1, a plasmid vector that allows in vitro transcription of the cloned DNA. The mRNA transcribed from the resulting plasmid (pGEM-6-1) yielded a primary translation product of ~65 kD (Fig. 3, lane *a*), the same size as that of the primary translation product of ribophorin I mRNA (Rosenfeld et al., 1984), which could be immunoprecipitated with anti-ribophorin I antibody (not shown). This indicates that the 2.3-kb insert contains the entire coding sequence for ribophorin I. When dog pancreas microsomes were present during translation, ribophorin I was efficiently inserted into the microsomal membranes, as indicated by its partial resistance to added proteases that converted the 65-kD polypeptide to a 55-kD form (Fig. 3, lane *c*). This decrease in molecular mass suggests that ribophorin I is a true transmembrane protein that has a segment of ~10 kD exposed on the cytoplasmic surface of the ER membrane. As expected, when the membranes were added after translation was completed, the in vitro–synthesized product was completely degraded by the added proteases (Fig. 3, lane *f*). Insertion of the in vitro–synthesized ribophorin into the membranes was accompanied by signal cleavage and co-translational glycosylation, although the apparent molecular mass of the polypeptide was not affected by these modifications. Glycosylation and signal cleavage were demonstrated by the fact that treatment of the polypeptide that was recovered with the membranes with endoglycosidase H yielded a product of higher electrophoretic mobility (Fig. 3, lane *g*) than the primary translation product. These observations are analogous to those that were obtained with the product of the natural ribophorin I mRNA (Rosenfeld et al., 1984). In the latter case, it was also shown that the product of endoglycosidase H digestion had the same electrophoretic mobility as the ribophorin polypeptide that accumulates in tunicamycin-treated cells, which undergoes signal removal but is not glycosylated.

Increase in Hepatic Ribophorin I mRNA Levels after Partial Hepatectomy

It has previously been shown (Rosenfeld et al., 1984) that synthesis of ribophorin I accounts for <0.001% of the total [^{35}S]methionine incorporation directed by normal rat liver mRNA. However, fivefold higher levels of translatable ribophorin I mRNA were found 15 h after partial hepatectomy (Rosenfeld et al., 1984). Using the nick-translated cDNA insert of clone 6-1 as a probe, we found that a commensurate increase in hybridizable ribophorin I mRNA had taken place 15 and 24 h after partial hepatectomy (Fig. 4). This clearly established that the increase in translatable mRNA observed after hepatectomy reflects a true rise in the concentration of ribophorin I mRNA. Northern blot analysis of mRNAs samples obtained from a variety of tissues and cultured cells (not shown) indicated that significant levels of ribophorin I mRNA are present in cell types other than the hepatocyte. Kidney and lung had substantially lower ribophorin I mRNA levels than normal liver, but relatively high levels of the mRNA were observed in mouse myeloma cells, which are proliferating secretory cells with a well-developed ER. These observations confirmed that ribophorin I is not a

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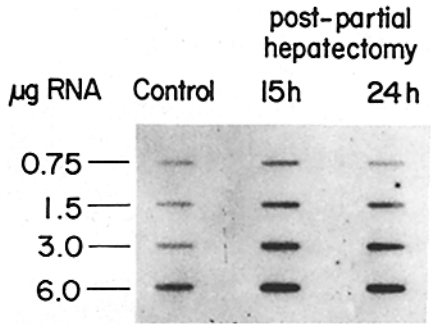

Figure 4. Increase in the hepatic level of ribophorin I in RNA after partial hepatectomy. Various amounts of RNA from livers of control or hepatectomized animals were applied to a nitrocellulose filter using a slot blot apparatus (Schleicher & Schuell) and the immobilized RNA was probed with the ^{32}P-labeled insert from pGEM-6-1. After hybridization and washing, the dried filters were exposed to X-ray film for 48 h.

tissue-specific gene product and, together with the aforementioned results with hepatectomized animals, suggest that there is a positive correlation between the abundance of ribophorin I mRNA and the secretory capacity of the cells or their proliferating activity. The increase observed after hepatectomy presumably reflects the fact that ribophorin I is a highly stable protein that, when cells are not growing, is synthesized at very low rates. Surprisingly, no correlation was observed (Lauffer et al., 1985) between the secretory capacity of different cell types and their levels of SRP receptor mRNA.

In Southern blot analysis of rat liver genomic DNA digested with a variety of restriction enzymes that do not cut the cDNA, including HindIII, BamHI, BglI, XhoI, and EcoRI

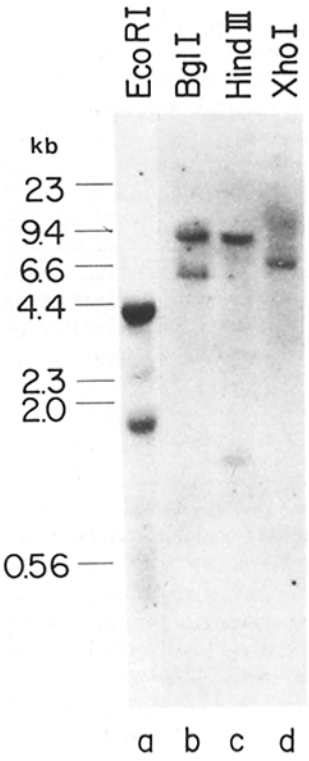

Figure 5. Southern blot analysis of rat liver genomic DNA. Rat liver genomic DNA (10 µg) was digested with restriction enzymes, as indicated. The digests were resolved on 0.8% agarose gels, transferred to nitrocellulose paper, and hybridized to the labeled inserts of pGEM-6-1. HindIII restriction fragments of lambda phage DNA detected by ethidium bromide staining were used as molecular weight markers.

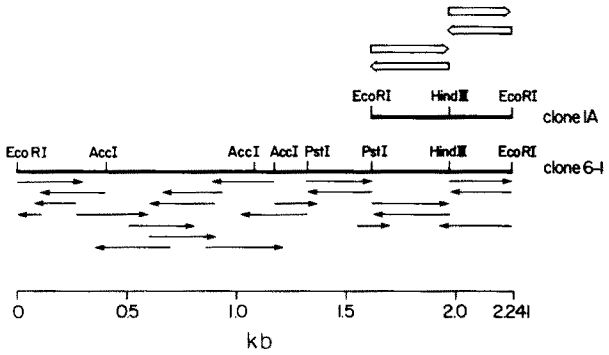

Figure 6. Strategy used for the sequencing of the ribophorin I cDNA clones. Restriction maps of the cDNA clones 1A and 6-1 were obtained by conventional procedures (Maniatis et al., 1982). The two ribophorin I clones were aligned by comparing the sequences. Clone 1A was sequenced (*open arrows*) by the chemical cleavage procedure (Maxam and Gilbert, 1980) and clone 6-1 by the enzymatic chain termination method (Sanger et al., 1977). For clone 6-1, the thin arrows that begin at restriction sites represent sequences determined from subcloned fragments using the universal M13 sequencing primer. The arrows that begin at other sites represent sequences determined using specific synthetic primers.

(Fig. 5), the 2.3-kb ribophorin I cDNA hybridized to only one or two bands. These results suggest that ribophorin I is not a member of a subfamily of closely related genes and that probably the ribophorin I gene is not larger than 10 kb.

Sequence of the Ribophorin I cDNA and of Its Encoded Polypeptide

Clone 1A was sequenced by the Maxam and Gilbert method (1980). The ribophorin I cDNA insert in clone pGEM-6-1 was analyzed by restriction endonuclease mapping, and convenient restriction fragments were subcloned into the M13mp18 or M13mp19 single stranded phage vectors for DNA sequencing of both strands (Fig. 6) by the enzymatic chain termination method (Sanger et al., 1977). The DNA sequences showed that the 600-bp insert in clone 1A represents sequences present at the extreme 3′ end of the insert in clone 6-1 (Fig. 7). The latter cDNA insert contains 2221 bp, and one strand has an open reading frame of 605 codons beginning with an ATG codon at the ninth nucleotide from the 5′ end. This initiation codon is present within the canonical sequence for translation initiation found in most eukaryotic mRNAs (Kozak, 1986).

Comparison of the amino acid sequence deduced for the long open reading frame with peptide sequences obtained by two different investigators (see legend to Fig. 7) for the amino terminal end of the mature protein purified by SDS gel electrophoresis suggested that ribophorin I is synthesized with a cleavable signal peptide that is removed during insertion of the polypeptide into the ER membrane. Although the two amino acid sequences available do not perfectly agree with each other or with the deduced amino acid sequence (see legend to Fig. 7), probably because of errors in the amino acid sequencing due to low repetitive yields, the best alignment (only two or three mismatches in 12 residues) indicated that the signal is 22 amino acids long. Although many ER proteins are known to be synthesized in membrane-bound ribosomes (see Kreibich et al., 1983), to date, ribophorin I,

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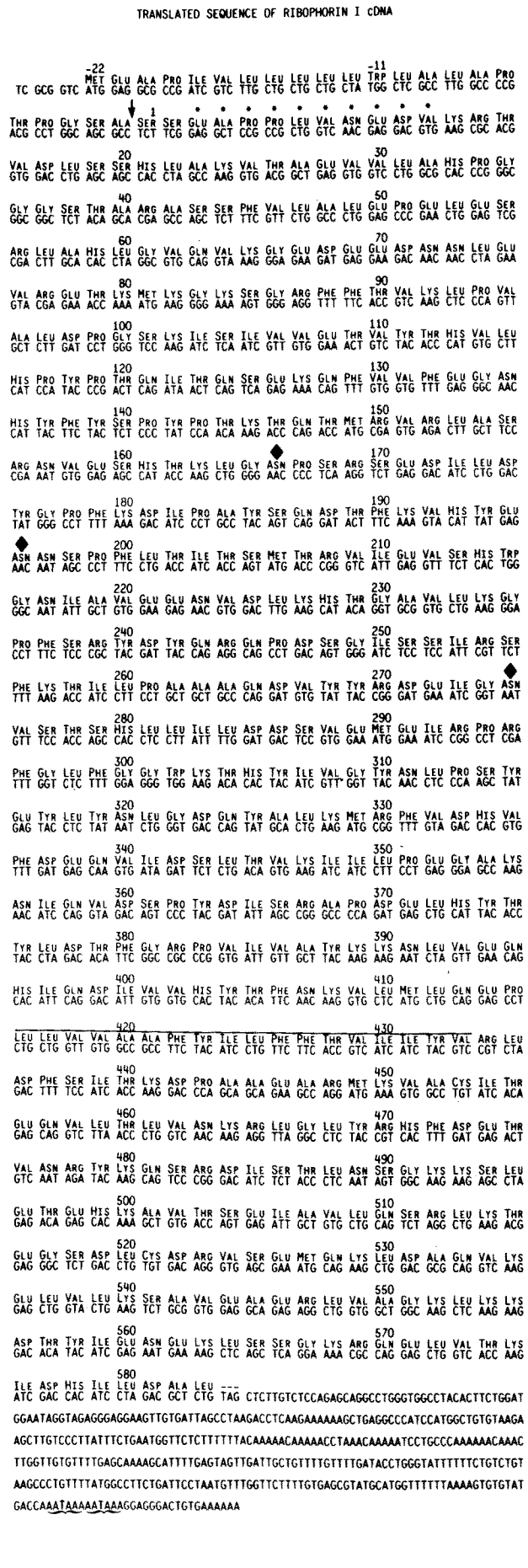

ribophorin II (Rosenfeld et al., 1984), and UDP-glucuronosyl transferase (MacKenzie, 1986) are the only ER membrane proteins for which cleavable signal sequences have been found. The insertion signal of ribophorin I has many of the features of the cleavable insertion signal present in secretory membrane proteins (von Heijne, 1981; Perlman and Halvorson, 1983). Thus, the hydrophobic core of the ribophorin signal extends from residues −20 to −7, is preceded by a charged amino acid (glu at position −21), and is followed by alpha helix-breaking residues (pro at −4 and −6 and gly at −3). Moreover, signal cleavage takes place after an alanine, which is an amino acid containing a small side chain. It is interesting, however, that cleavage just after the serine following this alanine residue would have been expected to be more likely on the basis of the statistical occurrence of different amino acids around the cleavage site in 78 known signal sequences (von Heijne, 1983).

The predicted molecular mass for ribophorin I is 68271 D, which is in close agreement with the apparent molecular masses (65 kD) estimated by SDS PAGE for the mature protein (Kreibich et al., 1978*b*) and for the ribophorin I mRNA primary translation product (Rosenfeld et al., 1984). The ribophorin I cDNA in clone 6-1 includes a 3′ untranslated segment of 389 nucleotides that contains two potential polyadenylation signals (AATAAA) (Proudfoot and Brownlee, 1976) in tandem. The presence of multiple poly (A^+) addition signals in eukaryotic mRNAs is not uncommon and as many as seven are found in the mouse procollagen mRNA (Meyer et al., 1986). The 3′ untranslated region of the ribophorin I cDNA is somewhat unusual, however, in that it contains several short stretches of T and A residues (Fig. 7).

The Amino Acid Sequence of Ribophorin I Predicts a Transmembrane Disposition

A[9] hydrophilicity analysis (Hopp and Woods, 1981) of the ribophorin I amino acid sequence (Fig. 8) revealed that, in addition to the signal sequence (residues −22 to −1), there is only one other hydrophobic stretch in the polypeptide (residues 415–433) sufficiently long to traverse the ER lipid bilayer. Since ribophorin I is an integral membrane protein and its amino-terminal insertion signal is cleaved, this hydrophobic segment must represent the membrane-anchoring domain of the protein, which during membrane insertion functions as a stop transfer signal to leave a carboxy terminal segment of 150 residues exposed on the cytoplasmic aspect

Figure 7. Nucleotide sequence of the ribophorin I cDNA and the deduced amino acid sequence. The complete nucleotide sequence (2221 nucleotides) of the insert in clone 6-1 is shown with the predicted amino acid sequence of the only long open reading frame it contains. The arrowhead marks the putative signal cleavage site and asterisks indicate those amino acids that match the amino-terminal sequence data. Two independent gas phase microsequencing determinations by Drs. K. Drickamer (University of Chicago) and N. Siegel (Monsanto Corp., St. Louis, MO) for ribophorin I purified by elution from an SDS polyacrylamide gel gave the following sequences, respectively: M T E A P Y L V N E D V and I I E A P P L V N E D. The three amino acid differences between those two sequences are probably due to the low repetitive yields during the automated degradation. The diamonds indicate the three possible glycosylation sites for Asn-linked oligosaccharides. The overlined segment corresponds to the transmembrane region. The two tandem poly A addition signals are indicated by brackets.

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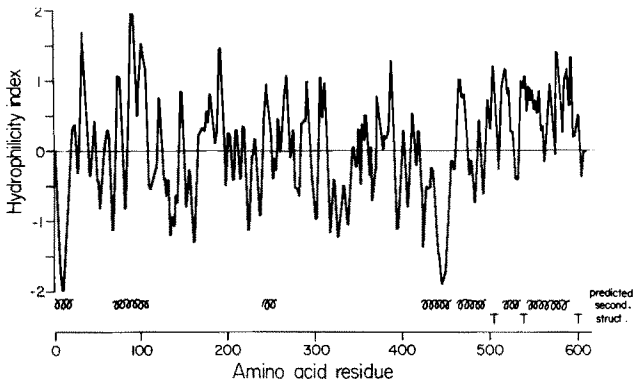

Figure 8. Hydrophilicity plot of ribophorin I. The plot was determined using a window of six residues (Hopp and Woods, 1981). Peaks above the horizontal axis represent hydrophilic regions and those below, hydrophobic portions of the molecule. Domains with a high probability to form alpha-helices were identified as described in Materials and Methods and are represented by coils above the corresponding regions of the amino acid axis. Helix-breaking regions are indicated by *T*s.

of the membrane. Indeed, the fact that exogeneous proteases reduced the apparent molecular mass of the membrane-inserted in vitro–synthesized ribophorin I from 65 to 55 kD is consistent with the exposure of >100 amino acids on the cytoplasmic surface of the ER membrane.

The amino acid sequence of ribophorin I and the proteolysis experiments, therefore, confirm our previous proposal (Rosenfeld et al., 1984) that this protein has a simple transmembrane disposition and demonstrates that the bulk (the amino terminal 414 residues) of the protein is located in the ER lumen. This luminal domain contains three potential glycosylation sites, Asn-X-Ser/Thr (Struck and Lennarz, 1980), at positions 166, 196, and 275. The small change in the electrophoretic mobility of ribophorin I caused by endoglycosidase H–catalyzed removal of high mannose oligosaccharides and the fact that no intermediates were observed during digestion with low concentrations of this enzyme, suggested that ribophorin I contains only one oligosaccharide chain (Rosenfeld et al., 1984). It is likely that this corresponds to the asparagine at position 275 since this is found within the triplet Asn-Val-Ser, which is much more frequently used for glycosylation than Asn-Pro-Ser or Asn-Asn-Ser (Struck and Lennarz, 1980). Indeed, in unpublished experiments using truncated mRNAs to program an in vitro translation–translocation system, glycosylation of the incomplete ribophorin polypeptides took place only when the site with an Asn at residue 275 was present.

The distribution of hydrophilic residues in ribophorin I is also worth noting. Within the cytoplasmic domain (151 residues), 36% of the residues are charged, with basic residues predominating, and 26% are neutral hydrophilic ones. The luminal domain is less hydrophilic (52%) and contains about equal numbers of positively- and negatively-charged residues. The overall hydrophilicity of the cytoplasmic domain of ribophorin I is significantly higher (48%) than that of the large cytoplasmic domain of the SRP receptor (Lauffer et al., 1985) although the latter has a more pronounced excess of basic residues that, it has been proposed (Lauffer et al., 1985), may play a role in the interaction of the receptor with the RNA component of SRP.

*Table I. Proteins with Regions of Homology to Ribophorin I**

	Identity	Ribophorin region	z value
	%		
Myosin heavy chain (147–239)	22.6	507–593	11.5
Tropomyosin β-chain (26–98)	32.4	511–584	10.95
Lamin C (101–247)	17.6	456–606	9.31
Keratin, type I (267–319)	26.4	506–558	11.48
Vimentin (198–271)	23.7	495–569	7.95

The data presented are for comparisons with rabbit skeletal muscle myosin heavy chain (Capony and Elzinga, 1981), rabbit skeletal muscle tropomyosin heavy chain (Mak et al., 1980), lamin C (McKeon et al., 1986), human cytoskeletal keratin type I (Hanukoglu and Fuchs, 1982), and hamster vimentin (Quax-Jenken et al., 1983). Comparable homologies to the same proteins from other species were observed but are not included in this table.

* The sequence of ribophorin I was compared with the sequences in the National Biomedical Research Foundation (NBRF) data bank and the Hoffman La Roche data bank using the FASTP program (Lipman and Pearson, 1985). After initial homologies to the carboxy terminal region of ribophorin I were detected, a second search was made using the sequence of the carboxy terminal 186 amino acid segment of ribophorin I. The numbers within the parenthesis adjacent to the name of each protein indicate the segment of homology within the reported sequence of that protein, which in some cases is a fragment of the complete molecule. The numbers in the columns indicate the percentage of sequence identity within the region of homology, the residues defining the region that showed homology within the complete ribophorin sequence, and the significance of the similarity expressed as a z value (Lipman and Pearson, 1985). This represents the number of standard deviations by which the optimized alignment score for the two proteins differs from the mean alignment score for randomly permuted sequences of the homologous protein with ribophorin. It has been proposed (Lipman and Pearson, 1985) that z values >10 are definitely significant, whereas those >6 are probably significant.

A conformational analysis of the sequence of ribophorin I (Garnier et al., 1978) indicates that, in contrast to the luminal domain, the cytoplasmic portion of the protein is mainly alpha helical (see Fig. 8). A computer search of data banks of known protein sequences using the FASTP program (Lipman and Pearson, 1985) led to the identification of several proteins that play a structural role in the cell and have significant homology to the cytoplasmic domain of ribophorin I (Table I). These include the muscle proteins myosin (Capony and Elzinga, 1981) and tropomyosin (McLachlan et al., 1975), as well as several proteins of the intermediate filament family such as type I cytoskeletal keratin (Hanukoglu and Fuchs, 1982), lamin C (McKeon et al., 1986), and vimentin (Quax-Jenken et al., 1983). The segments of homology are found within the alpha helical regions of these proteins and are indicated in Table I, which also gives an index (z value) of the statistical significance of each homology.

The homology of the cytoplasmic segment of ribophorin I with these proteins may be considered in the light of our previous suggestion that ribophorins play a structural role in the rough ER, providing a scaffolding within the ER membrane that restricts the ribosome-binding sites and their associated translocation apparatus to the rough domains of the ER (Kreibich et al., 1978*b*). We have also proposed that this scaffolding confers to the rough membranes their typical organization as flattened sacs that contrasts sharply with the convoluted tubular configuration of the contiguous smooth ER (Kreibich et al., 1978*a*, *b*). Although the cytoplasmic domain of ribophorin I does not contain the extensive series of heptad repeats within an alpha helical rod that enables the homologous proteins to form the coiled coils essential for their cytoskeletal function, other types of interaction may mediate the formation of a ribophorin network, perhaps in-

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volving an association with cytoskeletal proteins. A possible structural role for the ribophorins does not, of course, preclude their participation in ribosome binding either by directly providing ribosome binding sites or, as we have previously noted (Kreibich et al., 1978*b*), by contributing, in association with other membrane components, to the integration of supramolecular assemblies that act as ribosome receptors.

The availability of a cDNA clone containing the complete coding sequence for ribophorin I should facilitate studies on the functional role of this protein in the translocation process and on the mechanism for its segregation in the rough domains of the ER.

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