Human ribophorins I and II: the primary structure and membrane topology of two highly conserved rough endoplasmic reticulum-specific glycoproteins

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Ribophorins I and II represent proteins that are postulated to be involved in ribosome binding. They are abundant, highly-conserved glycoproteins located exclusively in the membranes of the rough endoplasmic reticulum. As the first step in the further characterization of the structure and function of these proteins, we have isolated and sequenced full-length human cDNA clones encoding ribophorins I and II using probes derived from a human liver expression library cloned into pEX1. The authenticity of the clones was verified by overlaps in the protein sequence of N-terminal and several internal fragments of canine pancreatic ribophorins I and II. The cDNA clones hybridize to mRNA species of 2.5 kb in length, and encode polypeptides of 68.5 and 69.3 kd, respectively. Primary sequence analysis, coupled with biochemical studies on the topology, indicates that both ribophorins are largely lumenally disposed, spanning the membrane once and having 150 and 70 amino acid long cytoplasmically disposed C termini, respectively. Both are synthesized as precursors having cleavable signal sequences of 23 (ribophorin I) and 22 (ribophorin II) amino acids. The topology suggested by the primary structure has been confirmed biochemically using proteolytic enzymes and anti-ribophorin antibodies. Proteolysis of intact microsomes with a variety of enzymes resulted in a reduction in the apparent mol. wt of ribophorin I that would correspond to a loss of its 150-amino acid cytoplasmic tail. In the case of ribophorin II, it is completely resistant to such proteolysis which is consistent with its luminal disposition and fairly hydrophobic C terminus. Sequence comparisons and immunoblotting using specific antibodies show both proteins to be highly conserved throughout a variety of species. No relationship between these two proteins could be deduced from their primary sequences.

Key words: rough endoplasmic reticulum/membrane proteins/secretion/membrane biogenesis

Introduction

Rough endoplasmic reticulum (ER) is defined by the presence of bound ribosomes (Palade, 1955). These ribosomes have been shown to be involved exclusively in the synthesis of secretory and membrane proteins (Redman, 1969). From these and other data the conclusion has been drawn that the binding of ribosomes to the membranes of rough ER is an integral part of the process of secretion and membrane assembly. Studies conducted in several laboratories indicate that ribosome binding is sensitive to proteases and therefore proteinaceous receptors that mediate this phenomenon must exist in the membrane (Shires *et al.*, 1974; Borgese *et al.*, 1974; Hortsch *et al.*, 1986). On the basis of cell

fractionation and cross-linking studies, Kreibich and collaborators have proposed that two integral rough ER-specific membrane proteins, named ribophorins I and II, fulfill the requirement for ribosome receptors (Kreibich *et al.*, 1978a,b). Moreover, the ribophorins are unique to the ER membrane system and are present in a stoichiometric relationship with the number of membrane-bound ribosomes (Marcantonio *et al.*, 1984).

Ribophorins represent the canonical rough ER-specific protein. They are abundant, appear conserved in a number of species and are part of the stubbornly insoluble complex of membrane

Fig. 1. Restriction map of ribophorins I and II. Shown are the restriction sites used in generating fragments that were subcloned for sequencing cDNA clones. Arrows indicate the length and location of the original expression clones isolated from the human liver cDNA library in pEX1. **Panel A:** ribophorin I; **panel B:** ribophorin II.

Fig. 2. Northern blot hybridized with ribophorin I and II probes. Electrophoresis and blotting of mRNA from HeLa and MDCK cells, radiolabeling of ribophorin I and II probes and hybridization are described in Materials and methods. Standards, whose lengths in base pairs are given on the left, were generated by the detection of *Hind*III-restricted bacteriophage lambda DNA with the appropriate radiolabeled probes. RI and RII refer to ribophorin I and II, respectively.

[illegible]

UCCAGCGGUUGCGAGACGGUCUGGUUCCAAGUGGGCCUGGCGCGGGGAGAGGGCGGGUCUGUCUCGGGAACUGCAAGGCCUGUGAGCGGGAGGACUGGGAUCCGGCCGCGG 114

115 CUGCUGGAAGCGUCGAAGCUCACGCGGGCCCGCGCAUGACCUGUGCUUAGAACUCUACCCUGGCCGCGAGAGCCUGGCCGCGAGUCCUGGCGUCCCUUGUGCGGGCUCUUGGAGCCACUU 234

MetAlaProProGlySerSerThrValPheLeuLeuAlaLeuThrIleIleAlaSerThrTrpAla -1
 235 UCCCCAGCGGAACUCAGCCCGCGGUCGGACUCCGGCGGGACUCUGCGGAGGAUUGCGGCCCGGGUUAAGCACUGUCUCCUGUUGGCCUGACAAUCAUAGCCAGCACCCUGGGCU 354

+1 10 20 30 40
 355 LeuThrProThrHisTyrLeuThrLysHisAspValGluArgLeuLysAlaSerLeuAspArgProPheThrAsnLeuGluSerAlaPheTyrSerIleValGlyLeuSerSerLeuGly 474
 CUGACGCCACUCACUACCUCACCAAGCAUGACGUGGAGAGACUAAAAGCCUCGUGGAGUCCCUUUCACAAAUUGGAUUCUGCCUUCUACUCCAUUGGGGACUCAGCAGCCUUGGU

50 60 70 80
 475 AlaGlnValProAspAlaLysLysAlaCysThrTyrIleArgSerAsnLeuAspProSerAsnValAspSerLeuPheTyrAlaAlaGlnAlaSerGlnAlaLeuSerGlyCysGluIle 594
 GCUCAGGUGCCAGAUCAAGAAAGCAUGUACCUACAUCAUGAUCAACCUUGAUCCAGCAUUGGGAUUCUUCUACCGUGCCAGGCCAGCCAGGCCCCUCUAGGAUGUGAGAUC

90 100 110 120
 595 SerIleSerAsnGluThrLysAspLeuLeuAlaValSerGluAspSerSerValThrGlnIleTyrHisAlaValAlaAlaLeuSerGlyPheGlyLeuProLeuAlaSerGln 714
 UCUAUUUCAAUAGACCAAAGAUUCUGCUUCUGGCAGCUGUCAGUGAGGACUCAUCUGUUACCCAGAUUCUACCAUGCAGUUGCAGUCUUAAGUGGCUUUGGCCUCCUUGGCAUCCCAA

130 140 150 160
 715 GluAlaLeuSerAlaLeuThrAlaArgLeuSerLysGluGluThrValLeuAlaThrValGlnAlaLeuGlnThrAlaSerHisLeuSerGlnGlnAlaAspLeuArgSerIleValGlu 834
 GAAGCACUCAGUGCCCUUACUGCUCGUCACGAAGGAGGAGACUGUGCGGCAACAGUCCAGGCUCUGCAGACAGCAUCCACCUGUCCAGCAGGCUGACCUAGGAGCAUCUGGAG

170 180 190 200
 835 GluIleGluAspLeuValAlaArgLeuAspGluLeuGlyGlyValTyrLeuGlnPheGluGluGlyLeuGluThrThrAlaLeuPheValAlaAlaThrTyrLysLeuMetAspHisVal 954
 GAGAUUGAGGACCUUGUUGCUCGCCUGGAUGAACUGGGGGCGUGUAUCCUCCAGUUUGAAGAGGACUGGAACACAGCGUUAUUUGGCGUGCCACCUACAAGCUCAUGGAUCUGUG

210 220 230 240
 955 GlyThrGluProSerIleLysGluAspGlnValIleGlnLeuMetAsnAlaIlePheSerLysLysAsnPheGluSerLeuSerGluAlaPheSerValAlaSerAlaAlaValLeu 1074
 GGGACUGAGCCAUCCAUUAAAGGAGGAUCAGGUCAUCCAGCUGAUGAACGCCGAUCUUCAGCAAGAGAACUUUGAGUCCUUCUCCAGGCCUUCAGCGUGGCCUCUGCAGCUGCUGGUC

250 260 270 280
 1075 SerHisAsnArgTyrHisValProValValValProGluGlySerAlaSerAspThrHisGluGlnAlaIleLeuArgLeuGlnValThrAsnValLeuSerGlnProLeuThrGln 1194
 UCGCAUAAUCCGUACCCAGUGCCAGUUGUGGUUGGCCUGAGGGCUCUGCUUCCGACACUCAUGAACAGGCUAUCUUGCGGUUGCAAGUACCAUUGUUCUGUCUACGCCUCUGACUCAG

290 300 310 320
 1195 AlaThrValLysLeuGluHisAlaLysSerValAlaSerArgAlaThrValLeuGlnLysThrSerPheThrProValGlyAspValPheGluLeuAsnPheMetAsnValLysPheSer 1314
 GCCACUGUUAAACUAGAACUGCUAAUUCUGUUGCUUCCAGAGCCACUGUCCUCCAGAGACAUCCUUCACCCUUGUAGGGGAGUUUUUUGAACUAAAUUUCUAGAACGCUAAAUUUUCC

330 340 350 360
 1315 SerGlyTyrTyrAspPheLeuValGluValGluGlyAspAsnArgTyrIleAlaAsnThrValGluLeuArgValLysIleSerThrGluValGlyIleThrAsnValAspLeuSerThr 1434
 AGUGGUUAUUAGUCAUUCCUUGUCGAAGUUGAAGGUGACAAACCGGUAAUUGCAAUACCGUAGAGCUCAGAGUCAAGAUUCCACUGAAGUUGGCAUACAAAUUGUUGAUUUUCC

370 380 390 400
 1435 ValAspLysAspGlnSerIleAlaProLysThrThrArgValThrTyrProAlaLysAlaLysGlyThrPheIleAlaAspSerHisGlnAsnPheAlaLeuPhePheGlnLeuValAsp 1554
 GUGGAUAGGAUCAGAGCAUUGCAUCCCAAACUACCCGGGUGACAUAUCCAGCCAAAGGCAAGGGGCAUUAUCGAGAGACGCCACCAAGAACUUGCCUUGUUCUCCAGCUGGUGAU

410 420 430 440
 1555 MetAsnThrGlyAlaGluLeuThrProHisGlnThrPheValArgLeuHisAsnGlnLysThrGlyGlnGluValValPheValAlaGluProAspAsnLysAsnValTyrLysPheGlu 1674
 AUGAACACUGGUGUGAACUCACUCCUACCCAGACAUUUGUCGACUCCAUAAACAGAGACUGGCCAGGAUGUGUGUUGUUGCCGAGCCAGACAAAGAAGGUGUACAGUUGAA

450 460 470 480
 1675 LeuAspThrSerGluArgLysIleGluPheAspSerAlaSerGlyThrTyrThrLeuTyrLeuIleIleGlyAspAlaThrLeuLysAsnProIleLeuTrpAsnValAlaAspValVal 1794
 CUGGAUACCUUGAAGAAAGAUUGAAUUGACUCUGCCUCUGGCAUCCACUCUCUUAUUAUUAUUGGAGUCCAUUUGAAGAACCAUCCUUGGAUUGUGGCUAGUGGUC

490 500 510 520
 1795 IleLysPheProGluGluGluAlaProSerThrValLeuSerGlnAsnLeuPheThrProLysGlnGluIleGlnHisLeuPheArgGluProGluLysArgProProThrValValSer 1914
 AUCAGAUUCCUGAGGAAGAGCUCUCCUGACUGUCUUGUCCGAAACUUAUCCUCCAAACAGGAAAUUCAGCACCUGUUCGCGAGCCUGAGAAGAGGCCCCCCCACCUGGUGUCC

530 540 550 560
 1915 AsnThrPheThrAlaLeuIleLeuSerProLeuLeuLeuPheAlaLeuTrpIleArgIleGlyAlaAsnValSerAsnPheThrPheAlaProSerThrIleIlePheHisLeuGly 2034
 AAUACAUUCACUGCCUGAUCCUCUGCCGUGUCUUCUGCUCUUCGUCUGUGGAUCCGGAUUGGUGCCAAUGUCUCCAACUUCACUUUUGCUUAGCAGGAUUAUUUACCUUGGA

570 580 590 600
 2035 HisAlaAlaMetLeuGlyLeuMetTyrValTyrTrpThrGlnLeuAsnMetPheGlnThrLeuLysTyrLeuAlaIleLeuGlySerValThrPheLeuAlaGlyAsnArgMetLeuAla 2154
 CAUGGUCUUAUGCUGGGACUCAUGAUUGUCUACUGGACUCAGCUCACAUGUUCACAGACCUUGAAGUACCUGGCCAUUUGGCGAGUGAGCUUUCUGGCGGCAUUGGGAUGCUGGCC

GlnGlnAlaValLysArgThrAlaHis
 2155 CAGCAGGCAGUCAAGAGAACAGCACAUUAGUCCAGAAAGAAAGAUUGGAAAUUCUGAAACUGAAUGUCAAGAAAGGAGUCAAGAAACAUAUACAGUAUGAGAAGAAAAUUGGAAAAAA 2274

2275 AAACUUUAUUAAAAAAGAAAAAGUCCAGAUUGAUGUUAUACUUUUGCUUGUUUUUACAGUUUCCCAACACACAGCAGAUACCUUGGUGAGCUAGAUAGUCUCUUUCUCUGACACUGUG 2394

2395 UAAGAAGCGUGAAUUAUCCUAAACUUAACCCAGAUUGCUUUUGAAAAAGUUGAAAUUGUUAUUUUGCAUAUAAAGAGGGUUAACAAUAGGA - (poly-A)

Fig. 3. Nucleotide sequence of cDNA clones encoding ribophorins and their deduced amino acid sequence. **Panel A:** ribophorin I; **panel B:** ribophorin II. Bars above the deduced amino acid sequences refer to fragments whose N termini were sequenced and found to generate homologous overlaps (see Figure 4). Arrowheads indicate putative glycosylation sites on lumenally-disposed sequences (see Figures 6 and 7). Underlined nucleotides represent consensus sites for polyadenylation.

membrane proteins whose subcellular distribution has been studied in the greatest detail and are the most unequivocal marker for this membrane system (Amar-Costesec *et al.*, 1984; Marcantonio *et al.*, 1984; Hortsch and Meyer, 1985). As a first step towards the elucidation of their precise function and mode of retention in the rough ER, we decided to clone cDNAs encoding ribophorins I and II. We report here the primary structure of these proteins and corroborate their deduced topology by biochemical means.

Results

Isolation of cDNA clones encoding ribophorins I and II

A human liver cDNA library (50 000 recombinants) cloned into pEX1 (Stanley and Luzio, 1984) was screened with monoclonal anti-ribophorin I and II antibodies (for details see Materials and methods). The longest inserts from this screening (shown in Figure 1) were 664 and 1437 bp for ribophorin I and II, respectively. Northern blot analysis (Figure 2) indicated that full-length clones should be ~2.5 kb for each cDNA in the case of mRNA derived from human (HeLa) cells. These probes were then used to screen a cDNA library derived from HeLa cell mRNA (100 000 recombinants) cloned into the lambda phage vector NM 1149. The longest clones obtained were 2397 bp and 2509 bp for ribophorins I and II, respectively. Restriction analyses of full-length cDNAs are shown in Figure 1.

Complete nucleotide and deduced amino acid sequences of ribophorins I and II

Restriction fragments of the cDNA clones were sequenced by the method of Sanger *et al.* (1977), and the nucleotide and deduced amino acid sequences are presented in Figure 3. The authenticity of these sequences was confirmed by comparison of the deduced amino acid sequence with the protein sequences of the N termini of the mature form and from cyanogen bromide-generated fragments of canine pancreatic ribophorins I and II (Figure 4). Proteins and CNBr fragments were purified on preparative SDS polyacrylamide gels and/or h.p.l.c. (see Materials and methods). An overall homology of 90% exists at the amino acid level between the fragments of human and canine ribophorins. These highly-conserved matches were not limited to specific regions, but rather were distributed throughout the sequences of each of the proteins (see Figure 3).

Ribophorin I sequence. There is a single open reading frame of 1821 bp that encodes 607 amino acids giving the precursor of ribophorin I a mol. wt of 68 550.8 daltons. The first 23 amino acids encode a cleavable signal sequence that corroborates the *in vitro* data of Rosenfeld *et al.* (1984) for rat liver ribophorin I. Interestingly, in contrast to most other cleavable signal sequences, there is no positive charge located N-terminal to the hydrophobic core (excluding the N-terminal methionine). In fact, the net charge in this region is negative. This is quite unusual, although not unheard of in eukaryotic cells (von Heijne, 1984). The mature protein would thus have a mol. wt of 66 272.2 daltons.

Ribophorin II. Ribophorin II is encoded by an mRNA having an open reading frame of 1896 bp. This corresponds to 632 amino acids and the mol. wt of the precursor is 69 283.4 daltons. It too has a cleavable signal sequence, consisting of 22 amino acids. In this case, there is also no positive charge on the N terminus of the signal. The mol. wt of the mature form of ribophorin II is 67 053.7 daltons. Both clones encode the consensus sequence for polyadenylation (AAUAAA) at their 3' ends.

A

Ribophorin I

B

Ribophorin II

Fig. 4. Matches between the amino acid sequence of canine ribophorins and the sequence deduced from the human cDNA. Generation of intact and fragmented canine ribophorins and their N-terminal sequencing was carried out as described (see Materials and methods). Numbers reflect the location of the fragment relative to the N terminus of the mature protein. Amino acids immediately below the nucleotide sequence represents the translation of the human cDNA. The second line of amino acids represent protein sequences of canine pancreatic ribophorins. Matches in amino acids are represented by vertical lines. **Panel A:** ribophorin I; **panel B:** ribophorin II.

Fig. 5. Hydropathy plot of ribophorins I and II. Hydropathy analysis was carried out according to Kyte and Doolittle (1982). Upper figure: ribophorin I; lower figure: ribophorin II. Darkened areas with stars represent sequences having sufficient length and hydrophobicity to represent membrane-spanning segments (Engelman *et al.*, 1986).

Structural and topological features of ribophorin I based on its deduced amino acid sequence and confirmed by biochemical analysis

Ribophorin I possesses the following characteristics from which its topology can be inferred. It has a cleavable signal sequence implying that the N terminus of the mature protein is lumenally disposed (Sabatini *et al.*, 1982; Wickner and Lodish, 1985). Based on hydropathy analyses carried out according to Kyte and Doolittle (1982) (Figure 5), only one 19 amino acid long segment has sufficient length and hydrophobicity to serve as a membrane-spanning domain (Engelman *et al.*, 1986). The fact that both ribophorins I and II are integral membrane proteins has been confirmed by their insolubility at pH 11 (Hortsch and Meyer, 1985) and their partitioning into the detergent Triton X-114 (Hortsch and Meyer, unpublished). Thus, this protein resides in the ER membrane with a single spanning sequence from amino acids 416 to 434, having a cytoplasmic C terminus of 150 amino acids and a luminal N-terminal domain consisting of 415 residues. The cytoplasmically-disposed portion of ribophorin I therefore represents about one quarter of the mass of the protein.

Ribophorin I is known to be N-glycosylated (Rosenfeld *et al.*, 1984), and an examination of the sequence shows consensus N-linked glycosylation sites (Asn-X-Thr/Ser) at residues 167, 197 and 276. Studies using endoglycosidase H have suggested that this protein is glycosylated only once (M.Hortsch, unpublished). Closer examination of the sites around Asn167 and Asn197 shows residues 168 and 200 to be Pro. Proline residues in the X position (168) as well as in the position C-terminal to Thr/Ser (200) prevent glycosylation (Bause, 1983). This leaves Asn276 as the sole putative site for N-linked oligosaccharide addition in ribophorin I. Since glycosylation is known to occur only on lumenally-disposed Asn residues, this provides further support for the topology suggested above.

The topology of ribophorin I suggested by the primary sequence can be confirmed by biochemical analysis. According to the predicted topology, ~75% of the protein should be in the membrane or the lumen of the rough ER, and thus resistant to digestion by proteolytic enzymes. To test this hypothesis, we have treated intact rough microsomes with a variety of proteases including trypsin, proteinase K, elastase and subtilisin, and examined the resistant forms of ribophorin I by immunoblotting using a polyclonal antibody that we know from protein fragmentation

Fig. 6. Immunoblotting with anti-ribophorin I of protease-treated intact and detergent-solubilized microsomes. Pancreatic microsomes, antibodies and immunoblots were prepared as described in Materials and methods. Microsomes were treated with proteases (200 μ g/ml) for 1 h at 0°C. Shown are immunoblots decorated with polyclonal anti-ribophorin I antibodies. On the right, the filled arrowhead denotes intact ribophorin I; open arrowhead the smallest degradation product that could be recovered from intact proteolyzed microsomes. Samples solubilized with Triton X-100 prior to digestion are indicated with +. Tryp, trypsin; ProK, proteinase K; Elas, elastase; Subt, subtilisin.

Fig. 7. Immunoblotting with anti-ribophorin II of protease-treated intact and detergent-solubilized microsomes. Identical procedures and materials were used as in Figure 6. Chym, chymotrypsin; V₈, *S. aureus* V₈ protease; Pap, papain; Tryp, trypsin.

analysis reacts with an N-terminal epitope (not shown). As can be seen in Figure 6, most of ribophorin I was resistant. Nonetheless, the smallest protease-resistant form obtained, regardless of which protease was used, had an apparent mol. wt of ~50 kD. In the presence of detergent, added to solubilize the rough microsomes, ribophorin I was further or completely degraded. As intact ribophorin I has an apparent mol. wt of 65 kD, a shift in size of 15 kD has occurred as a result of proteolysis. This is in good agreement with the topological prediction of a 150 amino acid long cytoplasmic domain.

Structural and topological features of ribophorin II based on its deduced amino acid sequence and confirmed by biochemical analysis

Ribophorin II is synthesized as a precursor having a cleavable signal comprising 22 amino acids. The Kyte–Doolittle hydropathy plot indicates, as with ribophorin I, only one putative membrane-spanning domain. This encompasses the 23 residue long stretch between amino acids 517 and 539. As the mature protein contains 610 amino acids, the cytoplasmic domain is maximally 70 residues in length. These values indicate that almost 90% of the protein is lumenally-disposed or within the membrane. Consensus sequences for N-glycosylation are at Asn residues 84, 544 and 547. Biochemical studies show about half of human ribo-

phorin molecules to be unglycosylated with the remainder having only one oligosaccharide chain (refer to Figure 8). As the membrane-spanning domain is located at residues 517–539, asparagine residues 544 and 547 would be cytoplasmically-disposed leaving only Asn84 as the putative site for oligosaccharide addition.

Biochemical data support these predictions. The same monoclonal antibody that enabled selection of the original ribophorin II clone (see Figure 1) was used to detect fragments of ribophorin II generated by V₈ protease cleavage on immunoblots. The cloning data show that the epitope for the antibody is on the C-terminal half of the ribophorin II molecule (Figure 1). A variety of smaller fragments that were generated by proteolysis and detectable by the antibody were not glycosylated; only large partially digested molecules with apparent mol. wts >41 kD had both the epitope and the glycosylation site (data not shown). This confirms the N-terminal location of the oligosaccharide chain at Asn84.

Ribophorin II is largely luminal in its orientation, and this was demonstrated by proteolytic digestion. The protein is completely resistant *in situ* to a variety of proteases, and not even the cytoplasmic tail of 70 residues is degraded (Figure 7). This total resistance can be explained by the fact that two somewhat hydrophobic and uncharged stretches of 21 and 14 residues, respectively (see Figure 5) are present in this domain. This may allow the C terminus of ribophorin II to 'dip' into the membrane rendering it insensitive to proteases. When detergent is added, an amount of ribophorin II, corresponding to the size of the cytoplasmic tail plus membrane-spanning segment, is digested by chymotrypsin and V₈, leaving the luminal domain intact. The fact that this undigested portion represents the luminal domain is demonstrated by the fact that it retains the appearance of the typical doublet indicative of the aforementioned incomplete glycosylation of ribophorin II. Using papain or trypsin, breakdown of the luminal domain can be achieved in the presence of detergent. These results serve to further localize the epitope of the monoclonal antibody to a region between amino acids 236 (the end of the original pEX clone) and 517 (the C terminus of the luminal domain), and the glycosylation site to the N terminus at Asn84.

Interspecies conservation of ribophorin epitopes

We have used immunoblots to demonstrate the broad species specificity of ribophorins. In the case of ribophorin I, our anti-canine monoclonal, used to select the original expression clone, recognized only human ribophorin I in addition to the canine form (not shown). A polyclonal antibody, that had been affinity purified on rat liver ribophorin I (see Materials and methods), was successfully used to decorate immunoblots of microsomes derived from chicken, mouse, rat and human sources in addition to its own canine antigen (Figure 8A). It even reacted weakly with microsomes from *Xenopus* liver. More striking was the result with the anti-canine ribophorin II monoclonal (Figure 8B). This antibody could recognize microsomes from all of the animal species mentioned above, and reacted strongly with ribophorin II from *Xenopus laevis* as well. These findings taken together with the close homology that we have observed between canine and human ribophorins at the amino acid level (Figure 4), attest to the fact that these proteins appear to have been highly conserved during evolution. Moreover, our biochemical studies on the topology of canine ribophorins were corroborated by the deduced sequence of their human counterparts. The nucleotide and the protein sequences of ribophorins I and II were compared with

Fig. 8. Species specificity of anti-ribophorin antibodies. Microsomes were prepared from: **lane 1**, yeast; **lane 2**, *Xenopus* liver; **lane 3**, chicken liver; **lane 4**, mouse MOPC-41 myeloma; **lane 5**, rat liver; **lane 6**, canine pancreas; and **lane 7**, HeLa cells, separated on polyacrylamide gels and blotted onto nitrocellulose as described in Materials and methods. **Panel A:** detection of ribophorin I with affinity-purified polyclonal anti-canine ribophorin I antibody. **Panel B:** detection with monoclonal anti-canine ribophorin II antibody.

sequences in various data libraries. No striking homology was found with any known proteins, nor between the ribophorins themselves.

Discussion

The data presented in this study detail the structural and topological features of the rough ER-specific membrane glycoproteins ribophorins I and II. These two proteins were the first ones to be demonstrated to be rough ER-specific (Kreibich *et al.*, 1978a), and represent two of the more abundant and well-conserved molecules of this type. Ribophorins have been postulated to be ribosome-binding sites. Two lines of evidence led to this conclusion. The first indicated that ribophorins remained associated with the ribosomal fraction when the microsomal membrane had been solubilized (Kreibich *et al.*, 1978a), and the second, using chemical cross-linking, showed that the complexes that were formed contained both ribosomes and ribophorins (Kreibich *et al.*, 1978b). It was on the basis of these data that the name ribophorin was given to these proteins. We have recently shown, however, that mild proteolysis of intact stripped microsomes eliminates ribosome binding with little or no effect on ribophorins (Hortsch *et al.*, 1986).

The cloning and sequencing of cDNA encoding ribophorins puts us in a position to address several questions concerning the function and localization of these proteins. We infer from their abundance, conservation, subcellular localization and their ability to form complexes with other translocation-related proteins that

they play a central role in rough ER function. Our results on their topology suggest that their functional role is probably played out in the lumen of the rough ER, but some form of participation in ribosome binding cannot therefore be ruled out. We are now in a position to prepare antibodies against precise regions of the ribophorins, such as their cytoplasmically-located C termini, using gene fusions for immunization. A direct effect of such antibodies on translocation, or on ribosome binding specifically, can be tested by *in vitro* assays. Should it prove possible to use the ribophorin probes to isolate their homologues in yeast, significant insights can be gained into their function by deletion and/or mutagenesis of the yeast ribophorins.

Ribophorins are resident proteins of the rough ER (Rosenfeld *et al.*, 1984). As the entry into the secretory pathway and the site of membrane assembly, the rough ER experiences a profuse amount of protein traffic through its membranes and cisternae (Palade, 1975). A central phenomenon in cell biology, in addition to that of protein transport through the various cellular compartments along the secretory pathway, is that of protein retention in these same organelles. In fact, protein transport and secretion has been suggested to be governed by the lack of specific retention signals (Wiedmann *et al.*, 1984). It is in the study of this retention aspect of transport that the ribophorins are exceptionally well suited as model proteins.

It is known that the resident proteins of rough ER have solubility characteristics different from rough ER proteins that are also found in smooth ER (Hortsch *et al.*, 1986). Non-ionic detergents can only be used to solubilize rough ER-specific proteins when high salt concentrations are used. In the absence of high salt, rough ER-specific protein complexes are formed upon solubilization, that not only contain a typical set of protein components, but that morphologically retain a membrane-like appearance, including the presence of bound ribosomes (Kreibich *et al.*, 1978c; Nagata, 1980). These data imply that a matrix or network exists within the rough ER whose functions would include the mediation of translocation and processing of nascent secretory and membrane proteins. Known members of such complexes include the docking protein (Hortsch *et al.*, 1986), the signal peptidase complex (Evans *et al.*, 1986) and the ribophorins (Hortsch *et al.*, 1986). By having cloned cDNA encoding the ribophorins, studies can commence that are designated to elucidate the nature of the interactions between proteins of this complex, or in other words, the structural features of ribophorins that allow their retention in the rough ER. The expression, *in vivo*, of fusions and deletions based on ribophorin sequences can be used to understand the molecular basis of rough ER specificity.

The importance of the ribophorins is underscored by their high degree of conservation. They represent glycoproteins of nearly identical mol. wt in a variety of vertebrates, that can be detected on immunoblots by a monoclonal antibody (see Figure 8). An antigenic similarity of this magnitude was never found for the docking protein, despite having tested many more mono- and polyclonal anti-docking protein antibodies on microsomes from the same variety of species (Hortsch and Meyer, 1985). Taken together, these observations and the results from other laboratories suggest that ribophorins are part of the complex of proteins that are specific to rough ER functioning either in translocation or in the maintenance of the structural uniqueness of this organelle.

Materials and methods

Library screening and clone selection

Initial clones were selected from a human liver cDNA library cloned into the expression vector pEX1 (Stanley and Luzio, 1984). Monoclonal anti-ribophorin

I (clone 18A4) and II (clones 3D1 and 7B3) antibodies were used in the screening procedure and have been described previously (Hortsch *et al.*, 1986). Immunoselection of positive colonies was carried out as described by Stanley (1983). Probes were generated from the inserts of positive clones and used to screen a HeLa cell cDNA library cloned into an NM 1149 lambda phage vector. This library was generously provided by Cladio Schneider (EMBL, Heidelberg). Radiolabeled probes were generated by nick-translation and colony or plaque hybridization on nitrocellulose filters was carried out according to standard procedures (Grunstein and Hogness, 1975). Pre-hybridizations and hybridizations were performed in $6 \times \text{SSC}$, $5 \times \text{Denhart's}$, 10 mM EDTA, 0.1% SDS at 65°C for 12–15 h. Washing was carried out in $0.1 \times \text{SSC}$, 0.1% SDS at 65°C .

Northern blot analysis

mRNA from HeLa and MDCK cells was prepared according to the guanidium isothiocyanate procedure described by Lizardi (1983). Formaldehyde-containing agarose gels were used to separate the mRNA (Lehrach *et al.*, 1977), and blotting onto Genescreen Plus^{TM} and hybridization was carried out according to the method of Church and Gilbert (1984). Probes were radiolabeled by extension of sequences randomly primed with oligonucleotides according to published procedures (Feinberg and Vogelstein, 1983).

Sequencing of cDNA clones

Fragments of cDNA were generated by restriction digestion and/or exonuclease treatment and subcloned into M13 mp8 and mp9 vectors (Messing and Vieira, 1982). Restriction fragments were chosen that would allow the complete sequencing of both strands of the cDNA (see Figure 1). Sequencing reactions were carried out according to the dideoxy method described by Sanger *et al.* (1977).

Purification and N-terminal sequencing of ribophorins I and II

Ribophorins I and II were isolated from alkali-treated (pH 11) canine pancreatic microsomes, and purified on preparative polyacrylamide gels (Hortsch *et al.*, 1985). Proteins were electroeluted from the gels (Knowles and Bologna, 1983), and aliquots were cleaved with saturated CNBr in 70% formic acid for 24 h at room temperature. Following lyophilization and washing, the peptides were dissolved in 70% formic acid. Fragments derived from ribophorin I were separated by h.p.l.c. on a Bakerbond wide pore butyl $5 \mu\text{m}$ column using an acetonitrile gradient (0–70%) in 0.1% trifluoroacetic acid (TFA). Fragments from ribophorin II were separated by preparative polyacrylamide gel electrophoresis on 15% gels and electroeluted. All samples (~ 50 – 200 pmol) were ultimately dissolved in 10% (v/v) TFA, and N-terminal sequencing was performed in a gas-phase sequencer as described by Frank and Trosin (1985). Fractions were subjected to amino acid analysis using the orthophthalaldehyde method of Ashman and Bosserhoff (1985).

Affinity purification of polyclonal antibodies and immunoblotting

Polyclonal anti-canine ribophorin II antibodies were affinity purified on rat liver ribophorin I that had been transferred to nitrocellulose according to the method of Smith and Fisher (1984). In this way, antibodies were immunoselected for the recognition of cross-homologous epitopes. Immunoblots were performed on samples of canine pancreatic (or other) microsomes that had been separated on gradient (10–15%) polyacrylamide gels and transferred to nitrocellulose filters. Anti-ribophorin antibodies were visualized with the appropriate second antibody coupled to peroxidase using diaminobenzidine as the substrate.

Protease digestion of rough microsomes

Canine pancreatic microsomes prepared as described (Shields and Blobel, 1977) were incubated with proteases (200 $\mu\text{g/ml}$) at 0°C for 1 h. The appropriate inhibitors were used to stop the reaction, and samples were loaded onto polyacrylamide gels (10–15%) for immunoblotting as described (Hortsch *et al.*, 1986).

Materials

Libraries in pEX were expressed in *Escherichia coli* strain K-12, libraries in NM 1149 in NM 514, and M13 in TG1. Proteases were purchased from the following sources: trypsin, elastase and proteinase K from Merck, Darmstadt; chymotrypsin, subtilisin and *Staphylococcus aureus* V8 from Sigma, St. Louis; and papain from Hoechst, Frankfurt. Radioisotopes were purchased from Amersham, UK.

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