Segregation of the Polypeptide Translocation Apparatus to Regions of the Endoplasmic Reticulum Containing Ribophorins and Ribosomes. II. Rat Liver Microsomal Subfractions Contain Equimolar Amounts of Ribophorins and Ribososmes

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ABSTRACT   Ribophorins I and II, two transmembrane glycoproteins characteristic of the rough endoplasmic reticulum (ER) are thought to be part of the translocation apparatus for proteins made on membrane bound polysomes. To study the stoichiometry between ribophorins and membrane-bound ribosomes we have determined the RNA and ribophorin content in rat liver microsomes or in microsomal subfractions of different density (i.e., ribosome content). The specificity of antibodies against the ribophorins was demonstrated by Western blot analysis of rat liver rough microsomes separated by 2-dimensional gel electrophoresis. The ribophorin content of microsomal subfractions was determined by indirect immunoprecipitation and for ribophorin I by a radioimmune assay. In the latter assay a molar ratio of ribophorin I/ribosomes approaching one was calculated for total microsomes as well as in the gradient subfractions. We therefore suggest that ribophorins mediate the binding of ribosomes to endoplasmic reticulum membranes or play a role in co-translational processes which depend on this binding, such as the insertion of nascent polypeptides into the membrane or their transfer into the cisternal lumen.

In the preceding paper we have shown that the capacity of microsomal membranes stripped of ribosomes to rebind in vitro inactive 80S ribosomes correlates well with the RNA content of the microsomal subfractions from which these membranes were derived (2). The capacity of the membranes to effect translocation of nascent polypeptides in vitro also showed a strong correlation with the ribosome content of the microsomal subfractions although only a small fraction of the translocation sites associated with the ribosomes was active in these in vitro assays. These observations suggested that elements of the apparatus necessary for the vectorial discharge of polypeptides synthesized in bound ribosomes are not distributed uniformly in the endoplasmic reticulum (ER),[1] but

[1] Abbreviations used in this paper: ER, endoplasmic reticulum; RIA, radioimmunoassay; RM, rough microsomes; SRP, signal recognition particle.

indeed are segregated to the rough portions of this organelle (10, 14, 25).

Biochemical and electrophoretic analyses have revealed the existence of protein compositional differences between rough and smooth microsomes which appear to be related to the function of the rough ER in protein synthesis (10, 11 14, 23). Two transmembrane glycoproteins (ribophorin I, M_r 65,000 and ribophorin II, M_r 63,000) have been shown to be characteristic of rough microsomes (RM) from several tissues of different species (18). These proteins are solubilized when microsomes are treated with ionic detergents but are quantitatively recovered in association with polysomes when neutral detergents are used to solubilize the microsomal membranes. These findings led to the suggestion (11,14) that ribophorins play a role in mediating the binding of ribosomes to ER membranes. Recently, a membrane protein of M_r 72,000 has

been identified in dog pancreas microsomes (8, 21, 33), which interacts with the signal recognition particle (SRP), associated with the signal peptide of nascent secretory and membrane polypeptide chains. The role of this protein appears to be in coordinating the elongation of newly synthesized polypeptide chains with their insertion into the membrane (29–32). It has been shown, however, that the amount of SRP receptor protein found in microsomes is much lower than the number of active ribosomes associated with the membranes (8).

In this paper we utilize monospecific antibodies against ribophorins I and II (18) for immunoprecipitation and radioimmune assays to study the distribution of the antigens in microsomal subfractions of different ribosome binding and translocation capacity. We demonstrate that there is a stoichiometric ratio between ribophorins and ribosomes close to one independently of the absolute ribosome load. These and previous results strongly support the notion that ribophorins are components of the translocation apparatus which remain associated with the active ribosomes throughout the process of vectorial discharge.

MATERIALS AND METHODS

Materials: Materials were obtained from the following sources: Bolton Hunter reagent (2,000 Ci/mmol) was purchased from New England Nuclear (Boston, MA), Ampholines was from LKB (Rockville, MD), and affinity-purified rabbit anti-goat IgG was from Cappel Laboratories (Westchester, PA); nitrocellulose filters (type BA85) were purchased from Schleicher and Schuell (Giessen, Federal Republic of Germany), BSA (fraction V) was from Sigma Chemical Co. (St. Louis, MO), X-ray films (XR-5) were from Kodak, Inc. (Rochester, NY), and Emulgen 913 was from Kao Atlas (Tokyo, Japan).

The cell fractionation procedures and methods for the determination of the reference constituents in microsomes are described in the preceding paper (2).

Purification of Ribophorins: Ribophorins I and II were purified by SDS PAGE and antibodies against these proteins were raised in goats as described previously (12, 18). In some experiments, ribophorin I was electroeluted from the stained gels (15). After dialysis against distilled H₂O, the protein was precipitated by adding 9 vol of ice-cold acetone and incubated at −20°C overnight. The precipitate was recovered by centrifugation (10 min, 5,000 rpm, Sorvall HB-4 rotor) and resolubilized by suspension in 2% SDS. Protein was measured (16) using BSA as a standard.

Gel Electrophoresis: Discontinuous SDS PAGE was carried out essentially as described by Maizel (17) using an apparatus similar to that described by Studier (27). For details see also Kreibich and Sabatini (13). For immunoblotting from gels proteins were transferred to nitrocellulose filters by electrophoresis (Hoefer Scientific Instrument, San Francisco, CA) at 0.2 A overnight according to Burnette (6). After transfer to the proteins, filters were incubated for 60 min at 37°C in PBS containing 5% BSA, then transferred to a fresh solution of PBS with 5% BSA and 0.1% Triton X-100 (AB buffer), to which specific antibodies were added (final dilution of 1:200). The filters were incubated overnight at 4°C in a rocking platform, then washed five times in 100 ml PBS containing 0.5% BSA and 0.1% Triton X-100. Bound IgG was identified by incubation in the same buffer for 2 h at 25°C, or overnight at 4°C, with ¹²⁵I-labeled affinity purified rabbit anti-goat IgG (200,000–500,000 cpm/ml). The nitrocellulose filters were then washed for 2 h in multiple changes of 500 mM NaCl, 10 mM NaHPO₄ pH 7.5, 0.1% Triton X-100 (2 liters total) (17). They were then air dried and exposed to X-ray film at −70°C using a Cronex intensifying screen (Dupont Photo Products, Wilmington, DE).

Two-Dimensional Electrophoresis of Rough Microsomal Proteins: This was performed using a modification (28) of the original O'Farrell technique (1975; reference 22). RM (50 μg protein) were sedimented (airfuge rotor 30, 135,000 g, 5 min, 4°C, Beckman Instruments, NJ), resuspended in lysis buffer (22) and electrophoresed in an isoelectric focusing tube gel (600 V/h). Afterwards, the tubes were loaded on SDS polyacrylamide gradient (6–11%) slab gels and electrophoresed overnight. The gels were silver stained by the method of Wray et al. (1981; reference 34) or used for immunoblotting to nitrocellulose filters. pH gradients were measured in gel slices (1 cm) after overnight incubation at 37°C in 1.0 ml of H₂O.

Immunoprecipitation of ¹²⁵I-labeled Rough Microsomes: Microsomes and microsomal subfractions were dialyzed against 0.25 M sucrose, 0.25 M Na borate pH 8.5, and iodinated using Bolton-Hunter reagent (4). This

was evaporated to dryness from anhydrous benzene (250 μCi/sample) and the microsomal sample was then added to the tube (100 μg of protein) which was incubated for 2 h at 0°C. The incubation medium was then diluted to 50 μl with 0.2 M Na borate pH 8.5, and layered over a 100-μl cushion of 0.5 M sucrose, 0.2 M Na borate pH 8.5 in a centrifuge tube. The labeled membranes were recovered by centrifugation (airfuge rotor 30 lbs/in², 5 min, 4°C), resuspended in 2% SDS, heated for 5 min at 100°C. Different microsomal subfractions incorporated similar amounts of ¹²⁵I (~7.5%). Aliquots containing 10⁶ cpm (corresponding to 1–2 μg protein), were diluted with 4 vol of 2.5% Triton X-100 and incubated overnight at 4°C with anti-ribophorins I or II, followed by Protein A Sepharose for 2 h at 25°C. Immunoprecipitates were analyzed using SDS polyacrylamide gradient (6–11%) gels and the labeled bands were identified by autoradiography of the dried gels using a Cronex intensifying screen. The radioactivity contained in the excised gel fraction was determined in a gamma counter.

RESULTS

Electrophoretic Analysis of Microsomal Subfractions

Rat liver microsomal subfractions of different density and ribosome content were obtained as described in the preceding paper (2), and equal amounts of membrane protein were analyzed by SDS gel electrophoresis (Fig. 1A). It is apparent that subfractions with the higher density have also higher concentrations of ribosomal proteins corresponding to some of the intensely stained bands below M_r 50,000 (Fig. 1A). The same microsomal subfractions showed prominent Coomassie Blue-stained bands corresponding in electrophoretic mobility to ribophorins I and II.

It has been previously shown that extraction of microsomes with certain nonionic detergents yields a sedimentable fraction in which membrane-bound ribosomes as well as ribophorins are quantitatively recovered (14, 18). Electrophoretic analysis of supernatants (Fig. 1C) and pellets (Fig. 1B) of detergent-extracted microsomal subfractions showed that ribophorins I and II as well as the proteins corresponding to bands of M_r 72,000 and 83,000, were recovered exclusively in the sedimentable fractions (Fig. 1B). For these four proteins, the trend of decreasing staining intensity with decreasing isopycnic density and ribosome load of the microsomal subfractions was more apparent in the analysis of the pellets obtained after many other microsomal proteins were solubilized. The intensely stained band of M_r 34,000 found in subfraction 12 (Fig. 1, A and B) corresponds to urate oxidase in the form of peroxisomal cores which contaminate the densest subfractions (see also reference 14).

In the corresponding supernatants, no stained bands were observed that showed a similar trend with respect to ribosome content. It should be noted, however, that the staining intensity of a band corresponding to rat serum albumin (as demonstrated by immunoprecipitation, data not shown) increased in inverse proportion to the ribosome content of the microsomal subfractions.

Characterization of Anti-ribophorin Antibodies

The specificity of goat antibodies raised against material extracted from bands corresponding to ribophorins I and II (18) was examined by immunoblotting of 2-dimensional gels of RM. The large variety of polypeptides found in total rough microsomes was reflected in numerous spots found in 2-dimensional gels (Fig. 2A). Autoradiographs showing the distribution of ¹²⁵I-labeled rabbit anti-goat IgG applied after immunoblotting demonstrated that anti-ribophorin I antibody reacted with a single spot (M_r 65,000) migrating at a pH
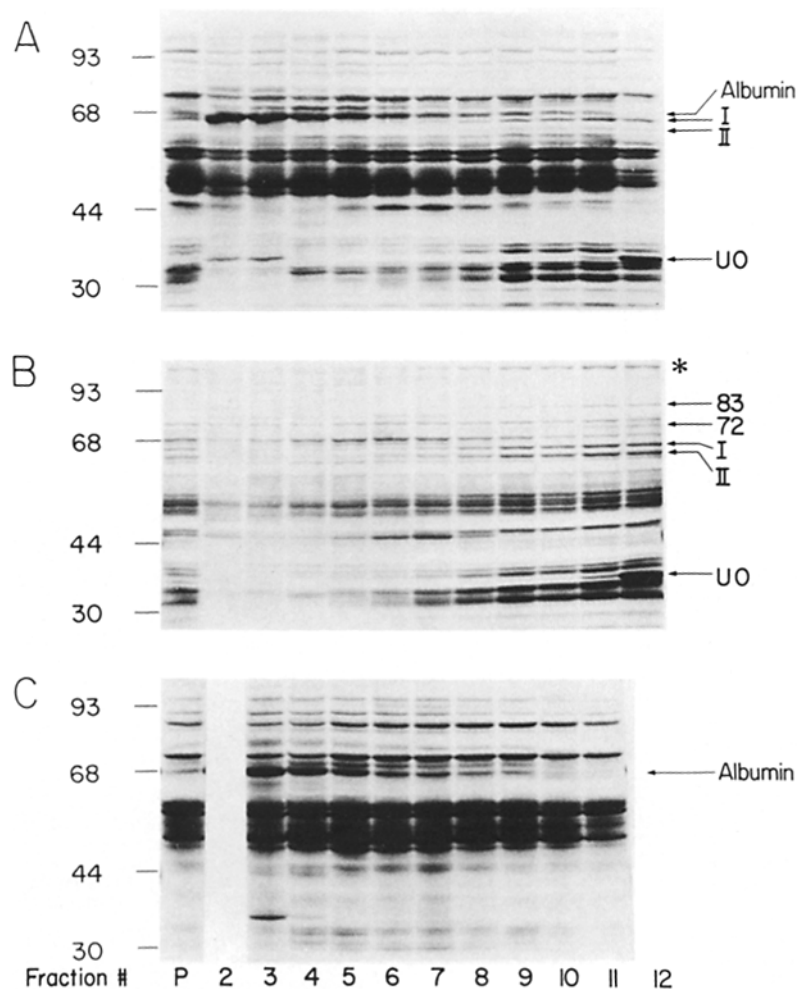
FIGURE 1 Electrophoretic analysis of microsomal subfractions. Microsomal subfractions were suspended in a low salt buffer (LSB) (50 mM KCl, 50 mM Tris-HCl [pH 7.2], 3 mg/ml membrane protein; corrections were made for the protein content of membrane-bound ribosomes) and 50-μl aliquots (containing 150 μg protein) were then directly analyzed (A) on a SDS polyacrylamide gradient (6–11%) slab gel. Alternatively, 16 μl of Emulgen 913 (15%) were added to 150-μl aliquots of the same suspension and supernatant and pellets were obtained after centrifugation for 10 min in a Beckman airfuge. 150 μl of gel sample buffer were added to both the pellets resuspended in 150 μl LSB (B) and supernatants (C). 50-μl aliquots were loaded onto a slab gel (6–11% polyacrylamide). Gels were stained with Coomassie Blue. Bands corresponding to serum albumin and ribophorins I and II, as well as the polypeptides of M_r 72,000 and 83,000, are indicated by arrows. The asterisk marks an unidentified Coomassie Blue-stained band which increases in intensity in the denser subfractions. UO indicates urate oxidase which contaminates the subfractions 11 and 12 in the form of peroxisome cores. A band seen in subfractions 4–8 (B) corresponds in electrophoretic mobility to actin. Fraction P denotes total microsomes before subfraction.
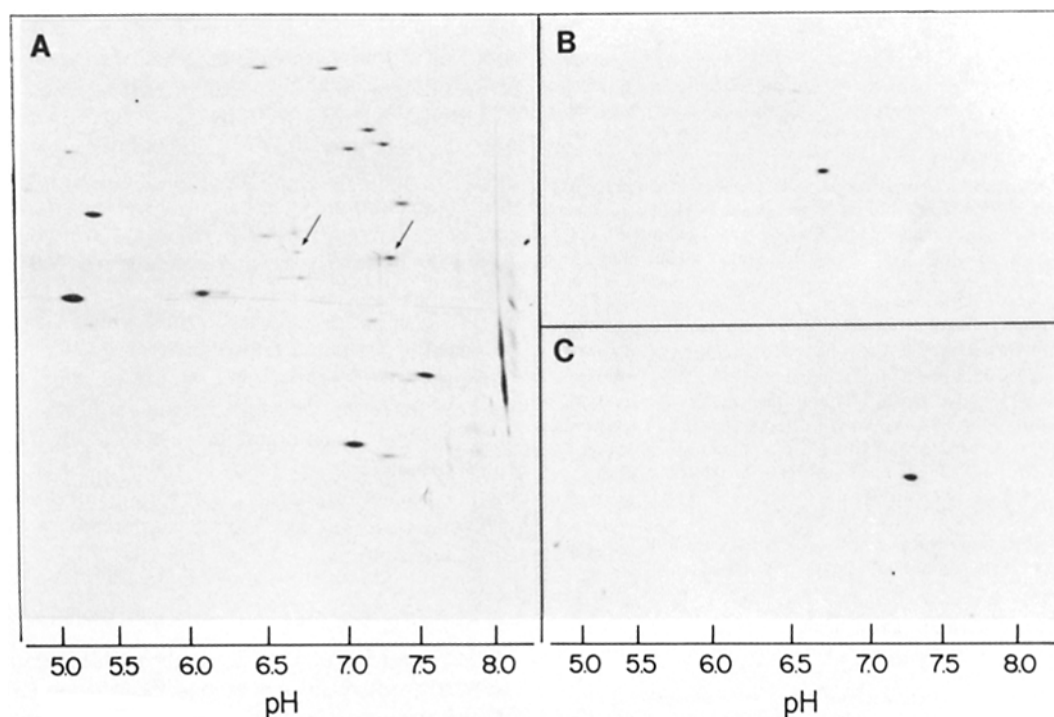
FIGURE 2 Characterization of anti-ribophorin IgG by immunoblotting of 2-dimensional-gels of RM. RM (50 μg protein of subfraction 10; see lower right of Fig. 1 in preceding article) (2) were separated by isoelectric focusing in polyacrylamide tube gels (see Materials and Methods). For analysis in the second dimension, the gel was placed on top of an SDS polyacrylamide slab gel. After electrophoresis, the gels were either silver-stained (A) or transferred to nitrocellulose filters for immunoblotting with anti-ribophorin I (B) or II (C) IgG. After washing, the bound IgG was visualized by incubation with ^{125}I-labeled rabbit anti-goat IgG, followed by autoradiography. The pH gradient is plotted on the abscissa. The arrows in A indicate the spots corresponding to ribophorins I and II.
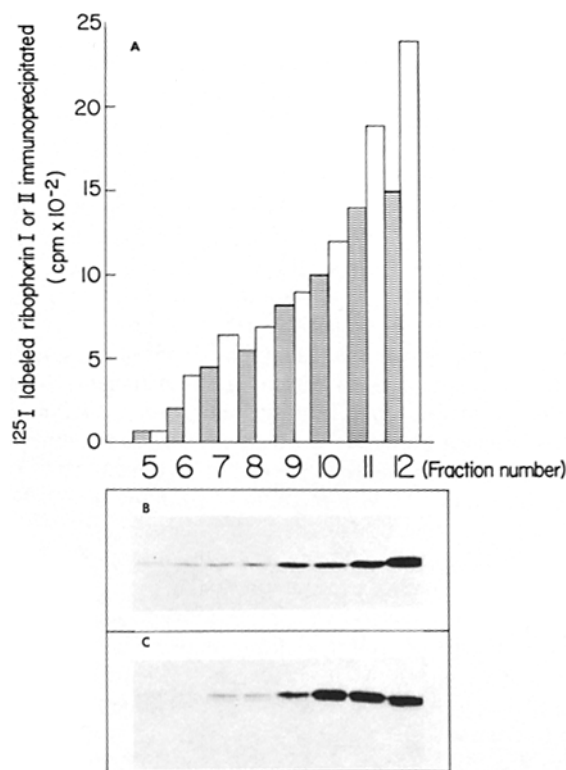
FIGURE 3 Immunoprecipitation of ribophorin I and II from ^{125}I-labeled microsomal subfractions. Microsomal subfractions 5–12 labeled with [^{125}I] (10^6 cpm; 1 µg protein) were incubated with 300 µg of either anti-ribophorin I or anti-ribophorin II IgG. Immunoprecipitates recovered after incubation with protein A-Sepharose were analyzed by SDS PAGE, followed by autoradiography. B and C show the autoradiographs of immunoprecipitates obtained using anti-ribophorin I (B) or anti-ribophorin II (C). The labeled bands were excised and the amounts of radioactivity was determined. The amounts of ^{125}I-labeled ribophorin I (empty columns), and II (shaded columns) recovered from each subfraction are shown in A.

of 6.7 (Fig. 2B), whereas anti-ribophorin II antibodies labeled a polypeptide (M_r 63,000) corresponding to a pH of 7.2 (Fig. 2C). The demonstration that both antibodies are monospecific makes them useful for the quantitation of ribophorins I and II using a radioimmune assay or by immunoprecipitation. Only the latter procedure was useful to measure the ribophorin II content, since this polypeptide was degraded to various degrees during purification involving extraction from SDS gels. Highly purified antigen is, however, a prerequisite for establishing a radioimmune assay standard curve.

Immunoprecipitation of Ribophorins I and II from ^{125}I-labeled Microsomal Subfractions

Microsomal subfractions iodinated with Bolton-Hunter reagent were used for immunoprecipitation and the ribophorin content of immunoprecipitates was determined by measuring the radioactivity in the excised labeled bands. The amount of antibodies required for maximal immunoprecipitation was established by incubating aliquots of a SDS-solubilized iodinated subfraction of high ribosome content (subfraction 11; see preceding paper), with increasing amounts of anti-ribophorin I IgG. Appropriate quantities of this antibody were used for immunoprecipitation. Accordingly immunoprecipitation of ribophorins I and II from SDS-solubilized, ^{125}I-labeled subfractions was performed by adding 300 µg of the respective antibody to subfractions containing equal amounts

of radioactivity. The results of these measurements (Fig. 3) clearly demonstrate that the amount of both ribophorins I (empty columns) and II (shaded columns) steadily increased with the ribosome content of the subfractions. Furthermore, similar amounts of radioactivity were recovered in each subfraction for each ribophorins. Since the specific activity of each polypeptide could not be determined this suggests, but does not prove, that the two ribophorins are present in equimolar amounts. Further support for this contention comes from the observation that ribophorin I and II show similar intensities when Coomassie Blue (Fig. 1) or silver staining (not shown) was used on polyacrylamide gels.

Radioimmune Assay of Ribophorin I

A direct measurement of the stoichiometric relationship between ribophorins and ribosomes was obtained from a radioimmune assay (RIA). Optimal concentrations were established from a calibration curve (Fig. 4), using ^{125}I-labeled RM which, after solubilization in SDS were mixed with known protein amounts of purified ribophorin I. Sufficient protein from each fraction was added to the RIA to decrease the recovery to 25–35% of the maximal recovery. The content of ^{125}I-labeled ribophorin in immunoprecipitates obtained using protein A-Sepharose was determined by SDS PAGE and autoradiography followed by the measurement of the ^{125}I-radioactivity in the excised band (Fig. 4B). This method allowed us to measure ribophorin content in the range of 2–30 ng, for most experiments the range between 5–15 ng was used.

It was found that total microsomes contained 2.80 µg of ribophorin I per mg protein. Considering a RNA/protein
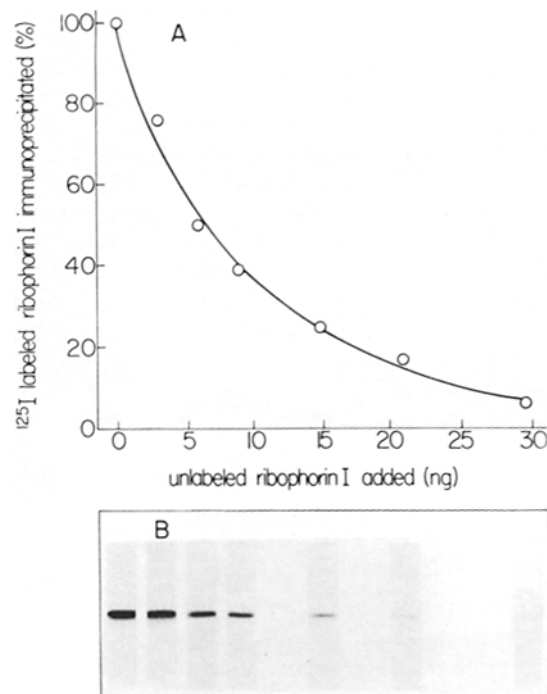

FIGURE 4 Standard curve for the ribophorin I radioimmune assay. Subfraction 11 was iodinated and aliquots containing 10^6 cpm were incubated with increasing amounts (2–29 ng) of purified ribophorin I. After addition of 75 µg of anti-ribophorin I IgG, immunoprecipitates were recovered using protein A-Sepharose. The immunoprecipitates were analyzed by SDS PAGE, followed by autoradiography (B). The radioactivity contained in the labeled bands was determined and plotted against the amounts of competing unlabeled ribophorin I added (A).

TABLE I

Density Distribution of Ribophorin I and Molar Ratio between Ribophorin I and Ribosomal RNA in Microsomal Subfractions

Subfraction no.[*]	RNA/protein	Ribophorin I/mb-protein[‡]	Molar ratio ribophorin I/ribosome[§]
	μg/mg	μg/mg	
5	23	0.81	1.51
6	32	1.12	1.52
7	60	1.41	1.02
8	99	2.26	0.99
9	176	3.53	0.87
10	205	3.47	0.73
11	248	4.51	0.78
12	234	3.92	0.72
Total microsomes	119	2.80	1.06

To the RIA for ribophorin I, (for details see legend to Fig. 4) 3–15 μg of subfractions 5–12 or total microsomes were added. The amounts of microsomal protein were chosen such that ~30% of the ^{125}I-labeled ribophorin was contained in the immunoprecipitate. The amount of ribophorin I contained in this competing amount of microsomes was determined using the standard curve shown in Fig. 4 which allowed us to establish the amount of ribophorin I per microgram of microsomal protein.
[*] The numbers refer to the microsomal subfractions defined in Fig. 1 of the preceding paper (2).
[‡] The amount of ribophorin I was determined using RIA (see also Fig. 4). The amount of membrane protein was obtained after correcting the content of ribosomal protein in the subfractions.
[§] The molar ratios were calculated assuming an M_r of 65,000 for ribophorin I and 2.35 × 10^6 for the ribosomal RNA. The raw data were corrected taking into consideration the recoveries of ribophorin I (83%) and RNA (100%) in the gradient.

ratio of 0.119 the molar ratio ribophorin I/ribosome was then determined to be 1.06. Since amounts of purified ribophorin I used to establish the standard RIA curve were determined by the Lowry procedure, using BSA as a standard, the actual molar ratio may well be even closer to one. An absolute determination of this ratio must be made with the knowledge of the amino acid composition of ribophorin I. In a similar fashion, the ribophorin I content in microsomal subfractions was established (Table I). Subfractions of intermediate density (7, 8) had ratios close to 1. The largest deviations from the mean value obtained for total microsomes were found in the lightest and heaviest subfractions. They were somewhat below one for subfractions 9–12. Molar ratios of 1.5 for the low density fractions (5 and 6) are, however, less reliable since the very low content of RNA makes the exact determination of this ratio rather difficult. A presentation of the normalized RNA and ribophorin content as a frequency distribution shows the good correlation between these two values (Fig. 5).

DISCUSSION

In the preceding paper (2), we used rat liver microsomes subfractionated by density gradient centrifugation to study the distribution of functions related to the vectorial transfer of polypeptides across the membrane in the rough and smooth domains of the ER. In an effort to identify the protein components of the translocation apparatus present in the ER, we have compared the electrophoretic patterns of the microsomal subfractions and correlated the presence of certain proteins with the respective ribosome content. A careful examination revealed that at least four membrane proteins have distributions similar to that of ribosomal proteins. In addition to the ribophorins I and II, a protein of M_r 72,000, was also present in higher concentrations in subfractions of higher

ribosome content. This component has the same M_r as the SRP receptor protein identified in dog pancreas RM (8, 21, 33). Since the SRP receptor has been characterized only in microsomes derived from dog pancreas, an organ that lacks a well developed smooth ER, a study of its distribution in the rough and smooth ER has not yet been possible. If the polypeptide of M_r 72,000 identified in these gels indeed corresponds to the rat liver SRP receptor, our results would indicate that it is also segregated to the rough portions of the ER. As is the case of the ribophorins, this protein was quantitatively recovered in a sedimentable fraction obtained after treatment of microsomes with nonionic detergents. This behavior is analogous to that of the dog pancreas SRP receptor which has also been recovered in a pellet sedimented after treatment with the nonionic detergent Triton X-100 (19, 20). The observation that a membrane protein of M_r 83,000 showed a similar behavior reinforces the previous identification of this component as characteristic of RM (9, 26). Thus it appears likely that also this protein is part of a complex which is not dissociated by the nonionic detergent containing in addition ribophorins and the putative SRP receptor. It cannot be excluded, however, that these ER membrane proteins share physical properties that renders them insoluble in certain nonionic detergents.

It is presently thought that initial steps in the translocation process are mediated by the SRP, a complex that recognizes the signal peptide of a growing nascent chain (32). Since binding of SRP to its receptor appears to be only necessary to relieve the elongation arrest caused by SRP and facilitate the initial steps in translocation, the interaction need only be a
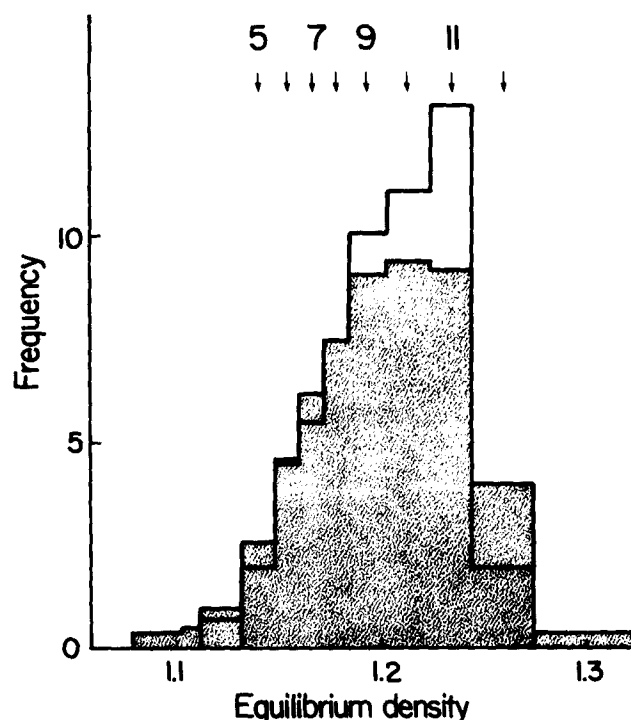

FIGURE 5 Comparison of the density patterns of ribophorin I and RNA in sucrose gradient. The density distribution curves were computed with the data given in Tables I and II. The subfraction numbers (arrows) are those introduced in Fig. 1 of the preceding paper (2). The shaded areas represent the ribosomal RNA distribution, while a solid line marks the distribution of ribophorin I. For details of the computations, see reference 3.

transient event in the vectorial discharge and therefore at any given time less than stoichiometric amounts of this protein with respect to the number of membrane-bound ribosomes may be found in microsomes (8).

Since a direct interaction between ribosomes and the underlying membranes is maintained throughout the translocation process (1, 5, 24, 25) at least the membrane components involved in this association should be present in microsomes in amounts stoichiometrically related to the number of membrane-bound ribosomes. This has now been proven using RIA. We found that the distribution of ribophorin I in microsomal subfractions of different ribosome loads closely followed that of the ribosomes and that for total microsomes and several microsomal subfractions the molar ratio between ribophorin I and ribosomes approached one.

Although we have not been able to carry out similar RIA determinations for the distribution of ribophorin II, indirect immunoprecipitation on ^{125}I-labeled microsomal subfractions showed that both ribophorins had parallel distribution curves, as would be expected if they were present in equimolar amounts. This and the quantitative recovery of this protein in a large complex obtained after extraction of RM with nonionic detergents suggests the existence of heterodimers of these two transmembrane polypeptides that define sites for ribosome attachment and/or translocation. Further work on the interaction of these proteins with the putative SRP receptor of liver microsomes and the 83-kd polypeptides is necessary for an understanding of the mechanism that effects the vectorial transfer.

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