Journal of Supramolecular Structure 8:279–302 (1978)

Characterization of the Ribosomal Binding Site in Rat Liver Rough Microsomes: Ribophorins I and II, Two Integral Membrane Proteins Related to Ribosome Binding

Gert Kreibich, Maria Czakó-Graham, Ruth Grebenau, Winnie Mok, Enrique Rodriguez-Boulan, and David D. Sabatini

Department of Cell Biology, New York University Medical Center, 550 First Avenue, New York, New York 10016

Rat liver rough endoplasmic reticulum membranes (ER) contain two characteristic transmembrane glycoproteins which have been designated ribophorins I and II and are absent from smooth ER membranes. These proteins (MW 65,000 and 63,000 respectively) are related to the binding sites for ribosomes, as suggested by the following findings: i) The ribophorin content of the rough ER membranes corresponds stoichiometrically to the number of bound ribosomes; ii) ribophorins are quantitatively recovered with the bound polysomes after most other ER membrane proteins are dissolved with the nonionic detergent Kyro EOB; iii) in intact rough microsomes ribophorins can be cross-linked chemically to the ribosomes and therefore are in close proximity to them.

Treatment of rough microsomes with a low Triton X-100 concentration leads to the lateral displacement of ribosomes on the microsomal surface and to the formation of aggregates of bound ribosomes in areas of membranes which frequently invaginate into the microsomal lumen. Subfractionation of Triton-treated microsomes containing invaginations led to the recovery of smooth and "rough-inverted" vesicles. Ribophorins were present only in the latter fraction, indicating that both proteins are displaced together with the ribosomes when these aggregate without detaching. Measurements of the ribosome-binding capacity of rough and smooth microsomal membranes reconstituted after solubilization with detergents sugest that ribophorins are necessary for in vitro ribosome binding. Ribophorin-like proteins were found in rough microsomes obtained from secretory tissues of several animal species. The two proteins present in rat lacrimal gland microsomes have the same mobility as hepatocyte ribophorins and cross-react with antisera against them.

Key words: rat liver endoplasmic reticulum, rough microsomes, membrane-bound polysomes, ribosome-binding sites

Received for publication December 22, 1977.

0091-7419/78/0803-0279$04.10

In parenchymal cells of the liver and of other organs engaged in the synthesis of secretory proteins, a large proportion of the ribosome population is found attached to membranes of the endoplasmic reticulum (ER) cisternae. In these specialized cells, membrane-bound ribosomes are frequently topographically segregated to specific regions of the cytoplasm which contain characteristic stacks of flat rough cisternae (Fig 1). In the hepatocyte the smooth endoplasmic reticulum (SER) consists of a system of highly convoluted tubules which permeate large portions of the cytoplasm and varies in its extension with the physiological state. Electron microscopy studies have provided numerous examples of continuities between rough and smooth ER membranes indicating that at the periphery of the stacks of rough cisternae the luminae of both systems become confluent. Biochemical studies with rat liver microsomal fractions have shown that aside from the presence of ribosomes, rough and smooth ER membranes of hepatocytes have similar protein and phospholipid compositions, sharing many enzymatic markers and biochemical functions [1–3]. Both types of ER membranes are also thought to be related biogenetically; regions of continuity allow for the direct flow of components between the rough and smooth compartments, ensuring the distribution of newly synthesized proteins released from bound ribosomes [4]. It is not yet known, however, how in spite of the fluidity of ER membranes [5] the segregation of bound ribosomes to the rough cisternae is maintained. The functional differentiation which arises from the presence of ribosomes

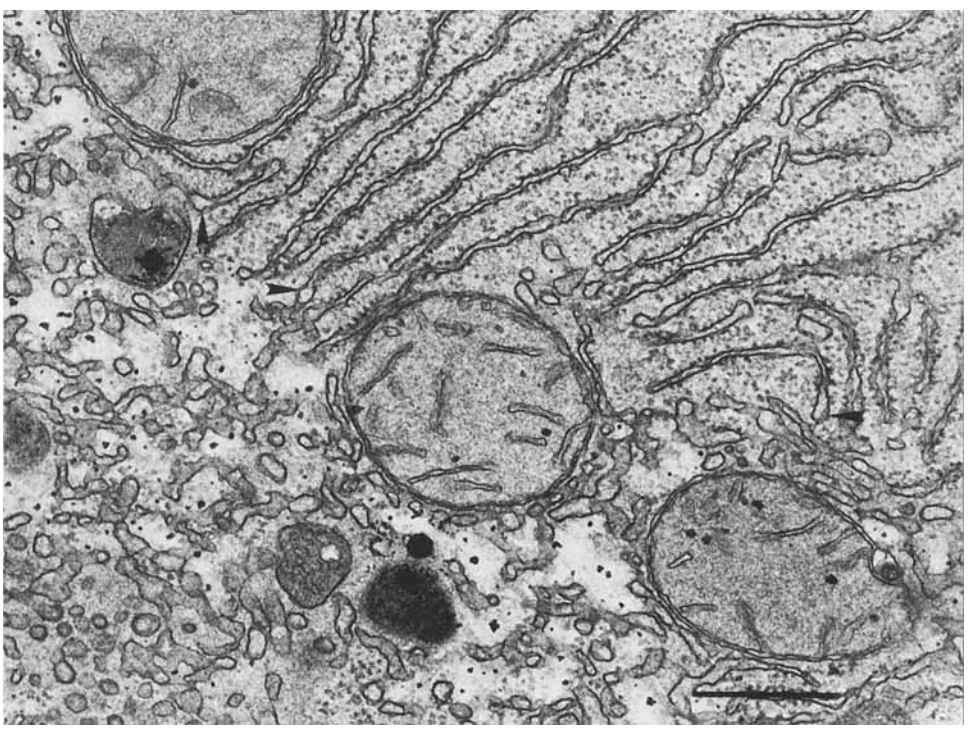

Fig 1. Transition between rough and smooth ER membranes in a hepatocyte from a male rat (150 g) starved for 18 h. Liver tissue was fixed at 4°C in 2% glutaraldehyde (2.5 h) and 1% OsO_4 (2 h) and stained in block with 0.5% uranyl acetate (15 min). The different morphologic appearance of the two portions of the ER is apparent. While RER membranes adopt a planar configuration showing flat stacked cisternae, smooth membranes (lower left) are arranged in contorted tubules. Arrowheads point to transitions between the two continuous membrane systems (41,000 ×).

is an important feature of subcellular organization in eucaryotic cells, which establishes the polarity necessary for the ordered sequence of postranslational steps involved in the production of secretory and membrane proteins.

IN VITRO RIBOSOME BINDING TO MICROSOMAL MEMBRANES

In vitro assays using radioactively labeled ribosomes have been used extensively to demonstrate the presence of specific sites for ribosome binding in membranes derived from the rough endoplasmic reticulum (RER) [6] (for review see Rolleston [7] and McIntosh and O'Toole [8]). These assays, in which ribosomes and membranes are mixed in a medium of physiological ionic strength, have shown that membranes of rough microsomes (RM) from which ribosomes have been removed (RMstr) by stripping procedures employing high salt and puromycin [9] become capable of accepting unprogrammed monomeric ribosomes to levels of saturation which approach the ribosome content of the original microsomes (see Figs 2 and 3). On the other hand, RM not treated for stripping have a negligible capacity to bind ribosomes specifically, which indicates that under normal conditions the ribosome-binding sites are fully occupied.

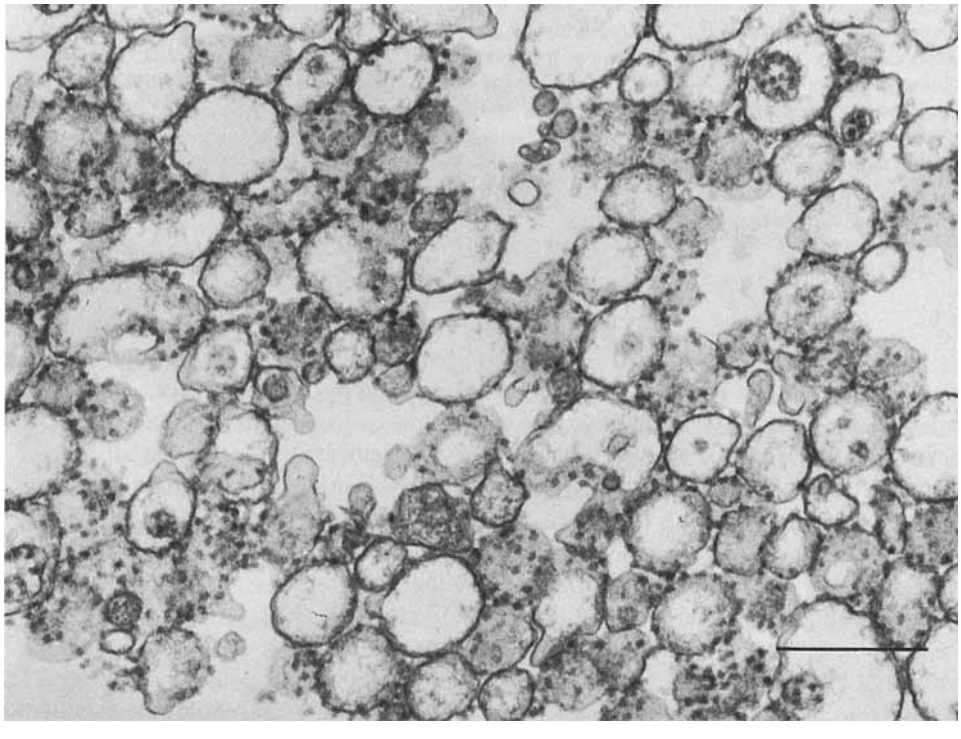

Fig 2. Microsomal vesicles bearing ribosomes rebound in vitro to binding sites exposed by previous removal of native ribosomes from RM. Ribosomes were detached from rough microsomes by incubation with puromycin-KCl [9]. The stripped vesicles (0.3 mg protein) recovered by centrifugation were incubated for 30 min at 0°C with 80 S ribosomes (2 OD units at 260 nm) in a medium containing 100 mM KCl, 50 mM Tris-HCl (pH 7.4), and 5 mM $MgCl_2$. Membrane vesicles with the rebound ribosomes were separated from unbound ribosomes by floatation (see also Fig 3), and the isolated membranes were fixed with glutaraldehyde (2%). After sedimentation (10 min at 8,000g) the pellet was postfixed with OsO_4, and processed for electron microscopy (63,000 ×).

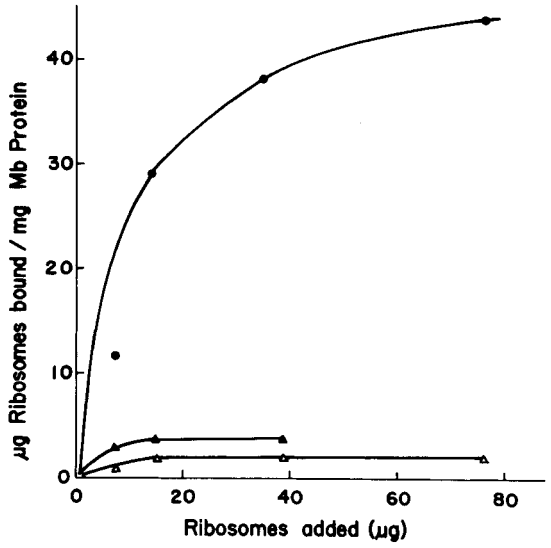

Fig 3. Specific binding of 80 S ribosomes to membranes derived from the rough endoplasmic reticulum. Rough and smooth microsomes prepared from rats which revealed phenobarbital (10 mg/100 g daily for 3 days, IP) were treated for stripping with puromycin-KC1. Membranes (0.3 mg) resuspended in 100 mM KC1, 50 mM Tris-HC1, 5 mM $MgCl_2$ were mixed with different amounts of ^{3}H-80 S ribosomes and were incubated at 0°C (final volume 0.12 ml). After 30 min 1.08 ml of 2,3 M sucrose was added and 0.8-ml aliquots were underlayered in gradients of the same ionic composition (1.0–1.9 M sucrose for SM and SMstr, 1.3 M–1.9 M sucrose for RMstr). Membranes with bound ribosomes were separated by floatation during centrifugation at 50,000 rpm in a SW56 rotor, for 60 min. The amount of tritium radioactivity in the bound ribosomes was measured and used to calculate micrograms of ribosomes bound per milligram of membrane protein (for detailed procedure see Borgese et al [6]). ●—●: RMstr; ▲—▲: SMstr; △—△: SM. Note that only RM show a significant number of binding sites which can be exposed by stripping.

Several investigators have measured the ribosome-binding capacity of smooth microsomes (SM) and shown that the number of binding sites in these fractions is significantly smaller than in RM treated for stripping but is not negligible [6]. It should be noted, however, that SM prepared by conventional procedures contain, in addition to membranes derived from the SER, fragments of other subcellular membranes of similar isopycnic density. It is also known that the composition of the smooth microsome fraction varies substantially with the cell fractionation procedure used and with the pretreatment of the animals (eg, starvation or administration of drugs). Actual levels of ribosome saturation with smooth microsome fractions are therefore variable, and the contribution of membranes derived from the SER is difficult to assess. Since plasma membranes, Golgi membranes, and mitochondrial outer membranes have a negligible capacity for ribosome binding [10], two main alternatives should be considered in interpreting the finding of binding sites in smooth microsome fractions: a) These sites are contributed exclusively by membranes derived from portions of the RER, which at the moment of fractionation bear no ribosomes and contaminate the smooth fractions; b) binding sites are indigenous to the SER and are uniformly distributed over the smooth membranes, although probably with a lower density than in the RER. In an attempt to resolve the question posed by these alternatives, we measured the binding capacity of smooth microsomes prepared

from rats treated with phenobarbital (PB) (Fig 3). This drug induces an extensive proliferation of the SER, which allows the preparation of fractions of SM with a well-defined subcellular origin. We found that these membranes from PB-treated rats were almost completely devoid of binding sites (Fig 3, △——△), although in rough microsomes from the same animals binding sites were present with a normal density (Fig 3, ●——●). Thus, our observations suggest the existence of mechanisms which maintain the segregation of ribosome-binding sites in spite of the continuity of rough and smooth endoplasmic reticulum membranes.

The demonstration that binding sites for ribosomes are restricted to only some portions of the ER led us to search for compositional differences between rough and smooth microsomes, which should reflect the presence of constituents of the sites in rough membranes. Previous studies demonstrating that the binding capacity of rough microsomes stripped of ribosomes is sensitive to heat treatment and to proteolysis ([6]; for review see McIntosh and O'Toole [8]) had suggested that membrane proteins are components of the binding sites.

IDENTIFICATION OF MEMBRANE PROTEINS CHARACTERISTIC OF ROUGH MICROSOMAL MEMBRANES

A comparison of high-resolution SDS acrylamide gels (Fig 4) from rough and smooth microsomes reveals that membranes of RM (Fig 4a) contain two proteins (molecular weight 65,000 and 63,000, arrows in Fig 4a, b) which are absent from smooth microsomes (Fig 4c) [11–14]. This compositional difference becomes most clearly apparent when rough microsomal membranes stripped of ribosomes (Fig 4b) are compared with the membranes of smooth microsomes, that is, when ribosomal proteins are removed. Several observations indicate that the two proteins present exclusively in rough microsomes are integral components of the ER membranes: 1) They are not released by treatment with low-detergent concentrations, as are proteins of the luminal content of the microsomes [15]; 2) the proteins remain associated with the microsomes when these are incubated in media of high salt concentration, in acid (pH 4) or alkaline (pH 9) media or with EDTA, conditions which remove peripheral membrane proteins [16].

Figure 4 shows that in addition to the proteins of MW 65,000 and 63,000 a protein of approximately 34,000 daltons is also present in rough but not in smooth microsomal fractions (compare gels a and c in Fig 4). In collaboration with Mr E. Nack we have demonstrated, however, that the presence of the latter protein represents contamination of the rough microsomes with urate oxidase, an enzyme contained in peroxisomal cores which cosediment with rough but not with smooth microsomes.

It is generally accepted that in addition to the nascent polypeptide chains, electrostatic interactions, which can be disrupted in media of high ionic strength or by anionic detergents, are involved in maintaining the direct binding of large ribosomal subunits to rough microsomal membranes [9, 17]. This suggested to us the possibility that by using nonionic detergents to dissolve the membranes it might be possible to isolate ribosomes still associated with membrane proteins of RM which are constituents of the binding sites. In an investigation which included a large series of detergents with different charges and hydrophobic-hydrophilic ratios, we found that treatment of RM with the nonionic detergent Kyro EOB allowed the recovery of the two proteins characteristic of RM in association with the sedimentable ribosomes, while most other membrane proteins

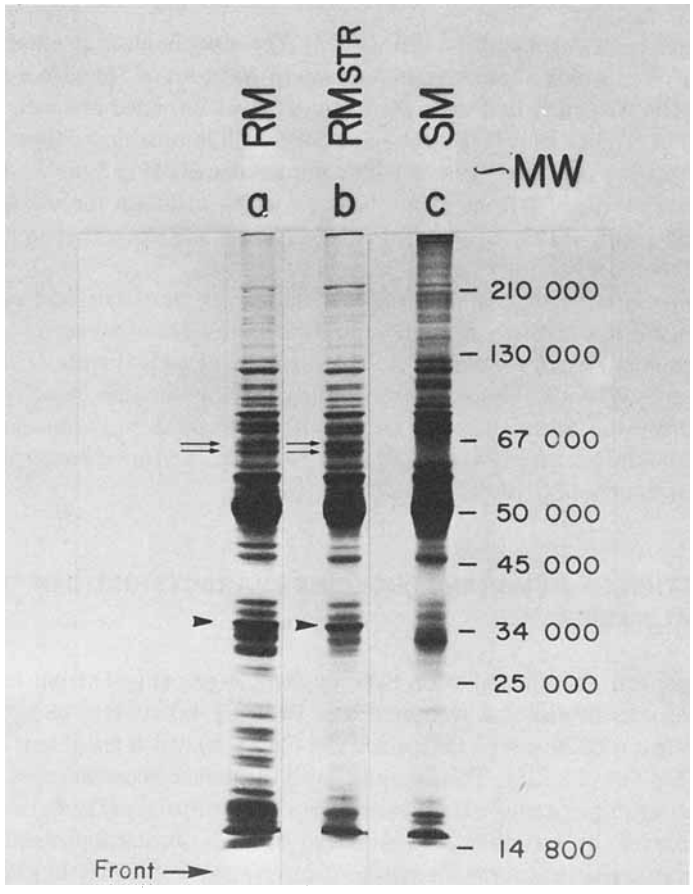

Fig 4. SDS acrylamide gel patterns of proteins in rough and smooth microsomal fractions. Freshly isolated RM (a) and SM prepared by floatation [35] (c) were washed by sedimentation in a low-salt buffer. A sample of RMstr (b) was prepared by removing ribosomes using the puromycin high-salt procedure [9]. Microsomal samples were analyzed by electrophoresis in SDS acrylamide gradient gels (7–12%) (for details see Kreibich and Sabatini [36]). Approximately 250 μg of protein from RM and 200 μg from RMstr and SM were loaded into the gel slots. Numbers on the right side indicate apparent molecular weights estimated from the mobility of marker proteins in a parallel track. Two proteins (marked by arrows; at MW 65,000 and 63,000; ribophorins I and II, respectively) are present in RM but not in SM. The position of urate oxidase in peroxysome cores which contaminate the RM fraction is indicated by an arrowhead (34,000 MW).

(approximately 80%) and phospholipids (approximately 90%) were solubilized. Electrophoretic analysis showed that the two proteins characteristic of RM are the only major microsomal proteins which are recovered completely with the ribosomes after treatment with Kyro EOB (Fig 5b). For this reason the proteins were designated ribophorins I and II (65,000 and 63,000 daltons, respectively). It should be noted that, as is shown in Figure 5a, both ribophorins are completely solubilized when RM membranes are dissolved with the anionic detergent deoxycholate (DOC), which is commonly used to purify membrane bound polysomes.

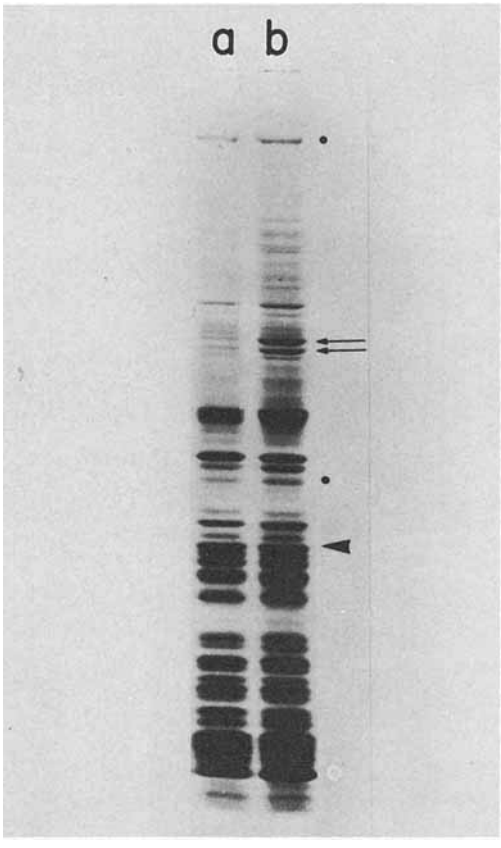

Fig 5. Ribophorins I and II are solubilized by DOC but remain associated with the ribosomes when rough microsomes are treated with the nonionic detergent Kyro EOB. RM (3.5 mg protein/ml) resuspended in LSB (low-salt buffer, 50 mM KCl, 50 mM Tris-HCl (pH 7.6), 5 mM MgCl) were incubated (30 min at 4°C) with a 2.5×10^{-2} M DOC (a) or Kyro EOB (b), and sedimentable fractions were recovered by centrifugation (60 min at 105,000g). Aliquots of resuspended fractions (derived from 350 μg of RM protein) were analyzed by electrophoresis in SDS acrylamide gradient gels (8–12%). The two proteins characteristic of RM (Ribophorin I and II, arrows in gel b) are recovered with the sedimentable ribosomes after Kyro EOB treatment. These proteins are not present in the ribosomal pellet obtained after DOC treatment (a) but are found in the supernate (not shown). Both sedimentable fractions contain the contaminating protein urate oxidase (arrowhead) and proteins with the mobilities of actin and myosin (dots in gel b).

STATE OF AGGREGATION OF RIBOSOMES AFTER EXTRACTION OF MEMBRANE PROTEINS WITH KYRO EOB

Because ribosome preparations obtained by differential centrifugation from RM treated with detergents may contain insoluble proteins which are not directly associated with the ribosomal particles, the association of ribophorins and ribosomes was also demonstrated by zone sedimentation analysis. Microsomes treated with Kyro EOB were

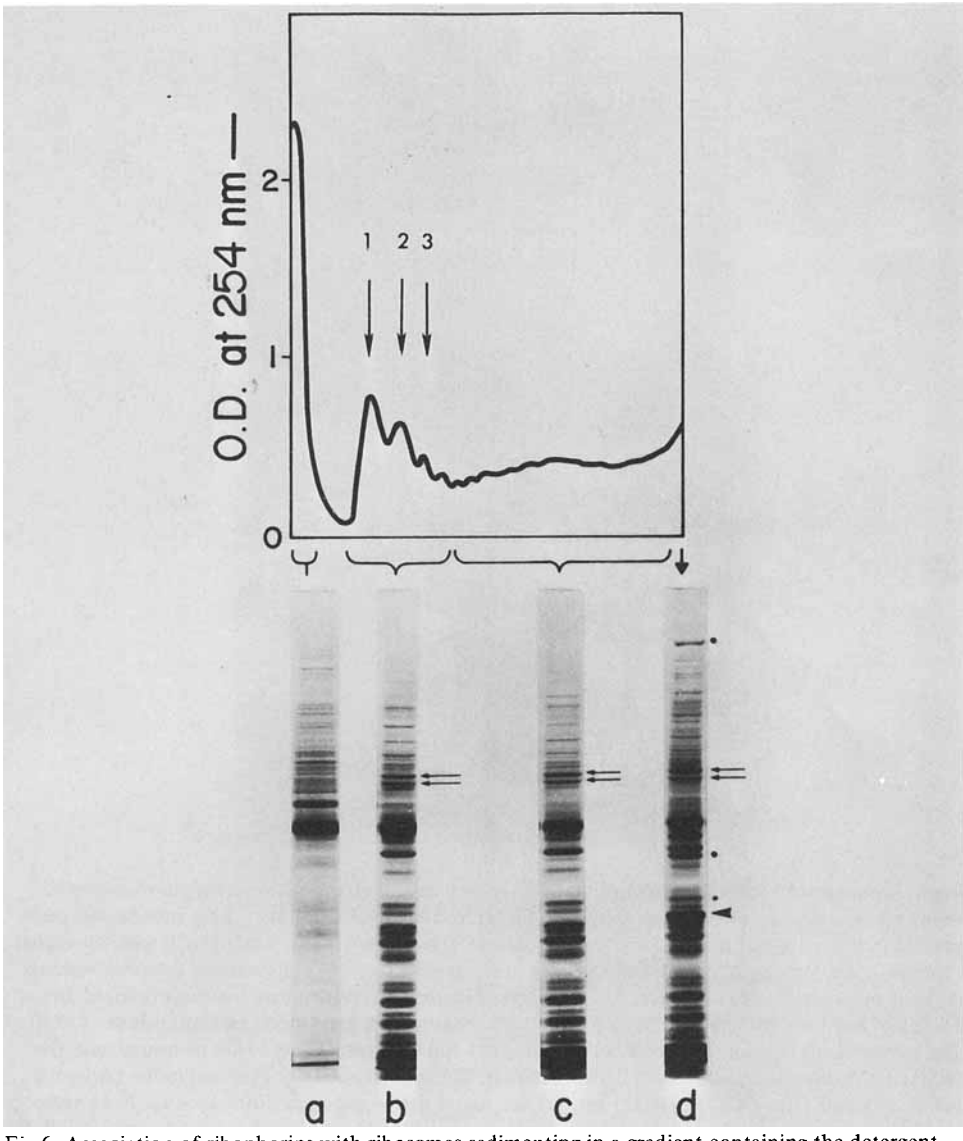

Fig 6. Association of ribophorins with ribosomes sedimenting in a gradient containing the detergent Kyro EOB. RM were resuspended in LSB (3.5 mg/ml) and incubated for 30 min at 4°C with Kyro EOB (2.5×10^{-2}M). A 0.5 ml sample was analyzed by centrifugation (60 min at 40,000 rpm in the Beckman SW41 rotor) in a 10–60% sucrose LSB gradient) containing 1.25×10^{-2} M Kyro EOB. The absorbance profile throughout the gradient was monitored at 254 nm. The position of monosomes (1), dimers (2), and trimers (3) is indicated by arrows in the upper panel. It should be noted that most of the ribosomes are found in larger aggregates which partially sediment to the bottom of the tube. Fractions from different regions of the gradient (a,b,c, indicated by brackets) were collected and pooled. Samples containing equal amounts of ribosomes (OD_{260} units) from fractions b and c and from the resuspended pellet (d) were analyzed by electrophoresis in SDS acrylamide gels (8–12%). The position of the ribophorins in gels from fractions b, c, and d is indicated by arrows. Proteins present only at the bottom of the tube are marked in the gel (d) by an arrowhead (urate oxidase) and dots (proteins with the electrophoretic mobility of myosin and actin). The OD_{254} profile (top) from the sucrose density gradient reveals the presence of ribosomal aggregates, resembling large polysomes. Densitometric tracings of the SDS gels were used to quantitate the amount of each ribophorin found in the ribosomal aggregates. A ribosomal protein marked by an asterisk in gel d was used as a reference to quantitate the number of ribosomes. It was found that aggregates contained less than two copies of each ribophorin per ribosome.

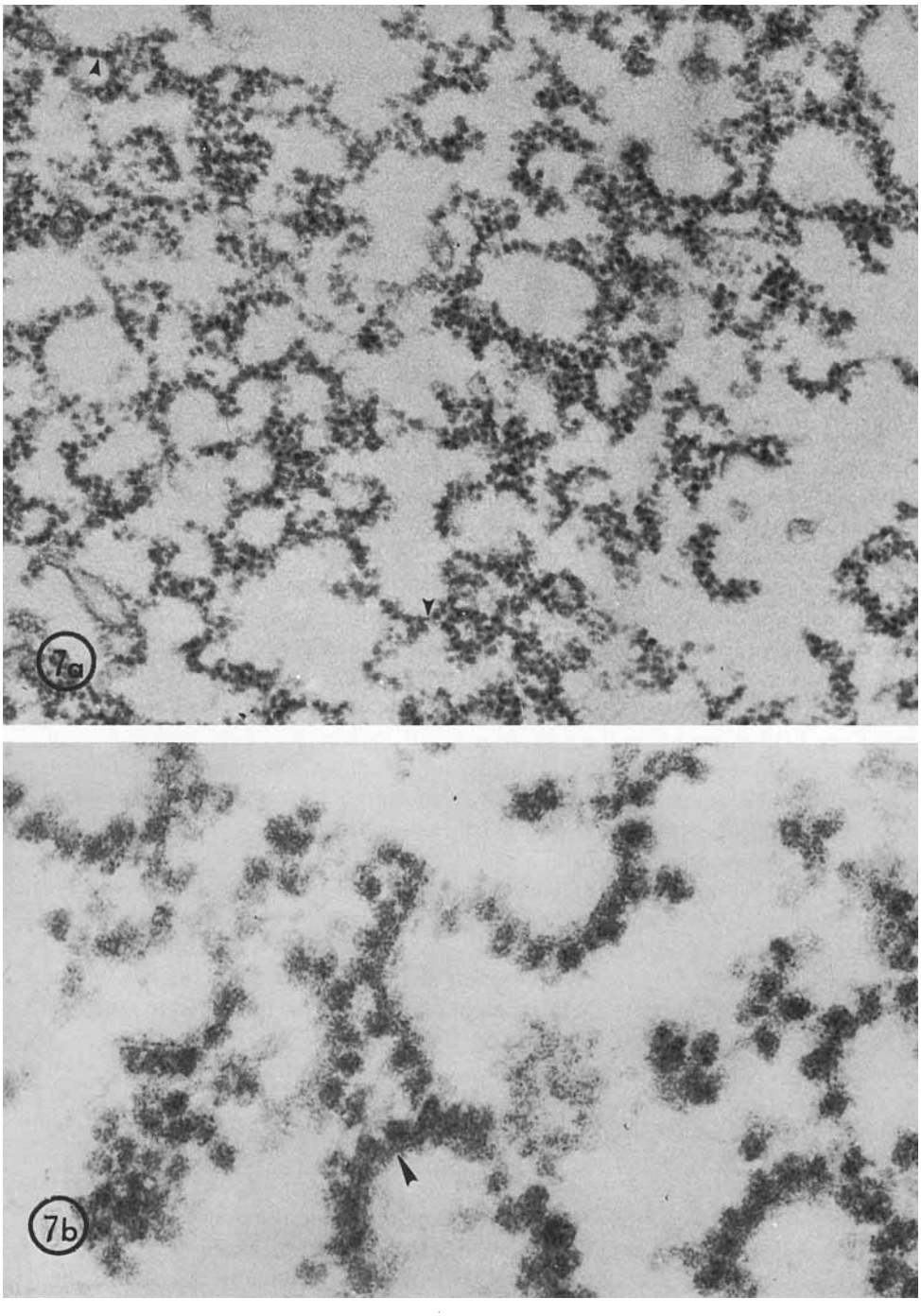

Fig 7. Electron microscopic appearance of sediments containing ribosomes and ribophorins obtained from RM treated with the nonionic detergent Kyro EOB. RM were treated with 2.5×10^{-2} M Kyro EOB in LSB. A sedimentable subfraction was obtained (see Fig 5), fixed in suspension with 1% glutaraldehyde, collected by filtration on a millipore filter [37] and postfixed with OsO_4. a) The thin-section electron micrograph shows that groups of tightly packed ribosomes remain attached to remnants of the microsomal membranes (arrows); magnification 50,000 ×. b) The same; magnification 265,000 ×.

sedimented in sucrose density gradients; fractions of the gradients, as well as of the sediments recovered at the bottom of the tubes, were analyzed by SDS acrylamide gel electrophoresis (gels a–d in Fig 6). These experiments demonstrated that in Kyro EOB-treated samples, ribophorins are present within large ribosomal aggregates, which produce sedimentation patterns resembling those of large polysomes (Fig 6). At difference with true polysomes, however, the ribosomal aggregates obtained by Kyro EOB treatment were not disrupted by treatment with RNase but were highly susceptible to incubation with proteases, DOC or media of high salt concentrations, which led to their disaggregation into short polysomes and ribosomes [13].

It was estimated from densitometric tracings of SDS acrylamide gels that the amount of each ribophorin present in the pseudopolysomal aggregates obtained from Kyro EOB-treated RM was related stoichiometrically to the number of ribosomes. Taking into account the molecular weight differences between ribosomal and microsomal membrane proteins, and assuming that the staining intensity of bands in gels is a measure of the amount of protein and that these two classes of proteins have similar specific staining intensities, it was calculated that there are approximately 1.4–1.9 molecules of each ribophorin per ribosome. The results in Figure 6 also show that other proteins are present in RM fractions which are not associated with the ribosomes but are poorly solubilized by Kyro EOB and therefore sediment to the bottom of the tubes during the sucrose density gradient sedimentation. The set of these proteins included urate oxidase, the contaminant present in peroxysomal cores, as well as two nonribosomal proteins which have been tentatively identified as myosin (approximate molecular weight 220,000) and actin (approximate molecular weight 42,000) (dots in Fig 6d).

Electron microscopic examination of sediments obtained after solubilization of RM with Kyro EOB showed that the structures which contain the ribophorins consist of groups of tightly packed ribosomes attached to the convex side of short (0.1–0.3 μ) curved membrane remnants (Fig 7a, b). These remnants consist of a layer of membrane material with an amorphous or frequently trilaminar appearance, reminiscent of that of the original ER membrane (Fig 7b).

We have also been able to isolate large complexes of the two ribophorins by cholate treatment of microsomes which had been previously stripped of ribosomes by incubation with puromycin-KC1 [13]. These findings suggest that in intact RM ribophorins may be arranged in an extended conformation forming an intramembranous network within the ER which helps to maintain the segregation of binding sites for ribosomes to rough areas of the endoplasmic reticulum.

CHEMICAL CROSS-LINKING OF RIBOPHORINS TO MEMBRANE-BOUND RIBOSOMES

To demonstrate that the association of ribophorins and ribosomes discussed in the preceding section does not result from an artifactual aggregation which occurs after other membrane components are extracted by Kyro EOB, we attempted to characterize the subset of membrane proteins which in intact rough microsomes is in close proximity to the ribosomes and therefore can be cross-linked to them by the reversible bifunctional reagent methyl-4-mercaptobutyrimidate (MMB) [18].

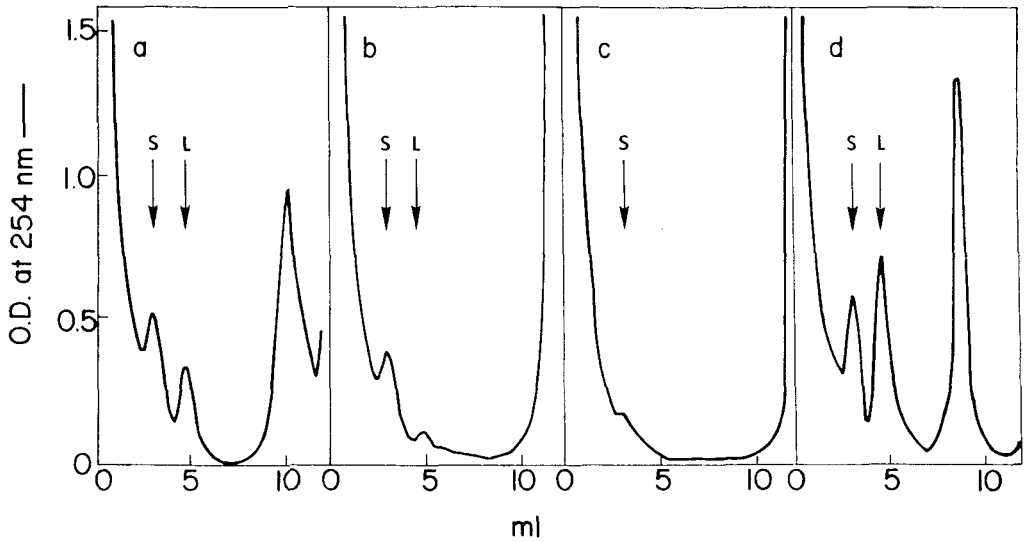

Fig 8. Reversible cross-linking of ribosomes to microsomal membranes with methyl-4-thiobutyrimidate (MMB). RM washed in high salt buffer (HSB) (500 mM KC1, 50 mM Tris-HC1 (pH 7.6), 5 mM $MgC1_2$) were suspended (3.5 mg protein/ml) in LSB (pH 7) containing Tris instead of triethanolamine (TEA) and incubated at 0°C with MMB: a) 0.04 MMB mg/ml; b) 0.26 MMB mg/ml; c) 1.7 MMB mg/ml. After 30 min H_2O_2 (40 mM) was added, samples were diluted ten times with LSB, and the microsomes were recovered by sedimentation (60 min at 100,000g). To assess the effectiveness of the cross-linking all samples were resuspended in HSB containing 2.5 mM $MgCl_2$ and treated with puromycin (10^{-3} M final concentration) for ribosome removal. Sample (d) was processed in the same way as (c) but received in addition dithiothreitol (10 mM) to reverse the disulfide cross-links. After incubation for 10 min at 37°C, aliquots (1.5 mg protein) were analyzed by sucrose density gradient (10–45% sucrose HSB) centrifugation (120 min at 40,000 rpm in a SW41 rotor). The arrows indicate the positions of the large (L) and small (S) ribosomal subunits in the OD_{254} profiles. Note that low concentrations of MMB (a,b) cross-link preferentially large subunits, since these are not proportionally released by puromycin. Treatment with dithiothreitol (d) completely reverses the cross-linking and allows the ribosome release by puromycin-KC1 (compare with c).

We found that very low concentrations (0.04 or 0.26 mg/ml) of MMB caused the selective cross-linking of large ribosomal subunits to the membranes and prevented their release by treatment with puromycin in a medium of high ionic strength (Fig 8a, b). Higher MMB concentrations were required to prevent the release of both subunits (Fig 8c), but at all MMB concentrations reversion of the cross-links restored the normal response to puromycin (Fig 8d). The finding that large subunits can be selectively cross-linked to the membrane by MMB complements previous studies indicating that large subunit proteins provide the ribosomal sites for interaction with the membranes [17, 19].

For the isolation of membrane proteins cross-linked to the ribosomes, a concentration of MMB was chosen (0.20%) which was sufficient to cross-link a large fraction of ribosomes to the membranes but still allowed the solubilization of the microsomes with DOC. Ribosomes not cross-linked were removed before the detergent treatment by incubation with puromycin-KC1, and cross-linked ribosomes were subsequently isolated by sedimentation from the DOC-treated membranes. Cross-linked proteins recovered with

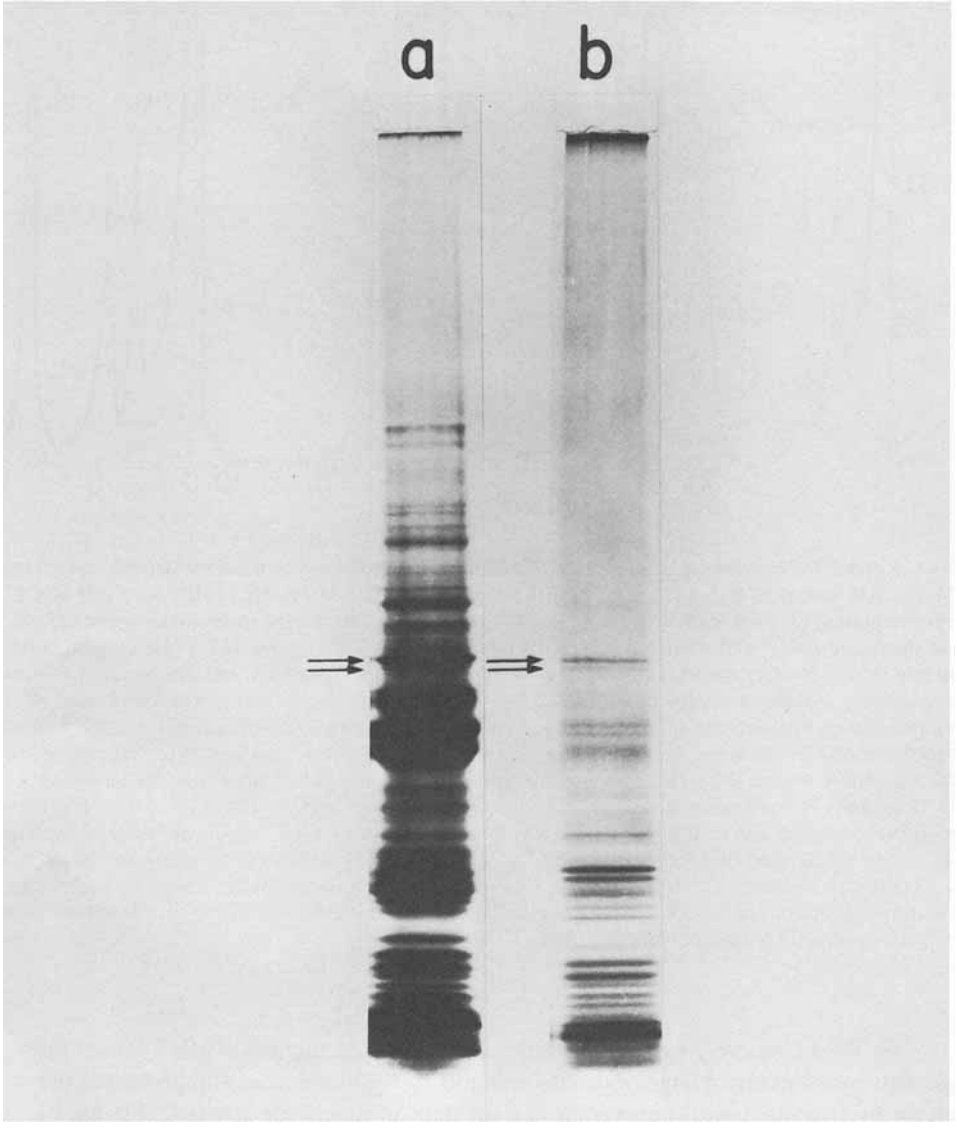

Fig 9. Cross-linking of ribophorins to membrane-bound ribosomes. RM (a) were processed for cross-linking with a relatively low concentration of MMB (0.20 mg/ml) as described in the legend to Figure 8. Ribosomes not cross-linked by this treatment were removed with puromycin-KC1 [9]. The microsomes with the remaining cross-linked ribosomes were treated with 1% DOC to solubilize the membranes. The ribosomes, with the membrane proteins cross-linked to them, were recovered by centrifugation (60 min at 100,000g) through a 2-ml cushion of 1.5 M sucrose HSB (b). Both samples were treated with mercaptoethanol to reduce disulfide cross-links before analysis in SDS acrylamide gels (5–13%). The position of bands corresponding to the ribophorins is indicated by arrows.

the ribosomes were identified electrophoretically after reversing the cross-links by reduction with dithiothreitol. The two ribophorins were found within the subset of membrane proteins cross-linked to the ribosomes (Fig 9b), although the intensity of the band representing ribophorin I was higher than that for ribophorin II. Since protein aggregates which did not penetrate the gel were present even after reversal of the cross-linking, it cannot be concluded that ribophorin I was preferentially cross-linked to the ribosomes. These experiments indicated, however, that as expected from components of the ribosome binding sites, in intact microsomes ribophorins are indeed in close proximity to the bound ribosomes [20].

IDENTIFICATION OF RIBOPHORIN-LIKE PROTEINS IN ROUGH MICROSOMES FROM DIFFERENT TISSUES AND ANIMAL SPECIES

Our observations with rat liver microsomes suggest that an important function of the ribophorins is to mediate the binding of ribosomes to membranes of the ER, thus facilitating the vectorial discharge of nascent polypeptides which characterizes protein synthesis in this organelle. The presence of ribophorins or ribophorin-like proteins may therefore be an essential feature of all RER membranes. We have analyzed electrophoretically the protein composition of rough microsomal membranes prepared from different tissues and from several animal species. Proteins similar in electrophoretic mobility and solubility in detergents to the ribophorins from rat hepatocytes were detected in RM from rat lacrimal glands, rabbit and chicken liver, chicken pancreas, and mouse myeloma cells [21]. Figure 10 demonstrates the results obtained with rough microsomes prepared from rat lacrimal glands by the procedure of Herzog and Miller [22]. The ribophorin-like proteins (indicated by arrows in Fig 10) in this case have electrophoretic mobilities identical to hepatocyte ribophorins I and II, although the overall protein composition of the membranes is clearly different in both organs as is apparent by comparing Coomassie Blue staining patterns of lacrimal gland and hepatocyte RM (compare Fig 10 with Fig 4a). As was the case with the ribophorins from rat liver (Fig 5), ribophorins from rat lacrimal gland were completely solubilized by 1% DOC, but remained associated with the sedimentable ribosomes when rough microsomes were treated with the nonionic detergent Kyro EOB. We have recently shown by double-diffusion immunoprecipitation in Ouchterlony plates that antibodies raised against electrophoretically pure ribophorin preparations obtained from rat liver RM, cross-react with the equivalent proteins present in the lacrimal gland microsomes.

RIBOPHORIN I AND II ARE TRANSMEMBRANE GLYCOPROTEINS

PAS staining patterns of SDS acrylamide gels indicated that ribophorins are membrane glycoproteins [23]. We therefore attempted their partial purification by affinity chromatography in columns of Con A Sepharose, a method which allows the separation of

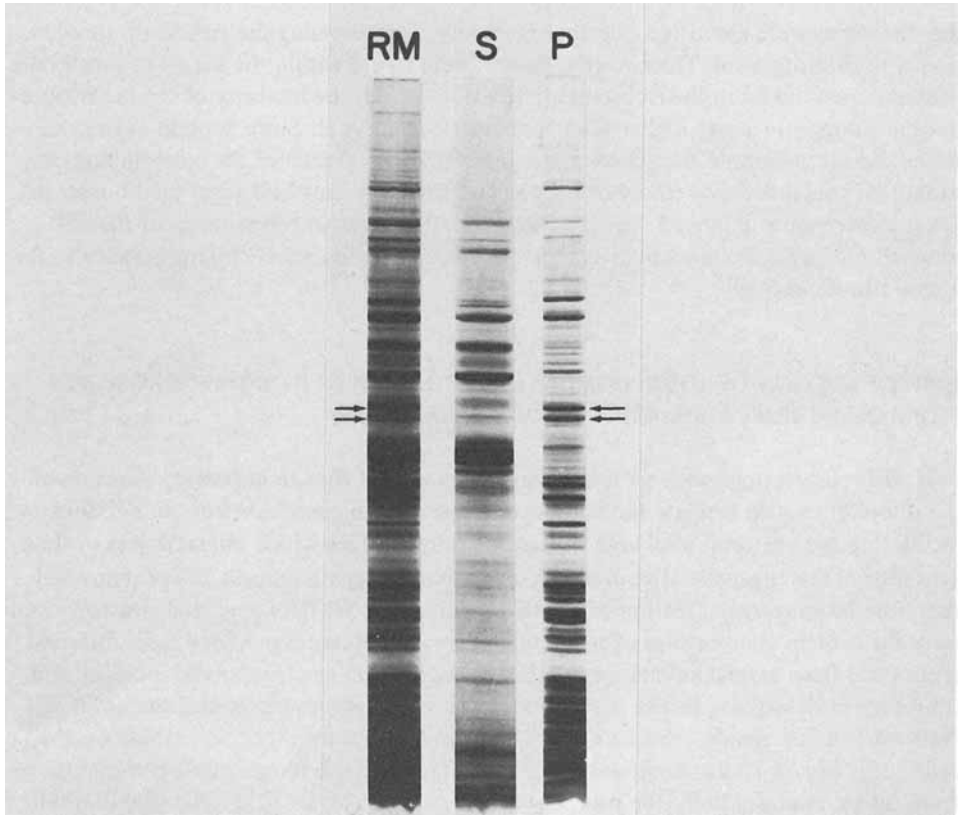

Fig 10. Identification of ribophorin-like proteins in RM from the lacrimal gland. RM isolated from rat lacrimal glands according to Herzog and Miller [22] were resuspended in LSB, treated with Kyro EOB (2.5×10^{-2}M) and fractionated by sedimentation (60 min at 100,000g). Total RM (RM) (400 μg protein) and equivalent amounts of the supernate (S) and pellet fractions (P) obtained after detergent treatment were loaded onto SDS acrylamide gels (8–13%). RM from lacrimal glands contain ribophorins with mobilities identical to those from rat liver (indicated by arrows). Note that the Coomassie Blue staining pattern, and therefore the overall set of proteins in lacrimal gland RM, is very different from that of rat liver RM (see Fig 4a). Bands corresponding to the ribophorins are present, however, in the lacrimal gland RM pattern. Ribophorin-like proteins from lacrimal gland RM are also recovered with the ribosomes after Kyro EOB treatment (P).

microsomal glycoproteins from other membrane proteins. Glycoproteins of the microsomal content fraction were first removed by treatment with a low DOC concentration (0.05%) [15]. The RM recovered by sedimentation were then solubilized in 1% DOC for fractionation of the proteins by lectin affinity chromatography. Electrophoretic analysis of the unbound protein fractions showed that the ribophorins were completely retained by the lectin column. Both proteins were, on the other hand, prominent members of the set of glycoproteins eluted by the specific competitor α-methyl mannoside [23].

Since the carbohydrate moieties of most microsomal membrane glycoproteins are exposed on the luminal side of the microsomes, where they can be visualized by cytochemical techniques [24, 25], a transmembrane disposition of the ribophorins was suspected. Double-labeling experiments using the lactoperoxidase-catalyzed iodination procedure [26–28] served to establish this disposition.

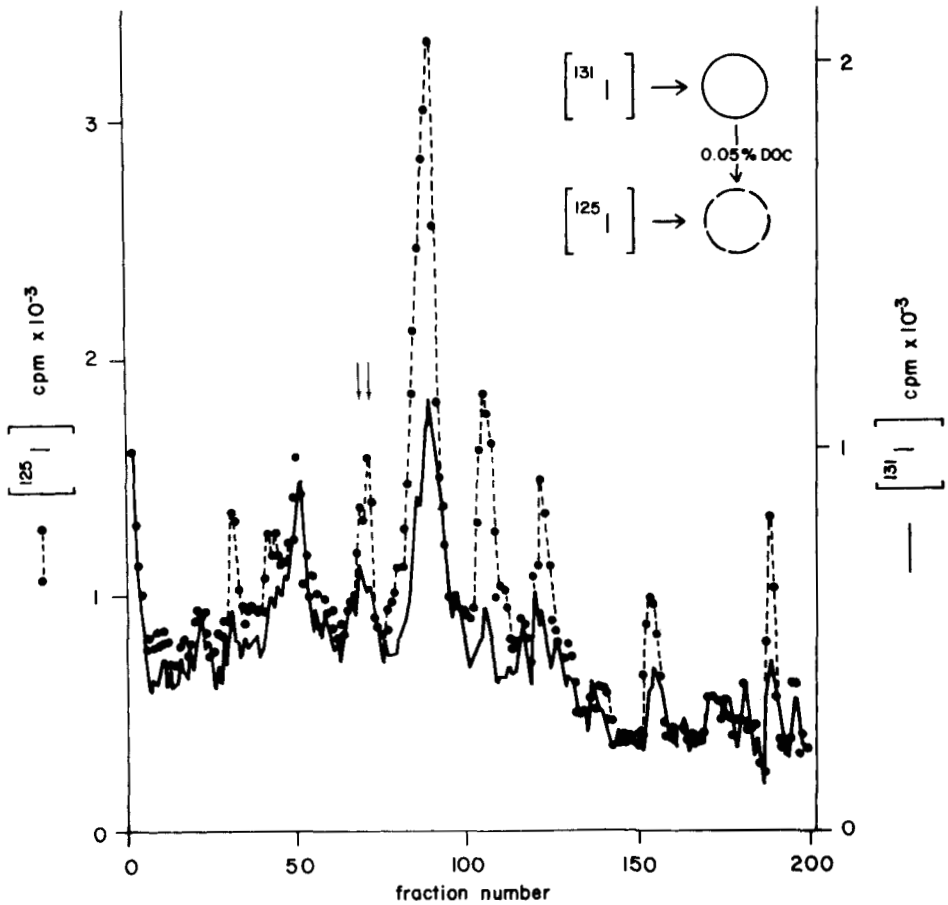

Fig 11. Exposure of ribophorins on both microsomal surfaces. Intact RM were iodinated by the lactoperoxidase-catalyzed iodination procedure [28], first with ^{131}I and then, in a second iodination cycle, with ^{125}I, in the presence of a low concentration of 0.05% DOC to make the luminal face of the microsomal vesicles accessible to the labeling system [15]. The double-labeled RM were resuspended in LSB and solubilized with 2% DOC. Microsomal glycoproteins were isolated by affinity chromatography on Con A sepharose [23]. Glycoproteins eluted from the column by 10 mg/ml α-methylmannoside were analyzed by SDS acrylamide gel (8–13%) electrophoresis. ^{125}I and ^{131}I radioactivity distribution throughout the gel was measured in 1-mm slices with a γ-counter calibrated for dual isotope counting. Double labeling of RM without DOC treatment led to a constant $^{131}I/^{125}I$ ratio throughout the gel (not shown). The high incorporation of ^{125}I in the peaks marked by the two parallel arrows demonstrates the luminal exposure of ribophorins I and II. This transmembrane disposition is a feature of most ER membrane glycoproteins.

RM were first prelabeled with ^{131}I as intact vesicles, recovered by sedimentation, and labeled a second time with ^{125}I, either as intact microsomes (controls, not shown) or in the presence of a sublytic concentration of DOC (0.025%), which makes the vesicles permeable to the iodinating system. An analysis of the distribution of ^{131}I and ^{125}I radioactivities in gels from glycoprotein fractions purified by affinity chromatography showed that both ribophorins were labeled with ^{131}I and are therefore exposed on the cytoplasmic face of the membranes (Fig 11). Both proteins (indicated by arrows in Fig

11) also showed an increased $^{125}I/^{131}I$ ratio when the second iodination was carried out in the presence of the sublytic concentration of detergent. These results demonstrated that ribophorins are exposed on both microsomal surface and therefore span the width of the ER membrane [23, 24].

Disappointingly, however, we observed that removal of ribosomes before the first iodination did not lead to a significant increase in the incorporation of ^{131}I into the ribophorins, or into any other microsomal membrane protein. An increase might have been expected from an arrangement in which the ribosomes, at least partially, protect the binding sites from lactoperoxidase (LPO). This observation indicates that if the ribophorins contribute directly to the binding sites, as suggested by the results described in the preceding sections, the sequences which are directly involved in this function do not contain iodinatable tyrosine residues. As was noted above, electron microscopic observations suggest an extended configuration for the ribophorins, which is compatible with the results of the labelling experiments.

We have also used proteases as probes for the exposure of the ribophorins on the surface of the microsomal membranes. Ribophorin I was found to be extremely sensitive to mild trypsinization (10 μg/ml at 0°C for 60 min) of RMstr under conditions which led to the digestion of few other proteins but completely abolished the in vitro ribosome-binding capacity of the membranes [10]. Although the iodination experiments showed that ribophorin II is also exposed on the cytoplasmic face of the membranes, this protein was degraded by trypsin only when the microsomes were made permeable to the enzyme by the addition of a low detergent concentration [20].

EXPERIMENTAL SEGREGATION OF RIBOPHORINS TO MEMBRANE AREAS BEARING RIBOSOMAL AGGREGATES

Recent experiments have shown that under experimental conditions bound ribosomes are capable of undergoing extensive lateral displacements in the plane of the microsomal membranes [5]. In these experiments it was shown that aggregates of bound ribosomes are formed when RM, previously treated with RNase or with antibodies against ribosomal proteins to promote the adhesion of ribosomes for each other, are incubated at temperatures above the transition temperature of the phospholipids. Ribosomes, binding sites, and/or membrane elements which interact with the nascent polypeptides were shown to be displaced together during the formation of ribosomal aggregates [5]. We have recently found an alternative procedure to produce large aggregates of membrane-bound ribosomes, which involves the treatment of RM with low concentrations of neutral detergents. We observed that at certain concentrations of neutral detergents (eg, 0.08% Triton X-100) membrane areas bearing the ribosomal aggregates (Fig 12b) frequently invaginate into the lumen of the microsomal vesicles (Fig 12e). At slightly higher detergent concentrations (eg, 0.16% Triton X-100), which cause the partial extraction of some proteins and phospholipids, a large proportion of the microsomes containing invaginations is fragmented into "rough inverted" and "smooth vesicles" (Fig 12f and g, respectively). Because of their different densities it has been possible to separate by centrifugation through a cushion of heavy sucrose the subfractions of "smooth and rough inverted" vesicles (Fig 13). An analysis of their protein composition showed that vesicles derived from smooth membrane areas are devoid of ribophorins (Fig 14b), which are completely recovered in the inverted ribosome-carrying vesicle population (Fig 14c). Since no other

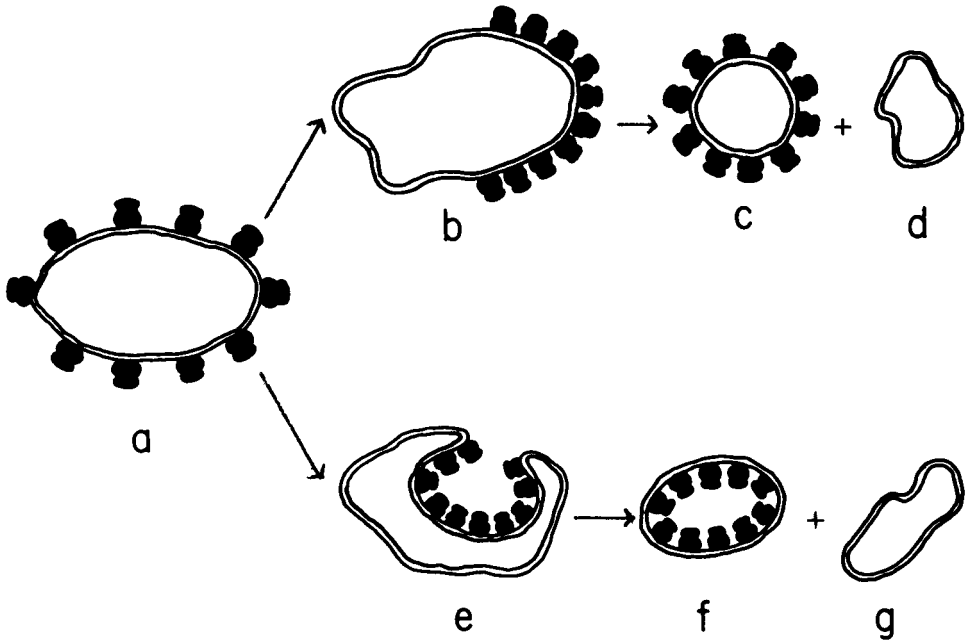

Fig 12. Formation of ribosome aggregates and fragmentation of RM into inverted rough vesicles and smooth vesicles by detergent treatment. Bound ribosomes spaced rather evenly in control RM (a) may form aggregates (b) in the presence of low concentrations of Triton X-100 (0.08%), which are frequently found in invaginated areas of the vesicular membrane (e). At slightly higher detergent concentrations (0.16% Triton X-100) RM may be fragmented preferentially into "inverted vesicles" (f) to some extent into "right side out" vesicles (c) and smooth-surfaced vesicles (d and g).

microsomal protein was partitioned so sharply with the bound ribosomes, the concomitant segregation of ribosomes and ribophorins demonstrated in these experiments provides independent support for the conclusion that these proteins are closely related to the binding sites.

The observation that membrane areas bearing ribosomal aggregates tend to form invaginations, which upon fragmentation of the microsomes lead to the formation of inverted vesicles, suggests that the displacement of ribophorins which occurs concomitantly with the ribosome movement directly affects the curvature of the membranes. As mentioned above, it has long been recognized that in spite of the continuity of their membranes (arrows in Fig 1) regions of the endoplasmic reticulum with attached ribosomes (RER) are morphologically distinct from smooth areas of the ER (see Fig 1). While, in general, membranes of the RER adopt a planar configuration which is most evident in the stacks of flat, ribosome-studded cisternae characteristic of many protein-secreting cells, SER membranes commonly form convoluted and narrow tubules which tend to vesiculate easily. The finding that ribophorins are absent from smooth microsomal membranes and also from smooth vesicles derived from ribosome-free areas of RM, together with the morphological changes which accompany the experimental displacement of ribosomes and ribophorins, suggest that the local morphological differentiations of the ER found within the cell may also be related to changes in the distribution of a porteinaceous intramembranous network, such as the one provided by the ribophorins in association with the ribosomal binding sites.

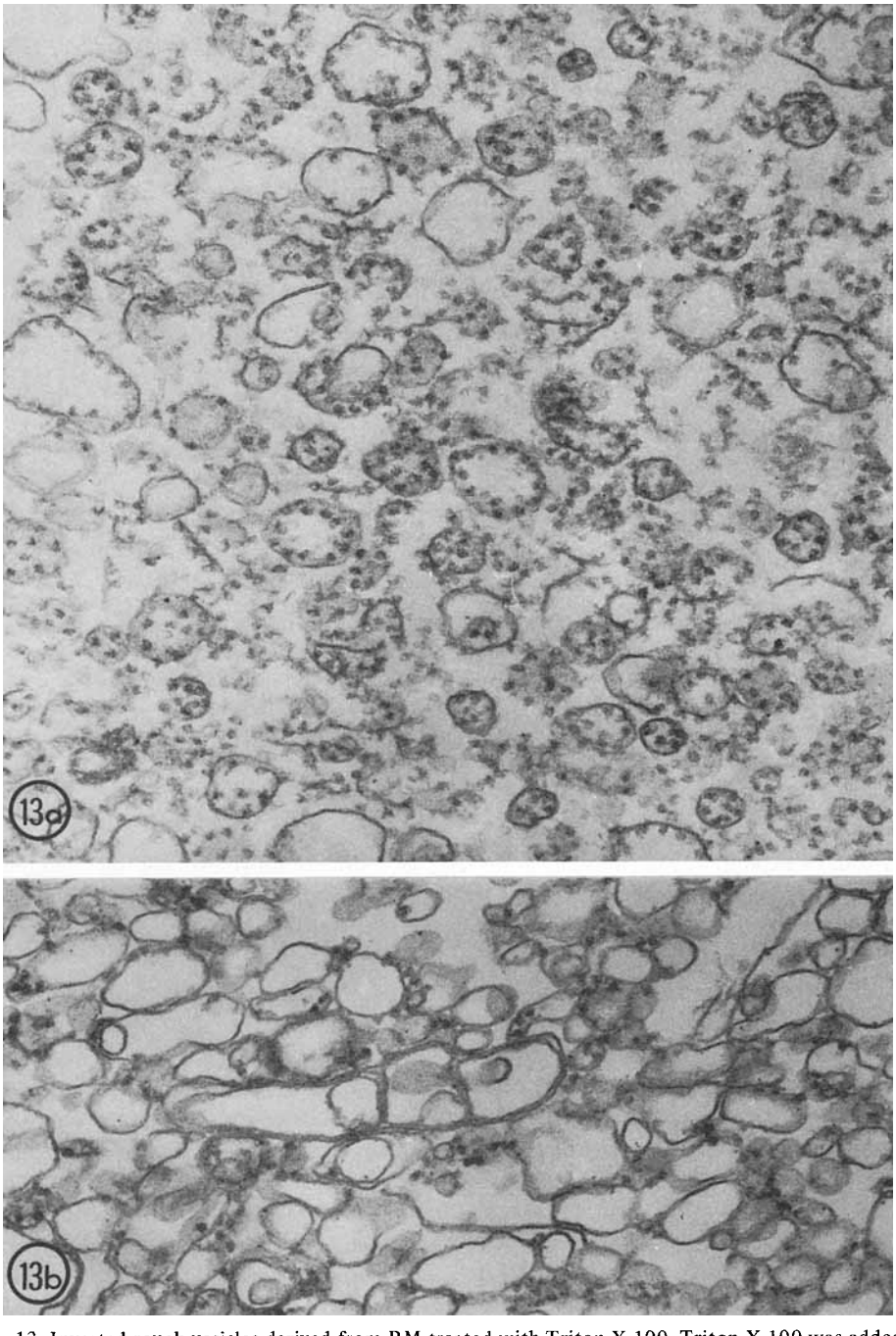

Fig 13. Inverted rough vesicles derived from RM treated with Triton X-100. Triton X-100 was added slowly to a suspension of RM in LSB (3.5 mg/ml), to a final concentration of 0.16%. The mixture was shaken for 30 min at 4°C, layered on top of a sucrose cushion (30% sucrose LSB), and centrifuged (60 min at 100,000g). The membrane fraction collected from the sucrose cushion (b) and the resuspended pellet (LSB-TEA) (a) were fixed with 2% glutaraldehyde and 1% OsO_4, and processed for electron microscopy. The sample (a) consists mainly of inverted vesicles with ribosomes on the inner face. This procedure leads to the release of microsomal content proteins, to the partial extraction of some membrane proteins, and also to a fraction of smooth vesicles (b) which are recovered on top of the cushion (65,000 ×).

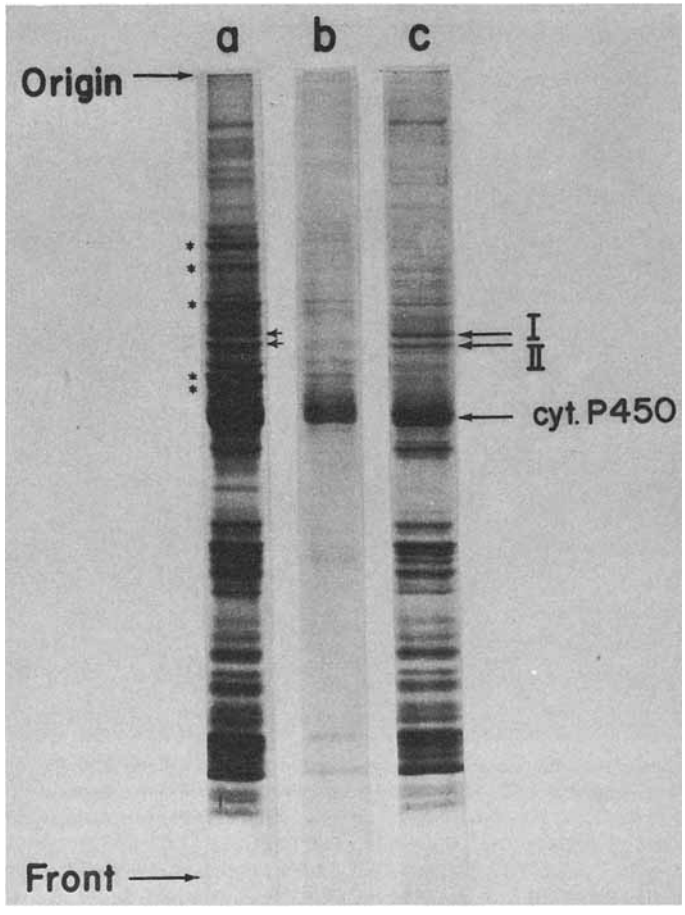

Fig 14. SDS acrylamide gel electrophoresis showing the segregation of ribophorins into inverted rough vesicles obtained from RM treated with Triton X-100; 150 μg of RM (a) and equivalent amounts of smooth vesicles (b) (see Fig 13b) and of inverted rough vesicles (c) (see Fig 13a) were loaded onto an SDS acrylamide gel (8–12%). The two ribophorins (marked by arrows) are quantitatively recovered in the fraction of the inverted vesicles (c). Major content proteins are marked by asterisks in gel a. Cytochrome P-450 is the main band in the smooth-surfaced membrane fraction (arrow in gel c).

RECONSTITUTION OF MEMBRANE VESICLES WITH RIBOSOME-BINDING CAPACITY

In experiments which involve the reformation of membranes from detergent-solubilized mixtures of microsomal proteins and phospholipids, we attempted to demonstrate directly a role of the ribophorins in ribosome binding. It has been shown in several systems that formation of closed vesicles from solubilized membrane preparations is facilitated when the detergent concentration was very slowly decreased (for example, see Meissner and Fleischer [29]; for review see Razin [30]). We have found that single-walled vesicle preparations can be obtained from DOC-solubilized RM stripped of ribosomes, if the detergent concentration is reduced gradually by continuous dilution (80 ×) over a period of 3 h (Fig 15). Membrane proteins including the two ribophorins (indicated by arrows in Fig 16) are selectively incorporated, while proteins of the microsomal content (marked by dots), present in the same samples, are excluded from the "recon-

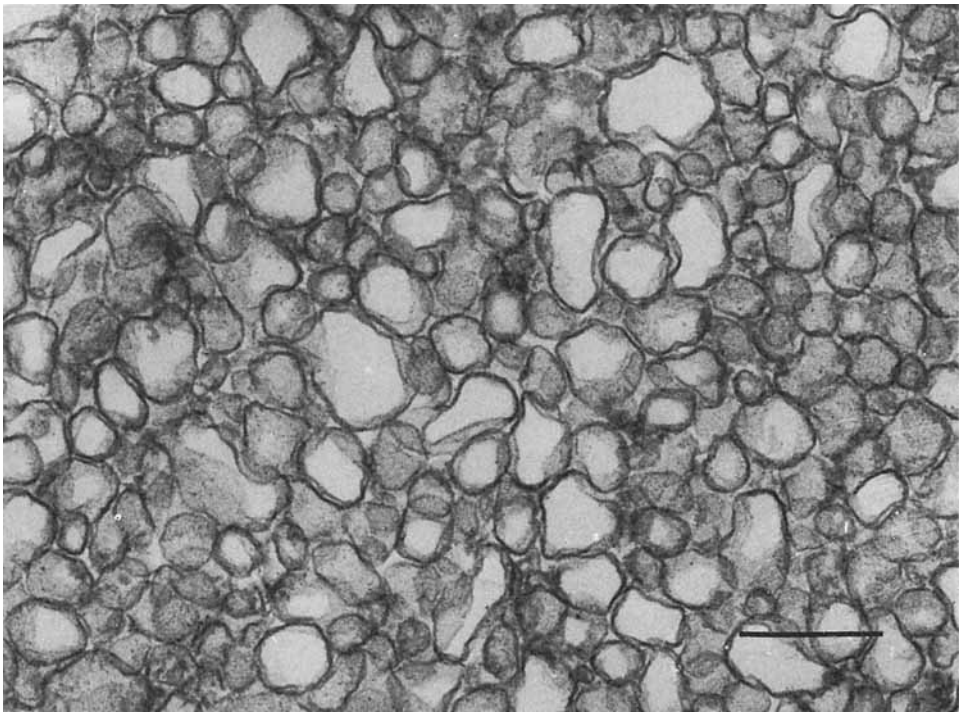

Fig 15. Thin-section electron micrograph of reconstituted membrane vesicles from detergent treated RM. RMstr were resuspended in LSB (3 mg protein/ml) containing butylated hydroxytoluene (BHT) (10^{-4}) and treated with DOC (1% final). The supernate obtained after centrifugation (60 min at 105,000g) was diluted 75 times during 3 h by addition of LSB-BHT containing 10% glycerol. After centrifugation the reconstituted vesicles were collected on the interface of a sucrose cushion (1.6 M). The membrane fraction was fixed with glutaraldehyde-OsO_4 and processed for electron microscopy (65,000 ×).

stituted microsomes." Scatchard plots of in vitro binding experiments (for details see legend to Figs 2 and 3) carried out with these reconstituted vesicles show (Fig 17; ●——●) that their affinity ($K = 2.6 \times 10^8 M^{-1}$) for ^{3}H-labeled 80 S ribosomes is comparable to that of RM stripped of ribosomes ($K = 6 \times 10^7 M^{-1}$), although the number of available binding sites (1.8×10^{-8} mole/g protein) in the reconstituted vesicles is lower than in RMstr (6.4×10^{-8} mole/g protein). The observation that reconstituted vesicles have a lower number of sites in the exposed surface than in native RM is not unexpected, since membrane proteins in the reformed membranes appear to be randomly oriented. This may be inferred from freeze-fracture preparations of reconstituted vesicles which showed a symmetrical distribution of intramembranous particles in both membrane halves (experiments in collaboration with Dr G. Ojakian). Ribosome-binding experiments were also carried out with vesicles which were reconstituted using a microsomal protein fraction recovered by sedimentation from RM treated with Kyro EOB (see Fig 4b) and a preparation of egg lecithin dissolved in 1% DOC, instead of native phospholipids (Fig 17; ▲——▲). These vesicles gave results equivalent to those obtained with vesicles recon-

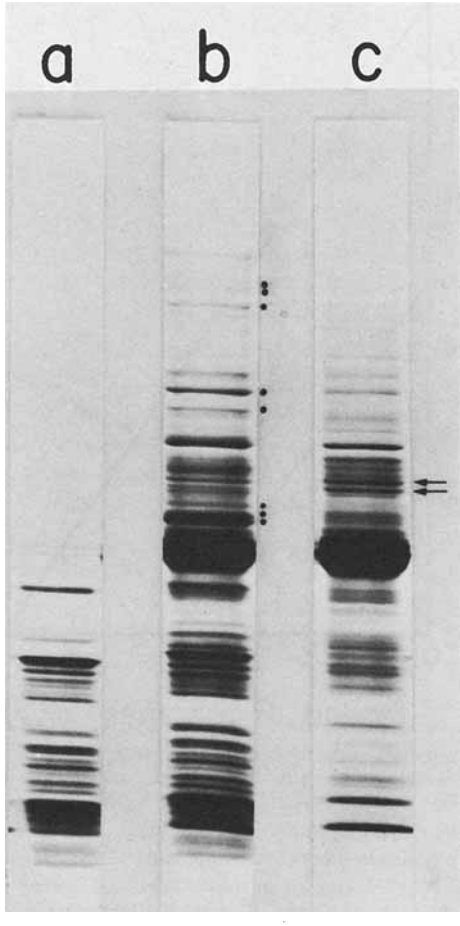

Fig 16. RM proteins in reconstituted membrane vesicles. Reconstituted vesicles (c) (prepared as described in the legend to Fig 15) and RM (b) were loaded onto SDS acrylamide gels (100 μg protein of each). Bound polysomes prepared from an equivalent amount of RM were analyzed for comparison (a). The two ribophorins (indicated by arrows) are present in the reconstituted vesicles but proteins of the microsomal content (marked by dots) are almost completely absent.

stituted from whole mixtures of native components. These preliminary results have encouraged us to design even more simple reconstitution systems, in which a direct role of the ribophorins in ribosomal binding may be demonstrated.

Possible roles of ribophorins during protein synthesis by membrane-bound polysomes should be discussed taking into account current models for the assembly of bound polysomes and the vectorial discharge of secretory proteins (Fig 18) [4, 31–34]. The establishment of a functional association between ribosomes and membranes which allows the vectorial transfer of nascent polypeptides is likely to involve a series of successive steps with increasing specificity, which follow an initial interaction determined by a

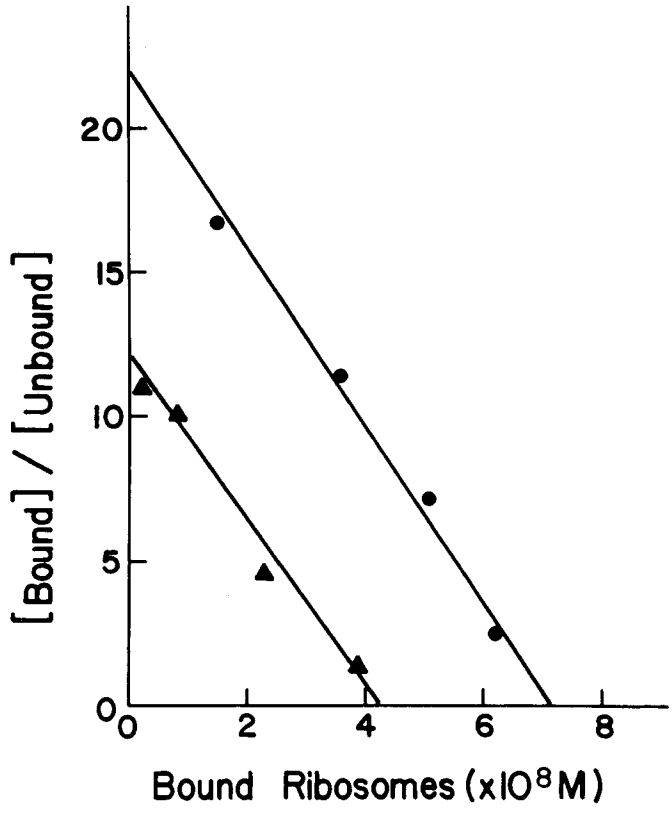

Fig 17. Ribosome-binding capacity of reconstituted membrane vesicles containing KM membrane proteins. Reconstituted vesicles were prepared by two different procedures: A) as indicated in the legend to Figure 15 from total membrane proteins dissolved by 1% DOC; B) using a set of membrane proteins including the ribophorins by extraction of Kyro EOB residues (see Fig 5b) with LSB (pH 8,5) containing DOC (1%). The set of proteins in this extract supplemented with egg lecithin (50 times the amount of protein) was diluted (75 times) during 3 h by addition of LSB containing 10^{-4}% BHT. The reconstituted single-walled vesicles were sedimented (3 h at 100,000g) onto a sucrose cushion (1.2 M). Reconstituted vesicles (0.33 mg protein of preparation A and 0.17 mg of preparation B) were incubated with increasing amounts of 80 S ribosomes (for details see legend to Fig 3). Both Scatchard plots describing ribosome binding to reconstituted vesicles show saturable sites with very similar affinities for ribosomes. Preparation A) ●——●; preparation B) ▲——▲.

specific signal sequence [31] present at the amino terminal segment of the nascent polypeptide. The demonstration that this signal consists of an amino acid sequence rich in non-polar amino acids suggests a role in facilitating the penetration of the chain into the hydrophobic interior of the endoplasmic reticulum membrane. This would be followed by a direct interaction of the ribosome with the ribophorins, which stabilizes the association of the ribosome with the membrane allowing the vectorial discharge of nascent polypeptides. The possibility should be considered that ribophorins also interact with the nascent polypeptide chain in a manner which facilitates its transfer across the hydrophobic barrier.

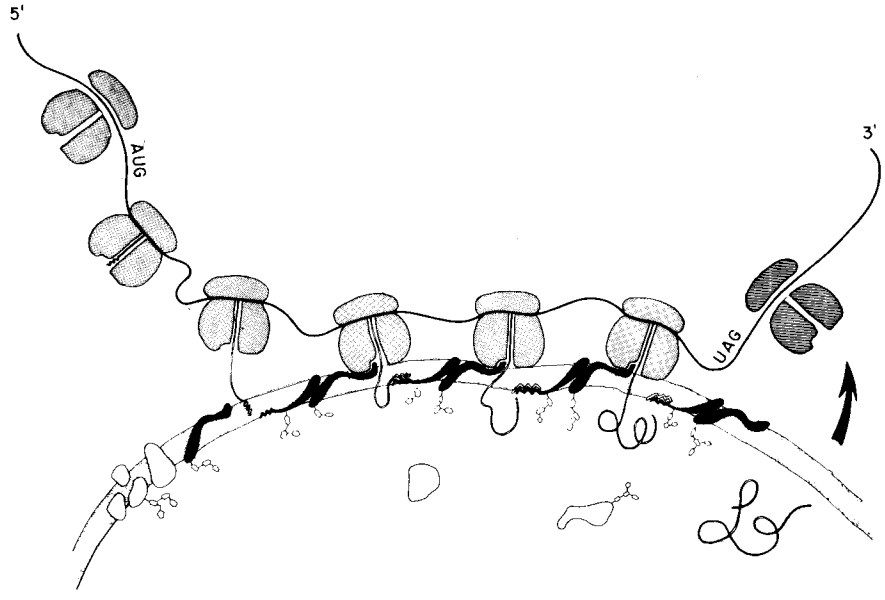

Fig 18. Tentative model for the spatial arrangement of the ribophorins in rough microsomal membranes. Features of the microsomal membrane in this diagram are suggested by our studies, which concern the orientation of the ribophorins and their relationship to each other and to the ribosomes. More speculative are possible functional roles for these proteins. It is possible that both proteins contribute directly to the binding site. Nevertheless in this diagram only a direct interaction between ribophorin I and the ribosome is represented. This possibility is suggested by the finding that ribophorin I is rapidly digested during mild trypsinization of intact microsomes, which simultaneously abolishes the ribosome-binding capacity of the membranes, even though ribophorin II remains intact. Although a role of ribophorin II as a receptor for the signal sequence is also depicted, this should be regarded as purely speculative.

ACKNOWLEDGMENTS

This work was supported by grants GM 21971, GM 20277 and AG 00378 from NIH. G.K is the recipient of a Research Career Development Award (GM 00232) from NIH. The nonionic detergent Kyro EOB (a polyethoxyalkylether) was a gift from Dr D.H. Hughes (Miami Valley Research Laboratory, Proctor and Gamble Co, Cincinnati, Ohio). The technical assistance of Ms Belinda Ulrich and Ms Theresa Feng is greatly appreciated. We thank Mr Brian Zeitlow for the photographic work and Ms Myrna Cort for typing the manuscript.

REFERENCES

1. Amar-Costesec A, Beaufay H, Wibo M, Thinès-Sempoux D, Feytmans E, Robbi M, Berthet J: J Cell Biol 61:201, 1974.
2. Colbeau A, Nachbaur J, Vignais PM: Biochim Biophys Acta 249:462, 1971.

3. Depierre JW, Dallner G: Biochim Biophys Acta 415:411, 1975.
4. Sabatini DD, Kreibich G: In Martonosi A (ed): "The Enzymes of Biological Membranes." New York: Plenum, 1976, vol 2, pp 531–579.
5. Ojakian GK, Kreibich G, Sabatini DD: J Cell Biol 72:530, 1977.
6. Borgese D, Mok W, Kreibich G, Sabatini DD: J Mol Biol 88:559, 1974.
7. Rolleston FS: Subcell Biochem 3:91, 1974.
8. McIntosh PR, O'Toole K: Biochim Biophys Acta 457:171, 1976.
9. Adelman MR, Sabatini DD, Blobel G: J Cell Biol 56:206, 1973.
10. Mok W, Freienstein C, Sabatini D, Kreibich G: J Cell Biol 70:1177a, 1976.
11. Kreibich G, Ulrich B, Sabatini D: J Cell Biol 67:225a, 1975.
12. Kreibich G, Grebenau R, Mok M, Pereyra B, Rodriguez-Boulan E, Ulrich B, Sabatini D: Fed Proc (Abstract) 36:656, 1977.
13. Kreibich G, Ulrich BL, Sabatini DD: J Cell Biol 77:406, 1978.
14. Sabatini DD, Ojakian G, Lande MA, Lewis J, Mok W, Adesnik M, Kreibich G: In Meints R, Davis E (eds): "Control Mechanisms in Development." New York: Plenum, 1975, pp 151–180.
15. Kreibich G, Debey P, Sabatini DD: J Cell Biol 58:436, 1973.
16. Steck TL, Yu J: J Supramol Struct 1:220, 1973.
17. Sabatini DD, Tashiro Y, Palade GE: J Mol Biol 19:503, 1966.
18. Traut RR, Bollen A, Sun T, Hershey JWB, Sunberg J, Pierce LR: Biochemistry 12:3266, 1973.
19. Lewis JA, Sabatini DD: J Biol Chem 252:5547, 1977.
20. Kreibich G, Freienstein CM, Pereyra BN, Ulrich BL, Sabatini DD: J Cell Biol 77:488, 1978.
21. Grebenau R, Sabatini DD, Kreibich G: J Cell Biol 75:234a, 1977.
22. Herzog V, Miller F: J Cell Biol 53:662, 1972.
23. Rodriguez-Boulan E, Sabatini DD, Pereyra BN, Kreibich G: J Cell Biol (In press).
24. Rodriguez-Boulan ER, Kreibich G, Sabatini DD: Fed Proc 34:582, 1975.
25. Rodriguez-Boulan ER, Kreibich G, Sabatini DD: J Cell Biol (In press).
26. Phillips DR, Morrison M: Biochemistry 10:1766, 1971.
27. Hubbard AL, Cohn ZA: J Cell Biol 55:390, 1972.
28. Kreibich G, Hubbard AL, Sabatini DD: J Cell Biol 60:616, 1974.
29. Meissner G, Fleischer S: J Biol Chem 249:302, 1974.
30. Razin S: Biochim Biophys Acta 265:241, 1972.
31. Blobel G, Sabatini DD: In Manson LA (ed): "Biomembranes." New York: Plenum, 1971, vol 2, pp 193–196.
32. Milstein C, Brownlee GG, Harrison TM, Mathews MB: Nature (New Biol) 239:117, 1972.
33. Blobel G, Dobberstein B: J Cell Biol 67:835, 1975.
34. Blobel G, Dobberstein B: J Cell Biol 67:852, 1975.
35. Kruppa J, Sabatini DD: J Cell Biol 74:414, 1977.
36. Kreibich G, Sabatini DD: J Cell Biol 61:789, 1974.
37. Baudhuin P, Evrard P, Berthet J: J Cell Biol 32:181, 1967.