Studies on membrane proteins involved in ribosome binding on the rough endoplasmic reticulum

Ribophorins have no ribosome-binding activity

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A membrane protein fraction showing affinity for ribosomes was isolated from rat liver microsomes (microsomal fractions) in association with ribosomes by treatment of the microsomes with Emulgen 913 and then solubilized from the ribosomes with sodium deoxycholate. This protein fraction was separated into two fractions, glycoproteins, including ribophorins I and II, and non-glycoproteins, virtually free from ribophorins I and II, on concanavalin A–Sephadex columns. The two fractions were each reconstituted into liposomes to determine their ribosome-binding activities. The specific binding activity of the non-glycoprotein fraction was approx. 2.3-fold higher than that of the glycoprotein fraction. The recovery of ribosome-binding capacity of the two fractions was about 85% of the total binding capacity of the material applied to a concanavalin A–Sephadex column, and about 90% of it was found in the non-glycoprotein fraction. The affinity constants of the ribosomes for the reconstituted liposomes were somewhat higher than those for stripped rough microsomes. The mode of ribosome binding to the reconstituted liposomes was very similar to that to the stripped rough microsomes, in its sensitivity to proteolytic enzymes and its strong inhibition by increasing KCl concentration. These results support the idea that ribosome binding to rat liver microsomes is not directly mediated by ribophorins I and II, but that another unidentified membrane protein(s) plays a role in ribosome binding.

INTRODUCTION

Many studies on the membrane protein components responsible for the direct binding of ribosomes to rough microsomal membranes have been performed (Jothy *et al.*, 1975; Takagi, 1977; Ohlsson & Jergil, 1977; Fujita *et al.*, 1977; Kreibich *et al.*, 1978a,b; Aulinskas & Scott-Burden, 1979). Kreibich *et al.* (1978a,b) reported two trans-membrane glycoproteins, ribophorins I and II, related to ribosome binding. These proteins are characteristic of rough microsomes (microsomal fractions), and are bound to polysomes after treatment of the microsomes with certain non-ionic detergents and can be cross-linked to ribosomes with bifunctional reagents. There is a stoichiometric ratio between ribophorins and ribosomes close to 1:1. On the basis of these facts, Kreibich *et al.* (1978a,b) suggested that the ribophorins may play an important role in mediating the binding of ribosomes to the membranes of rough microsomes. However, this ribosome-binding activity has not yet been confirmed.

In our previous study (Yamaguchi *et al.*, 1981), a membrane protein fraction showing high affinity to polysomes was isolated from rough microsomes of rat liver. This protein fraction when reconstituted into liposomes showed the ability to bind polysomes, and the mode of polysome binding was similar to that with stripped rough microsomes. In the present study, a membrane protein fraction showing high affinity to ribosomes was obtained from microsomes of rat liver by

the method of Kreibich *et al.* (1978a), and this fraction was further separated into two fractions, a glycoprotein fraction including a major part of the ribophorins and a non-glycoprotein fraction, by affinity chromatography on Con A–Sephadex columns. The two fractions were, respectively, reconstituted into liposomes, and then their ribosome-binding activities were compared. The results showed that ribosome-binding activity was predominantly found in the non-glycoprotein fraction that was virtually free from ribophorins.

EXPERIMENTAL

Materials

Phosphatidylcholine was prepared from hen eggs as described by Yamaguchi *et al.* (1981). Sodium deoxycholate, sodium cholate, SDS, α -methyl mannoside and orotic acid were purchased from Wako Chemical Co. (Osaka, Japan). Deoxycholate and sodium cholate were recrystallized from 90% (v/v) ethanol before use. Triton X-100 was obtained from Kishida Chemicals (Osaka, Japan), Emulgen 913 from Kao Atlas (Tokyo, Japan), puromycin and phenylmethanesulphonyl fluoride were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and Con A–Sephadex 4B was from Pharmacia (Tokyo, Japan). [5-³H]Orotic acid (19.2 Ci/mmol), isethionyl [1-¹⁴C]acetimidate (55 mCi/mmol) and L- α -di[1-¹⁴C]-palmitoyl phosphatidylcholine (115 mCi/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.).

Cell fractionation

Male Wistar rats weighing 200–230 g after starvation for 18 h were used to prepare liver microsomes by the procedure of Kreibich *et al.* (1978a), with a slight modification, i.e. their livers were homogenized with 2 vol. of 0.25 M-sucrose in TKM buffer (50 mM-Tris/HCl, 25 mM-KCl, 5 mM-MgCl₂, pH 7.6) containing 0.5 mM-phenylmethanesulphonyl fluoride and 7 mM- β -mercaptoethanol. A post-mitochondrial supernatant was obtained by centrifuging the homogenate for 15 min at 15000 g, adjusted to 1.35 M-sucrose and then layered over a step gradient consisting of 5 ml each of 2.0 M- and 1.55 M-sucrose in TKM buffer as described by Kreibich *et al.* (1978a). After centrifugation (16 h at 125000 g), rough microsomes were collected from the 1.55 M-/2.0 M-sucrose interface, and smooth microsomes from the 1.0 M-/1.35 M-sucrose interface. The microsomes were diluted 1:1 with TKM buffer and then recovered by sedimentation (40 min at 137000 g). Then the microsomes were resuspended in high-salt buffer (50 mM-Tris/HCl, 500 mM-KCl, 10 mM-MgCl₂, pH 7.6) (0.25 ml/g of tissue) and again recovered by sedimentation (40 min at 137000 g) through a layer of 0.6 M-sucrose in high-salt buffer. The rough microsomes obtained had a RNA/protein ratio of 0.202–0.219, and that of the smooth microsomes was 0.034–0.045.

Detergent treatment of rough microsomes

This was performed by the following two methods. The first method was based on the procedure of Kreibich *et al.* (1978a). In brief, rough microsomes were resuspended at a concentration of 3.5 mg of protein/ml in low-salt buffer (50 mM-Tris/HCl, 50 mM-KCl, 5 mM-MgCl₂, pH 7.5) containing 0.25 mM-phenylmethanesulphonyl fluoride and 5 mM- β -mercaptoethanol. Emulgen 913 was added to the microsomal suspension to a final concentration of 1.5% (w/v). After incubation for 30 min at 4 °C, the mixture was layered over 2 ml of 20% (w/v) sucrose in low-salt buffer containing 1.5% Emulgen 913, followed by centrifugation for 1 h at 125000 g. The pellet obtained is called the 'Emulgen-ribosomes' and the supernatant the 'Emulgen-soluble fraction'. The second method was a slight modification of the procedure of Fujita *et al.* (1977). Rough microsomes were resuspended at a concentration of 10 mg of protein/ml in low-salt buffer, and then Triton X-100 was added to a final concentration of 1% (w/v). After incubation for 30 min at 4 °C, the mixture was layered over a step gradient consisting of 2 ml of 1.6 M-sucrose in TKM buffer and 1.5 ml of 0.5 M-sucrose in TKM buffer, followed by centrifugation for 6 h at 136000 g (Hitachi RP 50 rotor). The pellet obtained is called the 'Triton-ribosomes'.

Preparation of the protein fraction (R-fr) from Emulgen-ribosomes

Emulgen-ribosomes were suspended in TK buffer (20 mM-Tris/HCl, 25 mM-KCl, pH 8.6) at a concentration of 10 mg of protein/ml, and then deoxycholate was added to a final concentration of 1% (w/v). After 30 min at 4 °C, the mixture was centrifuged for 5 h at 100000 g (Hitachi RPS 40 T-2 rotor). The supernatant obtained is called 'R-fr'.

Isolation of ³H-labelled ribosomes

A male Wistar rat weighing 170 g received 300 μ Ci of [^{5-³H}]orotic acid (0.64 Ci/mmol diluted with non-labelled orotic acid) intraperitoneally, 38 h before being killed. The rat was starved for 18 h before death. Isolation of total microsomes and detachment of bound ribosomes from the microsomal fraction were performed by the puromycin/KCl method of Borgese *et al.* (1974). The specific radioactivity of the ribosomes obtained was 2960 d.p.m./ μ g of RNA. Ribosomes suspended in TKM (3 mg of RNA/ml) were stored at –80 °C until use.

Preparation of ribosome-stripped rough microsomes

Rough microsomes were stripped of their ribosomes essentially as described by Borgese *et al.* (1974). The stripped rough microsomes obtained had a RNA/protein ratio of 0.030–0.041. The stripped rough microsomes suspended in TKM were diluted with 2 vol. of glycerol and then stored at –20 °C. Before use, a suspension was diluted with 2 vol. of TKM buffer, and then the microsomes were recovered by centrifugation (20 min at 100000 g).

Glycoprotein purification

Glycoproteins showing affinity for Con A were purified from R-fr as described by Rodriguez-Boulant *et al.* (1978). An 11 ml portion of R-fr (1.2 mg of protein/ml) in TND buffer (20 mM-Tris/HCl, 200 mM-NaCl, 1% deoxycholate, pH 8.6) was passed through a Con A-Sepharose 4B column (2 ml gel bed) pre-equilibrated with TND buffer. The column was washed thoroughly with TND buffer. Glycoproteins bound to the column were eluted with 3 ml of 100 mM- α -methyl mannoside in TND buffer.

Reconstitution of membrane proteins into liposomes

To 2.5 mg of phosphatidylcholine dissolved in chloroform/methanol (2:1, v/v) was added a suitable amount of [¹⁴C]phosphatidylcholine and an equal volume of benzene. The mixture was dried to a thin film in a glass tube. The dried phosphatidylcholine was redissolved in 0.5 ml of TKDC buffer [50 mM-Tris/HCl, 25 mM-KCl, 0.41% deoxycholate, 0.41% (w/v) sodium cholate, pH 7.6] containing 0.22 mg of the proteins to be reconstituted, followed by dialysis against two changes of 1000 ml of 50 mM-Tris/HCl/25 mM-KCl, pH 7.6, for 38–48 h. For the Emulgen-soluble fraction, 0.2 g of Amberlite XAD-2 was added to the fraction, followed by stirring for 2 h to remove the detergent before its reconstitution into liposomes.

Analysis of ribosome binding to reconstituted liposomes

The binding mixture contained the reconstituted liposomes (88 μ g of protein) and ³H-labelled ribosomes (30 μ g of RNA) in 0.5 ml of TKM buffer. After incubation at 4 °C for 30 min, the mixture was layered over a linear 0.1–1.3 M-sucrose gradient (3 ml) in TKM buffer on top of 0.5 ml each of 1.7 M- and 2.4 M-sucrose in TKM buffer, followed by centrifugation at 100000 g for 10 h in a Hitachi RPS 40 T-2 rotor. After the centrifugation, the sucrose gradient was collected as 20 equal fractions. Radioactivity was determined with an Aloka LSC-903 scintillation counter. For reconstituted liposomes alone, the mixture was made 1.0 M in sucrose,

and then layered under a linear 0–0.6 M-sucrose gradient (3.5 ml) in TKM buffer and centrifuged as above.

Analysis of ribosome binding to stripped rough microsomes

This was performed fundamentally by the method of Borgese *et al.* (1974). Stripped rough microsomes (174 μg of protein) and ^3H -labelled ribosomes (7.8–156 μg of RNA) were incubated at 4 °C for 30 min in 120 μl of TKM buffer. The mixture was made 2 M in sucrose by adding 1.08 ml of 2.22 M-sucrose in TKM buffer. A linear 1.3–1.9 M-sucrose gradient (3.42 ml) in TKM buffer was layered over 0.72 ml of the mixture laid at the bottom of a 5 ml tube (Hitachi RPS 40 T-2 rotor). Then 0.36 ml of TKM buffer was laid on top of the sucrose gradient. After centrifugation at 100 000 g for 5 h, the sucrose gradient was collected as 20 equal fractions to determine the radioactivity.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was carried out fundamentally by the method of Laemmli (1970) on 7–12% linear polyacrylamide gradient gels. Each sample was heated at 100 °C for 2 min in 7% (w/v) SDS/0.7 M-dithiothreitol/1.4 mM-EDTA/0.0035% (w/v) Bromophenol Blue/62.5 mM-Tris/HCl, pH 6.8.

Analysis

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. The concentrations of RNA were determined directly by using $A_{260}^{1\%} = 265$ at 260 nm (Wilson & Hoagland, 1965). RNA was determined by the method of Fleck & Munro (1962), by using $A_{260}^{1\%} = 312$ at 260 nm. The concentration of ribosomes was calculated by assuming that the ribosomes contain 52% RNA and M_r is 4.5×10^6 (Hamilton *et al.* 1971).

RESULTS

Identification of ribophorins I and II in the membrane-protein fraction

A membrane-protein fraction (R-fr) showing high affinity for ribosomes was isolated from the Emulgen-ribosomes as described in the Experimental section and subjected to electrophoresis. As shown in Fig. 1 (lane *d*), two protein components contained in R-fr were identified as ribophorins I and II, for the following reasons. The M_r values of these two components (65 000 and 62 000) were very close to those of ribophorins I and II (65 000 and 63 000 respectively). These components are contained in rough microsomes (lane *a*), but are not present or only found in very low amounts in smooth microsomes (lane *b*). They were recovered in association with the polysomes after the microsomes had been treated with Emulgen 913 (lane *c*), whereas the use of Triton X-100 in place of Emulgen 913 decreased the content of these proteins in the ribosomes from rough microsomes (lane *e*). Both components appeared to be glycoproteins on Con A-Sepharose column chromatography, as described below. These properties of the two components are consistent with the characteristics of ribophorins reported by Kreibich *et al.* (1978a) and Rodriguez-Boulán *et al.* (1978).

Fig. 1. SDS/polyacrylamide-gel electrophoresis of microsomes and microsomal subfractions

Rough (*a*) and smooth (*b*) microsomes were prepared as described in the Experimental section. Emulgen-ribosomes (*c*) and Triton-ribosomes (*e*) were obtained from rough microsomes treated with 1.5% (w/v) Emulgen 913 and 1% (w/v) Triton X-100 respectively. R-fr (*d*) was obtained from Emulgen-ribosomes treated with 1% (w/v) deoxycholate. Electrophoresis was carried out in SDS/polyacrylamide (7–12%) gels; approx. 60–80 μg of protein was loaded in each gel slot. The gels were stained with Coomassie Blue. The positions of ribophorins are indicated by arrows, I and II. The numbers on the left indicate the apparent M_r values estimated on the basis of the mobilities of marker proteins.

Ribosome-binding activity of R-fr

R-fr corresponded to approx. 10% of the total membrane proteins of rough microsomes. Residual membrane proteins were recovered as the Emulgen-soluble fraction. Ribosome-binding activity was exclusively observed in the liposomes containing R-fr, but virtually none was found in the liposomes containing the Emulgen-soluble fraction or in those made from phosphatidylcholine alone (Fig. 2). These results show that the ribosome-binding activity was concentrated in R-fr. In a previous study, we showed that R-fr isolated from Triton-ribosomes and reconstituted into liposomes exhibits binding activity toward polysomes (Yamaguchi *et al.*, 1981). In the present study (results not shown), it

Fig. 2. Binding of ribosomes to liposomes reconstituted with R-fr and the Emulgen-soluble fractions

^{14}C -labelled liposomes containing R-fr (a) or the Emulgen-soluble fraction (b) (protein/lipid ratio of 0.088), and ^{14}C -labelled liposomes made from phosphatidylcholine alone (c) were prepared as described in the Experimental section. Each binding mixture, containing 88 μg of protein of the reconstituted liposomes and 30 μg of RNA of ^3H -labelled ribosomes in TKM buffer, was layered over a 0.1–1.3 M-sucrose gradient on top of 1.7 M- and 2.4 M-sucrose in TKM buffer, followed by centrifugation at 100 000 g for 10 h. Direction of sedimentation was from right to left. In (c), phosphatidylcholine liposomes containing the same quantity of ^{14}C radioactivity as in (a) were incubated with 30 μg of RNA of ^3H -labelled ribosomes and centrifuged as above. ●, ^{14}C radioactivity; ○, ^3H radioactivity.

was found that the liposomes containing R-fr isolated from Triton-ribosomes showed a similar ribosome-binding activity to that of R-fr isolated from Emulgen-ribosomes.

Ribosome-binding activity of the Con A-bound and Con A-unbound fractions

It was reported that ribophorins are glycoproteins showing affinity for Con A (Rodriguez-Boulán *et al.*, 1978). Therefore, R-fr was separated into two fractions on a Con A-Sepharose column as described in the Experimental section. The recovery of protein was approx. 79%. As shown in Fig. 3, ribophorins were exclusively found in the Con A-bound fraction. Each of the Con A-bound and Con A-unbound fractions was reconstituted into liposomes, and then their ribosome-binding activities were determined. As shown in Fig. 4, the liposomes containing the Con A-unbound fraction exhibited a ribosome-binding activity much higher than that containing the Con A-bound fraction and a little higher than that of the R-fr. The specific binding activity of the Con A-unbound fraction was approx. 2.3-fold higher than that of the Con A-bound fraction (Table 1). The recovery of ribosome-binding capacity of the two fractions was about 85% of the total binding capacity of the R-fr applied to a Con A-Sepharose column, and about 90% of it was found in the Con A-unbound fraction (Table 1).

Confirmation of the reconstitution of ribophorins into liposomes

The data presented above showed that the Con A-bound fraction enriched in ribophorins exhibited a rather low ribosome-binding activity. Therefore whether

or not ribophorins were definitely reconstituted into liposomes was determined. The reconstituted liposomes were prepared with the Con A-bound fraction as described in the Experimental section and divided into two parts, one part being subjected to sucrose-gradient centrifugation to isolate the reconstituted liposomes. After the centrifugation, the liposome profile was depicted by measuring radioactivity over the whole sucrose gradient. The phospholipid/protein ratio of the isolated liposomes was close to that of the initial mixture. Then the liposomes were electrophoresed on a SDS/polyacrylamide gel together with the other part, which had been left to stand. They showed nearly identical electrophoretic patterns (results not shown), indicating that at least the major protein components, including ribophorins, in the Con A-bound fraction were all reconstituted into the liposomes. Next, to determine whether or not the incorporated ribophorins were exposed on the outer surface of the reconstituted liposomes, one part of the reconstituted liposomes isolated by sucrose-gradient centrifugation was labelled with isethionyl [1- ^{14}C]acetimidate, which was used to label proteins exposed on the outer surface of membranes (Whitely & Berg, 1974). Another part of the reconstituted liposomes was labelled similarly in the presence of 1% deoxycholate. As shown in Fig. 5, a good similarity was observed in the two fluorograms (lanes a and b). These fluorograms were also similar to the silver-stained electrophoretogram of the Con A-bound fraction as shown in Fig. 3 (lane b), with respect to the major protein components. These results show that most of the major proteins, including ribophorins, are incorporated into the liposomes and exposed on the outer surface of membranes of the reconstituted liposomes.

Fig. 3. SDS/polyacrylamide-gel electrophoresis of the Con A-bound and Con A-unbound fractions

R-fr(*a*) from Emulgen-ribosomes was applied to Con A-Sepharose columns and separated into two fractions, Con A-bound (*b*) and Con A-unbound (*c*), as described in the Experimental section. Electrophoresis was carried out in SDS/polyacrylamide (7–12%) gels; approx. 1–2 μ g of protein was loaded in each gel slot. The gels were silver-stained (Merrill *et al.*, 1981). The positions of ribophorins are indicated by arrows, I and II. The numbers at the sides of the gels indicate the apparent M_r values ($\times 10^{-3}$).

Quantitative measurement of ribosome binding

To determine ribosome binding quantitatively, increasing amounts of ^3H -labelled ribosomes were added to a fixed amount of the liposomes reconstituted with the Con A-bound or Con A-unbound fraction, and the amounts of ribosomes bound to the liposomes were determined as described in the Experimental section. As shown in Fig. 6(*a*), both fractions have a finite number of binding sites and become saturated when a sufficient amount of ribosomes is added. From the data in Fig. 6(*a*), Scatchard plots were drawn, as shown in Fig. 6(*b*),

from which the number of binding sites per g of protein and the affinity constants of ribosomes for these sites were calculated (Table 2). The Con A-unbound fraction possesses more than twice as many binding sites as the Con A-bound fraction does. This is compatible with the specific-binding-activity results shown in Table 1. On the other hand, the two fractions have rather similar affinity constants for ribosomes. However, with only these results it is uncertain whether or not similar kinds of binding sites are present in the two fractions.

The ribosome-affinity constants found for the two fractions are about one order of magnitude higher than that for the ribosome-stripped rough microsomes obtained by Borgese *et al.* (1974) (Table 2). We determined the affinity constant of the stripped rough microsomes with the same protein concentration as used by Borgese *et al.* (1974). As shown in Table 2, the affinity constant obtained was close to that reported by Borgese *et al.* (1974). However, a slightly higher value was obtained when the measurement was performed at a lower protein concentration of stripped rough microsomes (Table 2). A similar concentration-dependency was observed for the affinity constant of the stripped rough microsomes, which was determined with an intermediate protein concentration by Amar-Costesec *et al.* (1984) (Table 2). However, it appears that the concentration-dependency is not enough to explain the difference between the affinity constants of the two fractions and the stripped rough microsomes. The affinity constant might be altered by the deoxycholate treatment or by the incorporation into liposomes resulting in the change in membrane environment surrounding ribosome-binding protein.

Inhibition of ribosome binding to the reconstituted liposomes by mild proteolysis or by increasing KCl concentration

The ribosome-binding sites on stripped rough microsomes are known to lose much of their ability to bind ribosomes after preincubation for 15 min at 0 °C in the presence of trypsin and chymotrypsin (50 μ g/ml each) (Borgese *et al.*, 1974). Furthermore, increasing the KCl concentration in the binding mixture from 25 to 200 mM caused a marked decrease in the ribosome-binding activity of stripped rough microsomes (Borgese *et al.*, 1974). Similar results were obtained for the ribosome-binding activity of the liposomes reconstituted with the Con A-unbound fraction. As shown in Fig. 7, the ability of the reconstituted liposomes to bind ribosomes completely disappeared after mild treatment with trypsin (3 μ g/ml) at 0 °C for 60 min or in the presence of 200 mM-KCl in the binding mixture and in the sucrose gradient.

DISCUSSION

Ribophorins I and II have been thought to play a role in mediating the binding of ribosomes to endoplasmic-reticulum membranes. This postulate is supported by the following findings. Ribophorins are integral membrane glycoproteins found in rough microsomes, but are virtually completely absent from smooth microsomes prepared from several tissues of different species (Kreibich *et al.*, 1978a; Marcantonio *et al.*, 1982). When the microsomal membranes are solubilized with a non-ionic detergent, such as Kyro EOB or Emulgen 913,

Fig. 4. Binding of ribosomes to liposomes reconstituted with R-fr and Con A-bound and Con A-unbound fractions

Con A-bound and Con A-unbound fractions were obtained from R-fr by using Con A-Sepharose columns as described in the Experimental section. The ^{14}C -labelled liposomes containing R-fr (a), the Con A-bound fraction (b) or the Con A-unbound fraction (c) (protein/lipid ratio of 0.088) were prepared as described in the Experimental section. The binding reaction with ^3H -labelled ribosomes and the subsequent centrifugation for analysis of ribosome binding were carried out as described in the legend to Fig. 2. Direction of sedimentation was from right to left. ●, ^{14}C radioactivity; ○, ^3H radioactivity.

Table 1. Comparison of the ribosome-binding activities of the Con A-bound and Con A-unbound fractions

Specific binding activity was calculated from Fig. 4. Values for total binding capacity are computed from the protein amounts and the specific binding activity in each fraction. The recovery of ribosome-binding capacity is 84.9%.

Fraction	Protein (mg)	Specific binding activity (mg of RNA/mg of protein)	Total binding capacity (mg of RNA)
R-fr	20.9*	0.241	5.04 (100%)
Con A-bound	3.1†	0.124	0.38 (7.5%)
Con A-unbound	13.4†	0.291	3.90 (77.4%)

* Total amount of protein applied to Con A-Sepharose column.

† Protein amounts found in the two fractions. The recovery of protein is 78.9%.

Table 2. Affinity constants and numbers of binding sites in the ribosome binding with reconstituted liposomes containing the Con A-bound and Con A-unbound fractions and with stripped rough microsomes

The preparation of membrane protein fractions and analysis of ribosome binding with reconstituted liposomes containing these membrane protein fractions were performed as described in the Experimental section. The affinity constant, K , was calculated from the slope of the Scatchard plot (see Fig. 6b). The amount of binding sites was calculated from the Scatchard plot by extrapolation to zero ordinate value (see Fig. 6b).

Sample	Concn. of membrane protein (mg/ml)	K (M^{-1})	$10^8 \times$ Binding sites (mol/g of protein)	Reference
Con A-bound fraction	0.176	10.2×10^8	6.1	Present study
Con A-unbound fraction	0.176	13.2×10^8	15.2	Present study
Stripped rough microsomes	1.45	0.67×10^8	7.3	Present study
	0.363	2.91×10^8	4.6	Present study
	1.45	$(0.62-0.87) \times 10^8$	4.1-5.4	Borgese <i>et al.</i> (1974)
	0.83	1.7×10^8	—	Amar-Costesec <i>et al.</i> (1984)

Fig. 5. Exposure of ribophorins in the reconstituted liposomes

The Con A-bound fraction was reconstituted into liposomes (protein/lipid ratio of 0.088) as described in the Experimental section. The reconstituted liposomes were purified by centrifugation at 100000 *g* for 10 h on a 0–0.6 M-sucrose gradient. The reconstituted liposomes thus obtained were labelled at 23 °C for 2 h with 45 μ Ci of isethionyl [1-¹⁴C]acetimidate in the presence (a) or absence (b) of 1% (w/v) deoxycholate in 200 mM-triethanolamine buffer, pH 8.5. Each sample was precipitated by the methanol/chloroform methanol (Wessel & Flugge, 1984), and then treated as described in the Experimental section. Electrophoresis was carried out in SDS/polyacrylamide (7–12%) gels, followed by fluorography (Skinner & Griswold, 1983). The dried gel was exposed for 2 days to an X-ray film (Fuji AIF-RX; Tokyo, Japan). The positions of ribophorins are indicated by arrows, I and II. The numbers on the left indicate the apparent *M_r* values ($\times 10^{-3}$).

both proteins are recovered in association with polysomes (Kreibich *et al.*, 1978a; Marcantonio *et al.*, 1982). These proteins could be cross-linked to ribosomes by using bifunctional reagents (Kreibich *et al.*, 1978b). Good stoichiometry was seen between the amount of these

proteins in rough microsomes and the ribosome-binding capacity of rough microsomes (Kreibich *et al.*, 1980; Marcantonio *et al.*, 1984). However, direct evidence of the ribosome-binding activity of ribophorins has not yet been obtained.

In the present study a membrane protein fraction showing high affinity for ribosomes was isolated from rat liver rough microsomes, and separated into two fractions, Con A-bound and Con A-unbound, on Con A-Sepharose columns. These protein fractions were incorporated into liposomes to determine the ribosome-binding activities of the reconstituted liposomes. About 85% of the total binding capacity of the material applied to the Con A-Sepharose column was recovered as the two fractions, and about 90% of the recovered binding capacity was found in the Con A-unbound fraction (Table 1), which was virtually free from ribophorins. The ribosome binding depended on the amount of ribosomes added to the binding mixture, and became saturated when a sufficient amount of ribosomes had been added. Although the affinity constants of the two fractions were somewhat higher than that of the ribosome-stripped rough microsomes, similarity between the two binding systems was observed in the sensitivity to mild proteolysis and the suppression of ribosome binding by increasing KCl concentration. These results support the idea that ribosome binding to rat liver microsomes is not directly mediated by ribophorins, but that another unidentified membrane protein(s) plays a role in the ribosome binding. Exposure of ribophorins on the outer surface of the liposome membrane was represented by surface protein labelling with isethionyl [1-¹⁴C]acetimidate. However, as their orientation in the liposomal membrane was not examined in this study, the possibility could not be excluded that most of ribophorins may be oriented inverted with respect to microsomes and hence unable to interact with ribosomes. However, as shown in Table 1, the Con A-unbound fraction represented a slightly higher specific binding activity than that of the R-fr, and about 77% of binding capacity of the R-fr was found in the Con A-unbound fraction free from ribophorins. These results make the above possibility very improbable, and suggest the existence of a putative ribosome-binding protein other than ribophorins. The use of Triton X-100 in place of Emulgen 913 facilitated the dissociation of ribophorins from ribosomes, which is compatible with the observations by Kreibich *et al.* (1978a, 1982), and resulted in a decrease in the content of ribophorins in R-fr isolated from Triton-ribosomes. However, R-fr thus obtained showed a similar extent of ribosome-binding activity to that of R-fr isolated from the Emulgen-ribosomes. This also suggests the existence of putative ribosome-binding proteins that are different from ribophorins and are in more stable association with ribosomes than are ribophorins. The cross-linking of ribophorins to ribosomes and the stoichiometry between ribophorins and ribosomes may suggest the possibility that ribophorins are components of the translocation apparatus including ribosome-binding sites, and thus may play some role in the co-translational process, such as in the insertion of nascent polypeptide chains into the membrane or their transport into the cisternal lumen.

Marcantonio *et al.* (1984) reported that, when rough microsomes were solubilized with Emulgen 913, two proteins (*M_r* 72000 and 83000) in addition to ribophorins were recovered in association with ribosomes. They also

Fig. 6. Characterization of ribosome binding with reconstituted liposomes

(a) Saturation of binding sites by ribosomes. ¹⁴C-labelled reconstituted liposomes containing Con A-bound or con A-unbound fraction (88 μg of protein each) were incubated at 4 °C for 30 min with various amounts of ³H-labelled ribosomes in 500 μl of TKM buffer. Ribosomes bound to liposomes were separated from unbound material by centrifuging the binding mixture of 100000 g for 10 h on a 0.1–1.3 M-sucrose gradient on top of 1.7 M- and 2.4 M-sucrose in TKM buffer. (b) Scatchard plots of the data in (a). The points were fitted by means of a least-squares linear-fit program. The affinity constants (*K*) calculated from the slopes were $10.2 \times 10^8 \text{ M}^{-1}$ for the Con A-bound fraction and $13.2 \times 10^8 \text{ M}^{-1}$ for the Con A-unbound fraction.

indicated that these proteins are present in higher concentrations in microsomal subfractions with higher ribosome contents. In the present study, two proteins with similar *M_r* values (73000 and 83000) were also observed in R-fr (Fig. 3). The *M_r*-83000 protein was present in the Con A-bound fraction, suggesting that it is not involved in ribosome binding. On the other hand, the

M_r-73000 protein was found in the Con A-unbound fraction, showing ribosome-binding activity. The *M_r* of this protein is very close to that of the docking protein (Meyer *et al.*, 1982a; Gilmore *et al.*, 1982). Meyer *et al.* (1982b) reported that the docking protein does not bind to Con A. These facts provide a possibility that the *M_r*-73000 protein corresponds to the docking protein.

Fig. 7. Inhibition of ribosome binding to the reconstituted liposomes by mild proteolysis and by increasing KCl concentration

¹⁴C-labelled liposomes containing the Con A-unbound fraction (protein/lipid ratio of 0.088) were prepared as described in the Experimental section. In a control experiment (a), the binding mixture, containing 88 μg of protein of the reconstituted liposomes and 30 μg of RNA of ³H-labelled ribosomes, was layered over a 0.1–1.3 M-sucrose gradient on top of 1.7 M- and 2.4 M-sucrose in TKM buffer and then centrifuged at 100000 g for 10 h. Direction of sedimentation was from right to left. In (b), before the binding reaction the reconstituted liposomes were incubated at 0 °C for 1 h with 3 μg of trypsin/ml, followed by the addition of 30 units of trasylol/ml. In (c), the binding reaction was performed in the presence of 200 mM-KCl, followed by centrifugation through the above gradient containing 200 mM-KCl. ●, ¹⁴C radioactivity; ○, ³H radioactivity.

However, it was shown by Hortsch *et al.* (1986) that the docking protein is not involved in ribosome binding. During the preparation of this manuscript, a study on the ribosome-membrane interaction in dog pancreas endoplasmic reticulum was reported by Hortsch *et al.* (1986); they also concluded that ribosome binding is not directly mediated by ribophorins.

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