Isolation and Characterization of Membrane Proteins Responsible for Attachment of Polyribosomes to Rough Microsomal Fraction of Rat Liver

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A protein fraction which has a high affinity for polyribosomes was isolated from rough microsomal membranes of rat liver. The mode of polyribosome binding to this fraction (R-fraction) was studied by using CsCl equilibrium centrifugation and compared with that for stripped rough microsomal membranes. The following were found. (1) The polyribosome-binding capacity of the R-fraction was heat-labile and sensitive to trypsin, and was suppressed by increasing KCl concentration and addition of 0.1 mM-aurointricarboxylic acid. (2) Of the four subfractions obtained by gel filtration of the R-fraction on a Sephadex G-200, only the R_1 -fraction, eluted at the void volume, showed a high affinity for polyribosomes. The polyribosome-binding capacity of the R_1 -fraction decreased with time on storage at 4°C. (3) The R_1 -fraction contained three major proteins with mol.wts. 108 000, 99 000 and 65 000.

Evidence has been obtained that indicates that the interaction between polyribosomes and rough microsomal membranes occurs through the large ribosomal subunits (Sabatini *et al.*, 1966; Borgese *et al.*, 1973) or mRNA (Lee *et al.*, 1971) or its poly(A) segments (Lande *et al.*, 1975), and becomes more tightly bound by the nascent polypeptide chains (Adelman *et al.*, 1973). To explore the mechanism of polyribosome-membrane interaction, many studies have been carried out using a recombination system *in vitro* of polyribosomes and stripped rough microsomal membranes (Suss *et al.*, 1966; Ragland *et al.*, 1971; Shires *et al.*, 1971, 1973; Khawaja, 1971; Rolleston, 1972; Nolan & Munro, 1972; Burke & Redman, 1973; Borgese *et al.*, 1974). However, the polyribosome-binding site on the membranes remains to be characterized, but, up until now, few studies have been done to achieve this. It has been shown that phosphatidylcholine is not involved in the polyribosome-binding site (Jothy *et al.*, 1973; Borgese *et al.*, 1974). Other workers have shown that neuraminidase treatment of stripped rough microsomal membranes inhibits polyribosome-binding (Scott-Burden & Hawtrey, 1973). Partial proteolysis of stripped rough microsomal membranes with trypsin also eliminates their binding capacity (Shires *et al.*, 1971; Borgese *et al.*, 1974). Electrophoretic analysis of the rough microsomal membranes after proteolysis by low concentrations of trypsin and chymotrypsin have shown that three membrane

proteins with mol.wts. 120 000, 93 000 and 36 000 are correlated with the polyribosome-binding capacity of the membranes (Jothy *et al.*, 1975).

The objective of the present study was to isolate and characterize the membrane components that are responsible for the attachment of polyribosomes to the rough microsomal membranes. Olsnes (1971) showed that polyribosomes solubilized from microsomal membranes by Triton X-100 are bound by extraneous proteins at low salt concentration, which may be removed from the polyribosomes by treatment with deoxycholate. In the present study, the mode of polyribosome binding to this protein fraction was studied and compared with that for stripped rough microsomal membranes. This protein fraction was further fractionated into four subfractions by using gel filtration. The high affinity for polyribosomes was localized in one of these subfractions. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed the existence of three major proteins characteristic of this fraction, with mol.wts. 108 000, 99 000 and 65 000, besides several minor components.

Experimental

Materials

Sodium deoxycholate, puromycin and aurointricarboxylic acid were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pancreatic ribonuclease was from BDH Chemicals (Poole, Dorset, U.K.). Triton X-100 was from Wako Chemical Co. (Osaka, Japan). CsCl (DGD grade) was from Nakarai

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Chemicals (Osaka, Japan). Bio-Beads SM-2 were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-75, G-200 and Sepharose 6B were from Pharmacia (Uppsala, Sweden). [6-¹⁴C]Orotic acid (57mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Fractionation and labelling of liver cells

Postmitochondrial supernatant. Male Wistar rats weighing 200–230g were starved for 18h before death. The following procedures were carried out at 0–4°C, unless otherwise specified. Livers chilled in an ice-cold buffer were homogenized with 2.5 vol. of 0.25M-sucrose in TKM buffer (50mM-Tris/HCl, 25mM-KCl, 5mM-MgCl₂, pH7.6). The homogenate was centrifuged at 15000g for 15min to obtain a postmitochondrial supernatant.

Rough microsomal fraction and R-fraction. A 13ml portion of the postmitochondrial supernatant was layered over 10ml of 1.35M-sucrose in TKM buffer on top of 10ml of 2.0M-sucrose in the same buffer and centrifuged at 177000g for 4.5h in a Hitachi RP 50-2 rotor. The material at the 1.35M-/2.0M-sucrose interface was collected and diluted with 2 vol. of 0.25M-sucrose in TKM buffer and centrifuged at 126000g for 1h. The pellet (rough microsomal fraction) was suspended in 0.25M-sucrose in TKM buffer (0.5ml/g of tissue) and made 1% (w/v) with respect to Triton X-100. It was layered over an equal volume of 1.8M-sucrose in TKM buffer and centrifuged at 138000g for 3h. The supernatant was the Triton-soluble fraction. The pellet (Triton-polyribosomes) was suspended in high-pH buffer (50mM-Tris/HCl, 25mM-KCl, 1mM-MgCl₂, pH8.6) (0.2ml/g of tissue) and treated with 1% (w/v) deoxycholate. Then it was layered over a 0.5–1.0M-sucrose density gradient in high-pH buffer, containing 0.5% (w/v) deoxycholate, and centrifuged at 74800g for 12h in a Hitachi RPS 25-2 rotor. After the centrifugation, the region of the slowest-moving peak on the gradient (as indicated in Fig. 2) was pooled as the R-fraction. If needed, the R-fraction was concentrated by the use of a Diaflo cell equipped with PM-10 Diaflo membrane (Amicon, Lexington, MA, U.S.A.). Alternatively, the R-fraction was prepared from the Triton-polyribosomes, which were isolated from the postmitochondrial supernatant. In this case, the postmitochondrial supernatant was treated with 1% (w/v) Triton X-100 and centrifuged as described above through 1.8M-sucrose in TKM buffer to obtain the Triton-polyribosomes. The procedure for preparation of the R-fraction from the Triton-polyribosomes was the same as described above.

Fractionation of the R-fraction. Sephadex G-200, previously swollen in a large excess of distilled water

for 3h at 85°C, was packed in a column (2.6cm×100cm), and the gel was equilibrated with 20mM-Tris/HCl, pH8.6, containing 0.4% (w/v) deoxycholate. The R-fraction was applied to this column and eluted with the same buffer containing 0.4% (w/v) deoxycholate. Each peak, detected by its *A*₂₈₀, was pooled and concentrated with a Diaflo membrane. The first eluted peak, designated the R₁-fraction, was further purified on a Sepharose 6B column (2.6cm×100cm) equilibrated with the same buffer containing 0.4% (w/v) deoxycholate.

Preparation of stripped rough microsomal-membrane fraction. The rough microsomal membranes were stripped of their ribosomes essentially as described by Shires *et al.* (1971). The rough microsomal-membrane pellet was resuspended in 0.25M-sucrose in TK buffer (50mM-Tris/HCl, 25mM-KCl, pH7.0) (2ml/g of tissue). To the suspension were added ribonuclease and EDTA (final concns. 20μg/ml and 5mM respectively); the mixture was then incubated for 90min at 4°C. The resulting solution was layered over an equal volume of 0.5M-sucrose in TKM buffer, centrifuged at 106000g for 1h, washed twice with 0.25M-sucrose in TKM buffer and suspended in 0.25M-sucrose in TeaKM buffer (50mM-triethanolamine/HCl, 25mM-KCl, 5mM-MgCl₂, pH7.6).

Preparation of ¹⁴C-labelled polyribosomes. Male Wistar rats weighing 170–180g were injected intraperitoneally with 10 or 20μCi of [6-¹⁴C]orotic acid/rat, then starved for 16h, killed and their livers were excised. The postmitochondrial supernatant obtained as described above was treated with deoxycholate and Triton X-100 [final concns. 1.3% (w/v) and 0.5% (w/v) respectively]. Then a 4ml portion of the solution was layered over 3ml of 0.5M-sucrose in TKM buffer on top of 3ml of 2.0M-sucrose in TKM buffer and centrifuged at 127000g for 6h in a Hitachi RP 55T rotor. The polyribosomal pellet obtained was suspended in 0.25M-sucrose in TeaKM buffer (0.25ml/g of tissue) containing 0.1 vol. of ribonuclease-inhibitor fraction, which was prepared from rat liver by the method of Shortman (1961). The suspension of polyribosomes was stored at –20°C until use.

Removal of detergents

Deoxycholate was removed by gel filtration through a Sephadex G-75 column (1.9cm×21cm) equilibrated with 0.25M-sucrose in triethanolamine/HCl, pH7.6. A 2ml portion of the sample was applied to the column and eluted with the same buffer, and the fraction eluted at the void volume was collected. Deoxycholate was well separated from the fraction eluted at the void volume. Triton X-100 was removed by the use of Sephadex G-75 or Bio-Beads SM-2 (Holloway, 1973). The procedure using

the Sephadex G-75 column was the same as described above, but in this case it was found that some of the Triton X-100 was eluted at the void volume, probably because of the large size of the micelles of Triton X-100 (Makino *et al.*, 1973). The procedure using Bio-Beads SM-2 was as follows: to the sample containing Triton X-100 was added 0.2 vol. of Bio-Beads SM-2, which were previously treated as described by Holloway (1973), and the mixture was stirred for 90 min at 0°C, followed by centrifugation at 1000g for 15 min to remove the Bio-Beads. This procedure was repeated once more. A separate experiment showed that the concentration of Triton X-100 decreased from 1 to 0.016% after one treatment with Bio-Beads.

Binding reaction

The incubation mixture for the binding reaction contained membranes or membrane fractions and ^{14}C -labelled polyribosomes or monoribosomes in quantities as indicated in the legend of each

Figure in 1 ml of 0.25M-sucrose in TeaKM buffer. The incubation was for 30 min at 4°C, and then 30% (v/v) formaldehyde in 50mM-triethanolamine/HCl, pH 7.6, was added to give a final concentration of 4%; the mixture was kept in ice for 24 h or more.

CsCl-equilibrium-centrifugation analysis

To date, there are two assay procedures for separating the membrane-associated polyribosomes from the non-associated polyribosomes after the binding reaction: one is sedimentation and the other is the flotation procedure (Shires *et al.*, 1975). Both are based on the principle that the buoyant density of the associated complex of polyribosomes with membranes is less than that of high-sucrose medium (e.g. 1.8M). These assay procedures, however, are inadequate for the present study, since the complexes formed between polyribosomes and the R-fraction or its subfractions may have a higher density than 1.8M-sucrose. Therefore we used CsCl equilibrium centrifugation to assay the binding-

Fig. 1. CsCl equilibrium centrifugation of deoxycholate- and Triton-polyribosomes

Postmitochondrial supernatant prepared from rat liver was treated with 1% (w/v) deoxycholate (a) or 1% (w/v) Triton X-100 (b), followed by centrifugation through 2.0M- or 1.8M-sucrose in TKM buffer respectively at 127000g for 6h. Suspension of the resulting pellet in 0.25M-sucrose in TeaKM buffer was made 4% (v/v) with respect to formaldehyde and submitted to CsCl equilibrium centrifugation as described in the Experimental section. Values with arrows indicate the buoyant density (g/ml).

reaction mixture. Takagi & Hoagland (1974) successfully used CsCl equilibrium centrifugation to assay the polyribosome-binding capacity of membranes from phenobarbital-treated or regenerating rat liver.

CsCl equilibrium centrifugation was carried out essentially by the method of Brunk & Leick (1969). Usually, to 1 ml of the binding mixture, previously treated as described above, was added 1 ml of TeaKM buffer containing 4% (v/v) formaldehyde and then 0.9 g of CsCl. This was then layered over a CsCl solution which is made by adding 1.8 g of CsCl to 2 ml of TeaKM buffer containing 4% (v/v) formaldehyde. After centrifugation at 123 000g for 18 h in a Hitachi RPS 40-T-2 rotor, the CsCl gradient was collected in 10-drop fractions (about 250 drops per gradient). The refractive index was measured to determine the density. A portion of each fraction was used to measure the A_{260} and A_{280} , and to the rest was added 0.6 mg of protein in liver cytosol which was prepared from postmitochondrial supernatant by centrifuging it at 105 000g for 90 min. They were precipitated with 2 ml of ice-cold 5% (w/v) trichloroacetic acid. The precipitates were collected on Millipore HA filters (Bedford, MA, U.S.A.), washed with two additional portions of 5% (w/v) trichloroacetic acid, and the filters were transferred to a planchet to dry. The radioactivity was determined by using a gas-flow counter (Aloka model TDC-6, Nihon-Musen, Japan).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The procedure for electrophoresis was fundamentally based on the methods of Shapiro *et al.* (1967) and Weber & Osborn (1969), except for the use of urea. A sample for electrophoresis was suspended in 0.01 M-sodium phosphate, pH 7.2, containing 2% (w/v) sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol and 0.5 mM-EDTA, incubated for 2 min at 100°C. Electrophoresis was carried out at 8 mA/gel for 4 h in a 7.5% (w/v) polyacrylamide gel (0.6 cm × 8 cm) containing 0.1% (w/v) sodium dodecyl sulphate, 0.1 mM-EDTA, 6 M-urea and 0.1 M-sodium phosphate, pH 7.2. The gel was fixed overnight with 10% (v/v) acetic acid in 50% (v/v) methanol, stained for 5 h with 0.25% (w/v) Coomassie Blue in a mixture of 10% (v/v) acetic acid and 50% (v/v) methanol, and destained with the same solvent mixture.

Analysis

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (crystallized; Sigma Chemical Co.) as standard. Samples containing triethanolamine buffer were washed

twice with 5% (w/v) trichloroacetic acid before the determination of protein. RNA was determined by the method of Fleck & Munro (1962), by using $A_{1\text{cm}}^{1\%} = 313$ at 260 nm. Alternatively, concentrations of ribosomes as RNA were determined directly, by using $A_{1\text{cm}}^{1\%} = 263$ at 260 nm.

Results

Characterization of Triton-polyribosomes

Although sodium deoxycholate has been used most commonly to release polyribosomes from the membranes of the endoplasmic reticulum, a non-ionic detergent such as Triton X-100 can also be used

Fig. 2. Preparation of the R-fraction from Triton-polyribosomes

A suspension of Triton-polyribosomes in the high-pH buffer was treated with 1% (w/v) deoxycholate, layered over a 0.5–1.0 M-sucrose gradient (50 ml) in the high-pH buffer containing 0.5% (w/v) deoxycholate, and centrifuged at 78 000g for 12 h. A portion of each fraction (1.8 ml) was analysed for A_{260} (—) and A_{280} (---) after dilution with water. (a) Triton-polyribosomes from rough microsomal membrane; (b) Triton-polyribosomes from postmitochondrial supernatant. M, L and S indicate the positions of monoribosomes and of large and small ribosomal subunits respectively.

Fig. 3. *Binding of polyribosomes to the R-fraction and to the Triton-soluble fraction*

The R-fraction and the Triton-soluble fraction were prepared from rough microsomal membranes as described in the Experimental section. A binding mixture contained ^{14}C -labelled polyribosomes ($100\mu\text{g}$ of RNA) and R-fraction or Triton-soluble fraction ($200\mu\text{g}$ of protein). After incubation for 30 min at 4°C , analysis on a CsCl gradient was performed as described in the Experimental section. Values with arrows indicate the buoyant density (g/ml). (a) R-fraction; (b) Triton-soluble fraction treated with Bio-Beads SM-2 to remove Triton X-100; (c) Triton-soluble fraction prepared by passage through a Sephadex G-75 column to remove Triton X-100.

Fig. 4. *Binding of monoribosomes to the R-fraction*

A binding mixture contained R-fraction ($130\mu\text{g}$ of protein) and (a) ^{14}C -labelled polyribosomes or (b) monoribosomes ($100\mu\text{g}$ of RNA) in 0.25M -sucrose in TeaKM buffer. After incubation for 30 min at 4°C , the mixture was submitted to CsCl equilibrium centrifugation, details of which are given in the Experimental section. The ^{14}C -labelled monoribosomes were prepared as follows; ^{14}C -labelled polyribosomes were treated with $2\mu\text{g}$ of pancreatic ribonuclease for 2 min at 37°C , followed by centrifugation at $130000g$ for 4 h on 0.5M -sucrose in TeaKM buffer. Values with arrows indicate the buoyant density (g/ml).

Fig. 5. Binding of ribosomal subunits to the R-fraction

A binding mixture contained R-fraction (360 μ g of protein) and (a) 14 C-labelled 60S subunits (21 μ g of RNA) or (b) 14 C-labelled 40S subunits (9.0 μ g of RNA) in 0.25M-sucrose in TeaK₁₀₀M buffer (50mM-triethanolamine/HCl, 100mM-KCl, 5mM-MgCl₂, pH7.6). After incubation for 30min at 4°C, the mixture was submitted to CsCl equilibrium centrifugation, details of which are given in the Experimental section, except that radioactivities were measured by an automatic low-background gas-flow counter (Nuclear-Chicago, background count of about 2.0 c.p.m.). Values with arrows indicate the buoyant density (g/ml). The 14 C-labelled polyribosomes suspended in TeaK₅₀₀M₂ buffer (50mM-triethanolamine/HCl, 25mM-KCl, 2mM-MgCl₂, pH7.6) were treated with KCl and puromycin (final concns. 500mM and 1mM respectively) and incubated for 1h at 0°C, followed by 15min at 37°C (Blobel & Sabatini, 1971). The mixture was layered over a 15–35% (w/v) linear sucrose gradient (25ml) in TeaK₅₀₀M₂ buffer (50mM-triethanolamine/HCl, 500mM-KCl, 2mM-MgCl₂, pH7.6) and centrifuged at 63600g for 10h at 20°C; 0.9ml fractions were collected and the A_{260} was read. Fractions containing 60S or 40S subunits were pooled, concentrated with a Diaflo membrane and dialysed against 400vol. of 0.25M-sucrose in TeaKM buffer before use.

for the same purpose, owing to its mild action on cellular components (Hunter & Korner, 1966; Murty *et al.*, 1975). Some workers have reported that polyribosomes prepared by using Triton X-100 bind more protein than do those prepared by using deoxycholate (Olsnes, 1971; Dissous *et al.*, 1974). To confirm this finding, the polyribosomes isolated from postmitochondrial supernatant by treatment with 1% (w/v) Triton X-100 were submitted to CsCl equilibrium centrifugation for comparison with polyribosomes isolated with deoxycholate. As shown in Fig. 1, the polyribosomes isolated with Triton X-100 (Triton-polyribosomes) were composed of two

components with buoyant densities (ρ) 1.55 and 1.48g/ml, whereas those isolated with deoxycholate (deoxycholate-polyribosomes) were a homogeneous population with $\rho = 1.57$ g/ml. In a separate experiment the polyribosomes with $\rho = 1.55$ g/ml were confirmed as being derived from free polyribosomes, which always showed a slightly lower buoyant density than deoxycholate-polyribosomes. On the other hand, the component with $\rho = 1.48$ g/ml in the Triton-polyribosomes was considered to be the polyribosomes that are associated with a special class of membrane proteins with high affinity for polyribosomes (Olsnes, 1971). The

Fig. 6. Effect of aurintricarboxylic acid on the binding of polyribosomes to the R-fraction and to stripped rough microsomal membranes

In (a) and (b), ^{14}C -labelled polyribosomes ($120\mu\text{g}$ of RNA) were incubated with stripped rough microsomal membranes ($240\mu\text{g}$ of protein) without (a) or with (b) 0.1 mM -aurintricarboxylic acid. In (c) and (d), ^{14}C -labelled polyribosomes ($100\mu\text{g}$ of RNA) were incubated with R-fraction ($200\mu\text{g}$ of protein) without (c) or with (d) 0.1 mM -aurintricarboxylic acid. The incubation for binding and the subsequent analysis on CsCl gradient were carried out as described in the Experimental section. In (a) and (b), the unbound ^{14}C -labelled polyribosomes were detected as the precipitates [470c.p.m. for (a) and 1180c.p.m. for (b)] after CsCl equilibrium centrifugation. Preparations of the R-fraction from postmitochondrial supernatant and of the stripped rough microsomal membranes are also described in the Experimental section. Values with arrows indicate the buoyant density (g/ml). In (b) ----, A_{280} .

Triton-polyribosomes indeed showed a lower A_{260}/A_{280} ratio (1.64, compared with 1.76 for deoxycholate-polyribosomes) and a higher protein/RNA ratio (1.21, compared with 0.90 for deoxycholate-polyribosomes). It should be added that the Triton-polyribosomes after treatment with deoxycholate exhibited a CsCl-centrifugation profile similar to that of deoxycholate-polyribosomes (results not shown).

Preparation of the R fraction from Triton-polyribosomes

Fig. 2(a) shows the profile of sucrose-density-gradient centrifugation of the Triton-polyribosomes

after treatment with deoxycholate. The Triton-polyribosomes in this case were obtained from the rough microsomal fraction. The R-fraction was pooled as indicated in the Figure. Fig. 2(b) shows the case in which the Triton-polyribosomes were isolated directly from the postmitochondrial supernatant. The latter method seems to be advantageous for the following reasons: (a) the recovery of the R-fraction is high; (b) the time for the preparation is shortened, this being desirable to avoid the inactivation of the R-fraction by aging as described below; (c) in the former method, as suggested by the higher A_{260}/A_{280} ratio, the R-fraction may be contaminated with RNA,

Fig. 7. Effect of KCl concentration on the binding of polyribosomes to the R-fraction

The R-fraction was prepared from Triton-polyribosomes, which were isolated from postmitochondrial supernatant as described in the Experimental section. A binding mixture contained ^{14}C -labelled polyribosomes ($100\mu\text{g}$ of RNA) and R-fraction ($200\mu\text{g}$ of protein) in 0.25M -sucrose in TeaK_xM buffer (x indicates the millimolar concentration of KCl specified in each panel). The incubation for binding and the subsequent analysis on CsCl gradient were carried out as described in the Experimental section. Values with arrows indicate the buoyant density (g/ml).

probably resulting from polyribosome degradation by ribonuclease which is solubilized from the rough microsomal fraction by the treatment with Triton X-100 (Sugano *et al.*, 1967).

Polyribosome-binding capacity of the R-fraction

As shown in Fig. 3, the polyribosomes after incubation with the R-fraction showed a new peak during CsCl centrifugation with $\rho = 1.42\text{g/ml}$, as

well as the peak of unbound polyribosomes ($\rho = 1.57\text{g/ml}$), whereas no significant change was observed in the polyribosomes incubated with Triton-soluble fraction. To exclude the possibility that the lack of polyribosome-binding capacity in the Triton-soluble fraction may result from the incomplete removal of Triton X-100 from the latter fraction by the use of the Sephadex G-75 column, that fraction was treated with Bio-Beads SM2, which ensured more complete removal of Triton X-100, as described in

Fig. 8. Elution profile of the R-fraction from Sephadex G-200

The R-fraction from Triton-polyribosomes isolated from postmitochondrial supernatant was applied to a Sephadex G-200 column (2.6cm $\times$ 100cm) and eluted with 20mM-Tris/HCl, pH8.6, containing 0.4% (w/v) deoxycholate; 5ml fractions were collected, and A_{260} (—) and A_{280} (---) measured. A, B, C and D represent the V_e values for the protein standards: γ -globulin (mol.wt. 147000), serum albumin (mol.wt. 68000), γ -globulin light chain (mol.wt. 23500) and cytochrome *c* (mol.wt. 11700). V_0 , void volume.

the Experimental section. But after this treatment also, no significant interaction was observed between ^{14}C -labelled polyribosomes and the Triton-soluble fraction (Fig. 3b).

Binding of monoribosomes and ribosomal subunits to the R-fraction

The R-fraction was also able to bind monoribosomes and ribosomal subunits. As shown in Fig. 4, the binding complex of the R-fraction with monoribosomes showed a slightly lower density than did those with polyribosomes. Moreover, the higher radioactivity of the binding complex with polyribosomes than of that with monoribosomes indicated that polyribosomes bound to the R-fraction to a greater extent than did monoribosomes. This is compatible with a hypothesis that some of the monoribosomes assembled in the polyribosomes are not bound directly to the R-fraction but are connected by a strand of mRNA.

The binding of 60S and 40S ribosomal subunits to the R-fraction is shown in Fig. 5. The binding reaction was carried out in 0.25M-sucrose in $\text{TeaK}_{100}\text{M}$ buffer (50mM-triethanolamine/HCl, 100mM-KCl, 5mM-MgCl₂, pH7.6) containing R-fraction and 60S or 40S subunits in the amounts specified in the legend of Fig. 5. The amounts of

each subunit bound to 360 μg of R-fraction protein were 14.3 μg of RNA for 60S subunits and 5.1 μg RNA for 40S subunits. As calculated on the basis of the molecular-weight ratio (Hamilton *et al.*, 1971) of 60S/40S subunits (nearly 2), the molar quantity of 60S subunits bound to the R-fraction is about 40% higher than that of 40S subunits. Several workers reported different effects of increasing KCl concentration on the binding of both subunits to the stripped rough-microsomal-membrane fraction (Rolleston, 1972; Borgese *et al.*, 1974).

Similarity of the modes of polyribosome binding to the R-fraction and to the stripped rough-microsomal-membrane fraction

Studies on the polyribosome-membrane association *in vitro* have so far been largely carried out with rough endoplasmic reticulum stripped of endogenous polyribosomes, so it was decided to compare the mode of polyribosome binding to the R-fraction with that to this stripped rough-microsomal-membrane fraction. First, the effect of aurintricarboxylic acid, which is known to inhibit strongly the binding of ribosomes to stripped rough microsomal membranes (Borgese *et al.*, 1974), was examined. As shown in Fig. 6, addition of 0.1mM-aurintricarboxylic acid to the binding mixture resulted in the disappearance of the peak of the binding complex ($\rho = 1.33\text{g/ml}$) between polyribosomes and stripped rough microsomal membranes; instead, the peak of stripped rough microsomal membranes, containing no radioactive label, appeared at $\rho = 1.28\text{g/ml}$, and the unbound polyribosomes were recovered as the pellet. A similar result was obtained when the stripped rough microsomal membranes were replaced by the R-fraction (Figs. 6c and 6d). The peak of the binding complex of the R-fraction and polyribosomes with $\rho = 1.43\text{g/ml}$ virtually disappeared after the addition of 0.1mM-aurintricarboxylic acid to the binding mixture.

Further, the effect of raising the KCl concentration on the binding of polyribosomes to the R-fraction was tested. As shown in Fig. 7, the polyribosome-binding capacity of the R-fraction decreased linearly with increasing KCl concentration and at a concentration above 200mM was approx. 10% of that in a KCl-free buffer. Borgese *et al.* (1974) reported that increasing the KCl concentration from 25mM to 200mM caused an essentially linear decrease in the ribosome-binding capacity of stripped rough microsomal membranes. Thus the suppression of polyribosome binding caused by the addition of aurintricarboxylic acid and by raising KCl concentration was very similar for the R-fraction and for stripped rough microsomal membranes.

Fig. 9. Polyribosome-binding capacity of subfractions R_1 – R_4

The R_1 (a), R_2 (b), R_3 (c) and R_4 (d) subfractions were prepared from the R-fraction as described in Fig. 8, followed by passage through a Sephadex G-75 column to remove deoxycholate. A sample of each (400 μ g of protein) was incubated with 14 C-labelled polyribosomes (200 μ g of RNA) in 0.25M-sucrose in TeaKM buffer. The incubation for binding and subsequent analysis on CsCl gradient were carried out as described in the Experimental section. Values with arrows indicate the buoyant density (g/ml).

Borgese *et al.* (1974) reported that the ribosome-binding sites on stripped rough microsomal membranes lost much of their capacity to bind ribosomes after preincubation for 15 min at 55°C or after treatment at 0°C with a mixture of trypsin and chymotrypsin (50 μ g/ml each). A similar situation was observed for the binding capacity of the R-fraction. The polyribosome-binding capacity of the R-fraction virtually disappeared after treatment with trypsin (10 μ g/ml) for 15 min at 37°C or after preincubation for 5 min at 60°C (results not shown).

From these results it is conceivable that the binding mechanism of polyribosomes to the R-fraction is much like that for stripped rough microsomal membranes.

Fractionation of the R-fraction, and the polyribosome-binding capacity of the subfractions obtained

The R-fraction was fractionated by gel filtration on a Sephadex G-200 column. In the elution profile shown in Fig. 8, four peaks were identified, referred

Fig. 10. Elution profile of the R_1 -fraction from Sepharose 6B

The R_1 -fraction isolated on the Sephadex G-200 column as shown in Fig. 8 was applied to a Sepharose 6B column (2.6 cm $\times$ 100 cm), which was previously equilibrated with 20 mM-Tris/HCl, pH 8.6, containing 0.4% (w/v) deoxycholate, and eluted with the same medium; 5 ml fractions were collected, and A_{260} (—) and A_{280} (---) measured. A, B, C and D represent the V_e values for the protein standards apoferritin (mol.wt. 480000), γ -globulin (mol.wt. 147000), serum albumin (mol.wt. 68000) and cytochrome *c* (mol.wt. 11700) respectively. V_0 , void volume.

to as R_1 -, R_2 -, R_3 - and R_4 -fractions respectively in the order of elution from the column. The molecular weight of each subfraction was calculated from a plot of V_e/V_0 against $\log(\text{molecular weight})$ of protein standards, except for the R_1 -fraction which was eluted at the void volume. Thus mol.wts. of 180000, 33000 and 8000 were found for R_2 -, R_3 - and R_4 -fractions respectively. Deoxycholate was then removed from each fraction by passing them through a Sephadex G-75 column to test their polyribosome-binding capacities. Of the four subfractions only the R_1 -fraction exhibited polyribosome-binding activity, as shown in Fig. 9. The R_1 -fraction, which was eluted at the void volume of the Sephadex G-200 column, was further fractionated on a Sepharose 6B column. As shown in Fig. 10, the bulk of the R_1 -fraction appeared at about fraction 47 as a large symmetrical peak (designated below as the $R_{1,6B}$ -fraction), and four minor peaks were also detected. Two minor peaks eluted later than the $R_{1,6B}$ -fraction might be the product of dissociation or degradation of the R_1 -fraction, since they had an A_{260}/A_{280} ratio different from those of the R_3 - and R_4 -fractions depicted in Fig. 8. The apparent mol.wt. of the $R_{1,6B}$ -fraction was estimated as 320000 from a linear relationship between $\log(\text{molecular weight})$ of protein standards and their V_e/V_0 . As shown in Fig. 11, the $R_{1,6B}$ -fraction also exhibited polyribosome-

Fig. 11. Binding of polyribosomes to the $R_{1,6B}$ -fraction

The $R_{1,6B}$ -fraction was obtained by applying the R_1 -fraction to a Sepharose 6B column as shown in Fig. 10. A binding mixture contained ^{14}C -labelled polyribosomes (100 μg of RNA) and the R_1 -fraction (a) or the $R_{1,6B}$ -fraction (b) (200 μg of protein). The R_1 -fraction was stored at 0°C until the $R_{1,6B}$ -fraction was prepared for the binding experiment. Values with arrows indicate the buoyant density (g/ml).

Fig. 12. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of R-, R₁-, R₂- and R₃-fractions

The R-fraction was prepared from Triton-polyribosomes isolated from postmitochondrial supernatant. The R₁- to R₃-fractions were obtained by applying the R-fraction to a Sephadex G-200 column as shown in Fig. 8. Each fraction was pooled, passed through a Sephadex G-75 column to remove deoxycholate, and concentrated by using a Diaflo membrane. Details of the procedure for gel electrophoresis are given in the Experimental section. The three bands marked with asterisks indicate the major bands characteristic of the R₁-fraction. O, Origin; Dye, Bromophenol Blue. The arrow indicates the direction of electrophoretic migration.

binding capacity, although the buoyant density of the binding complex shifted to a higher value, owing to the loss of binding capacity by aging, as described below. A similar situation was observed with the R₁-fraction, which was stored in the cold until the R_{1,6B}-fraction was prepared for the binding experiments.

Fig. 12 shows the results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the subfractions R₁ to R₃. The R₄-fraction showed a fast-moving band, near the position of the marker dye, which was not detected with Coomassie Blue but was stained by Sudan Black, suggesting that a large part of the R₄-fraction consisted of phospholipids (Lopez & Siekevitz, 1973). In the gel for the R₁-fraction, as well as the bands also observed in the gels for the R₂- and R₃-fractions, were found nine bands characteristic of the R₁-fraction, of which three are major and the others minor. Mol.wts. of

108000, 99000 and 65000 were estimated for these three major proteins characteristic of the R₁-fraction.

Aging of polyribosome-binding capacity of the R₁-fraction

Shires *et al.* (1971) reported that the capacity of ribonuclease-conditioned rough membranes to accept polyribosomes declined with time of storage at 0°C at a rate giving a half-life of about 5 days. To test whether the R₁-fraction also exhibits this loss of polyribosome-binding capacity on aging, a preparation of the R₁-fraction was stored at 0°C and samples were withdrawn at various times for binding to ¹⁴C-labelled polyribosomes. As shown in Fig. 13, the binding capacity of the R₁-fraction significantly declined during storage at 0°C. Even after 20h the profile on a CsCl gradient of the binding complex showed a shift to the higher density. In parallel to this, the proportion of unbound polyribosomes in the binding mixture increased with time of storage. Addition of dithiothreitol to the R₁-fraction before the start of storage failed to prevent the aging of polyribosome-binding capacity of the R₁-fraction.

Discussion

The present study shows that the polyribosomes isolated from the rough microsomal membranes by treatment with Triton X-100 are bound by a group of membrane proteins, which could be detached from the polyribosomes by treatment with deoxycholate. These proteins had high affinity for polyribosomes after the deoxycholate had been removed. These findings agree with the observations of Olsnes (1971), who indicated that the extraneous proteins bound to Triton-polyribosomes may be removed from the polyribosomes by treatment with a high concentration of KCl in the presence of Triton X-100 as well as with deoxycholate, and that reabsorption of the detached proteins on polyribosomes was inhibited by increasing KCl concentration. He further indicated that these proteins with high affinities for polyribosomes did not originate in the soluble fraction of the cytoplasm but came from the endoplasmic reticulum, and considered that these membrane proteins have some function in the specific attachment of polyribosomes to the membranes (Olsnes, 1971).

In the present study, no polyribosome-binding capacity could be found in the Triton-soluble fraction. It was entirely localized in the R-fraction. The binding mode *in vitro* of polyribosomes to the R-fraction was very similar to that to rough microsomal membranes stripped of endogenous polyribosomes. First, the binding capacity of the R-fraction was heat-labile and sensitive to proteolytic

Fig. 13. Aging of polyribosome-binding capacity of the R_1 -fraction

From a large pool of R_1 -fraction stored at 0°C in the presence of 0.4% (w/v) deoxycholate, samples were withdrawn (a) immediately and after (b) 20h, (c) 70h and (d) 100h, passed through a Sephadex G-75 column to remove deoxycholate and immediately submitted to the binding experiment. Each binding mixture contained ^{14}C -labelled polyribosomes ($100\mu\text{g}$ of RNA) and R_1 -fraction ($200\mu\text{g}$ of protein). Other procedures are given in the Experimental section. Values with arrows indicate the buoyant density (g/ml).

enzymes. Similar results were reported for stripped rough microsomal membranes (Shires *et al.*, 1971; Borgese *et al.*, 1974). Secondly, 0.1mM-aurointricarboxylic acid strongly inhibited the binding of polyribosomes to the R -fraction. A similar inhibition for ribosome binding was observed with stripped rough microsomal membranes (Borgese *et al.*, 1974). Finally, the effects of KCl concentration on the polyribosome-binding capacity also indicated the similarity of the binding mode between the R -fraction and the stripped rough microsomal membranes

(Borgese *et al.*, 1974). These results strongly suggest that the R -fraction contained the binding-site proteins that are responsible for the binding of polyribosomes to rough microsomal membranes.

The polyribosome-binding capacity was found only in the R_1 -fraction of the four subfractions obtained by further fractionation of the R -fraction. Jothy *et al.* (1975) showed, by electrophoretic analysis of the stripped rough microsomal membranes, after proteolysis by low concentrations of trypsin and chymotrypsin, that three membrane proteins with mol.wts.

120000, 93000 and 36000 can be correlated with the polyribosome-binding capacity of the membranes. Our data also showed the existence of three major proteins characteristic of the R_1 -fraction, the molecular weights of two of which (except the smallest one) were similar to those reported by Jothy *et al.* (1975).

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