The Binding of Ribosomal Subunits to Endoplasmic Reticulum Membranes

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The binding of ribosomes and ribosomal subunits to endoplasmic reticulum preparations of mouse liver was studied. (1) Membranes prepared from rough endoplasmic reticulum by preincubation with 0.5M-KCl and puromycin bound 60–80% of added 60S subunits and 10–15% of added 40S subunits. Membranes prepared with pyrophosphate and citrate showed less clear specificity for 60S subunits particularly when assayed at low ionic strengths. (2) Ribosomal 40S subunits bound efficiently to membranes only in the presence of 60S subunits. The reconstituted membrane–60S subunit–40S subunit complex was active in synthesis of peptide bonds. (3) No differences in binding to membranes were seen between subunits derived from free and from membrane-bound ribosomes. (4) It is concluded that the binding of ribosomes to membranes does not require that they be translating a messenger RNA, and that the mechanism whereby bound and free ribosomes synthesize different groups of proteins does not depend on two groups of ribosomes that differ in their ability to bind to endoplasmic reticulum.

Two pools of cytoplasmic ribosomes can be isolated from eukaryotic tissues, the free ribosomes and ribosomes bound to endoplasmic reticulum. It has been established that, in liver, at least some proteins are synthesized preferentially on one or the other group of ribosomes. Thus many serum proteins (e.g. albumin) are preferentially synthesized on rough endoplasmic reticulum, whereas some soluble tissue proteins (e.g. ferritin) are synthesized mainly on free ribosomes (Ganoza *et al.*, 1965; Takagi & Ogata, 1968; Ganoza & Williams, 1969; Redman, 1969; Hicks *et al.*, 1969; Takagi *et al.*, 1970). Exceptions to the above are the report that serine dehydratase is synthesized on rough endoplasmic reticulum (Pitot & Jost, 1968) and that bound ribosomes also synthesize some ferritin (Puro & Richter, 1971). In addition, it has been reported that one endoplasmic reticulum membrane protein, NADP cytochrome *c* reductase (EC 1.6.2.3) and one soluble protein, catalase (EC 1.11.1.6), are synthesized on both membrane-bound and free ribosomes (Ragnotti *et al.*, 1969; Takagi *et al.*, 1970); the sites of synthesis of other membrane proteins are not known.

Studies on the attachment of ribosomes to membranes are essential to an understanding of the mechanism of segregation of ribosomes into bound and free pools synthesizing different proteins. Sequential dissociation of ribosomal subunits from rough endoplasmic reticulum has shown the 40S subunit to be more easily removed than the 60S subunit by increasing concentrations of EDTA (Sabatini *et al.*, 1966) and KCl (Lawford & Kaiser, 1970). The conclusion that ribosomes are attached to membranes

through the 60S subunit is supported by electron microscopy (Sabatini *et al.*, 1966) and by the observation (Baglioni *et al.*, 1971) that, in intact plasmacytoma cells, 60S subunits can attach to membranes in the absence of 40S subunits and of protein synthesis. It has also been shown that puromycin treatment facilitates removal of ribosomes from membranes (Sabatini *et al.*, 1966; Lawford & Kaiser, 1970), indicating that nascent peptides play a role in holding ribosomes to membranes. However, the facts that pretreatment of tissue with puromycin does not decrease the number of bound ribosomes (Blobel & Potter, 1967*b*; Andrews & Tata, 1971), and that 60S subunits can attach to membranes in the absence of protein synthesis in intact cells (Baglioni *et al.*, 1971) both suggest that nascent peptides are not required to attach ribosomes to membranes.

Studies of reattachment of ribosomes to membranes *in vitro* have shown that rough endoplasmic reticulum stripped of its ribosomes binds added labelled polyribosomes more efficiently than untreated rough or smooth endoplasmic reticulum (Suss *et al.*, 1966; Ragland *et al.*, 1971; Khawaja & Raina, 1970; Khawaja, 1971). Two groups have reported that, in a comparison between polyribosomes derived from rough endoplasmic reticulum and free polyribosomes, those from the membrane-bound fraction reattached more efficiently to membranes (Scott-Burden & Hawtrey, 1971; Shires *et al.*, 1971*a*). Effects of ionic composition and of spermine or spermidine on polyribosome binding to membranes have also been reported (Shires *et al.*, 1971*a*; Khawaja, 1971). Effects of steroid hormones and structurally similar carcinogens on

polyribosome binding, assayed by the indirect method of masking of disulphide interchange enzyme, have also been reported (Williams *et al.*, 1968; Williams & Rabin, 1969, 1971; Sunshine *et al.*, 1971).

There have been few reports on the binding of ribosomal subunits to membranes. Both subunits have been reported to bind efficiently (Scott-Burden & Hawtrey, 1971; Khawaja, 1971). Pretreatment of the membrane with unlabelled large subunits decreases the binding of labelled small subunits, whereas pretreatment with unlabelled small subunits has no effect on the binding of large subunits (Scott-Burden & Hawtrey, 1971). In the present paper I report a study of the binding *in vitro* of ribosomal subunits to endoplasmic reticulum. Mouse liver preparations were used to avoid problems of dimerization and aggregation of ribosomes seen with rat liver preparations (Reader & Stanners, 1967; Martin *et al.*, 1969). It is shown that the 60S subunit binds much more efficiently to rough endoplasmic reticulum membranes than the 40S subunit, that 40S-subunit binding to membranes is dependent on the 60S subunit, and that no differences in binding between subunits obtained from free and bound ribosomes could be seen.

Experimental

Animals

Male Swiss mice, weight 15–20g, maintained on standard laboratory diet and starved overnight with full access to water were used throughout this study. Mice were killed by cervical fracture, then the livers were exposed by ventral midline incision, and the gall bladders removed intact with forceps. The livers were removed and immediately immersed and rinsed in homogenization buffer at 0°C. For preparation of labelled ribosomes, mice were injected with [³H]-orotic acid (100 μ Ci of >15 Ci/mmol) and starved overnight.

Materials

[5-³H]Orotic acid, specific radioactivity >15 Ci/mol and [2,3-³H]phenylalanine were purchased from Amersham/Searle Corp., Toronto, Ont., Canada. ATP (disodium salt, grade I), GTP (sodium salt, type IIS), phosphoenolpyruvate (potassium salt), pyruvate kinase (type I) and dithiothreitol were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Stripped *Escherichia coli* B transfer RNA was from General Biochemicals, Chagrin Falls, Ohio, U.S.A. and puromycin dihydrochloride was from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Sucrose, density-gradient grade and ribonuclease free, obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A., was used throughout this work. Aquasol

liquid scintillation fluor was obtained from New England Nuclear Corp., Montreal, P.Q., Canada.

Methods

Preparation of membranes, ribosomes and ribosomal subunits. Livers were homogenized in 2 vol. of medium A [50mM-tris – HCl buffer (pH 7.8) – 25mM-KCl – 5mM-MgCl₂ (Blobel & Potter, 1967a)] containing 0.25M-sucrose with 10 strokes of a loose-fitting Teflon pestle in a glass Potter–Elvehjem homogenizer (0.5mm differential in radius with a tube radius of 1.25cm). All centrifugations were at 0–4°C.

A post-mitochondrial supernatant was obtained by centrifugation for 10min at 5020g (r_{av} , 6.9cm) in the Sorvall SS34 rotor and filtration of the supernatant through glass-wool to remove floating fat. Total liver ribosomes were obtained from this supernatant by adding 0.11 vol. of 10% (w/v) sodium deoxycholate, pH 8.0, layering 6ml portions over 6ml of 1.0M-sucrose in medium A and centrifuging at 165000g (r_{av} , 5.9cm) for 2h in the Beckman 50Ti rotor. Pellets were stored at –20°C.

Ribosomal subunits were prepared by a combination of the methods of Blobel & Sabatini (1971) and Falvey & Staehelin (1970). Ribosomes suspended in medium B [20mM-tris–HCl buffer (pH 7.8)–100mM-NH₄Cl–5mM-MgCl₂] containing 0.25M-sucrose (Falvey & Staehelin, 1970) were adjusted, in the order given, to 2mM-dithiothreitol, 0.5M-KCl and 25 μ g/ml of puromycin by addition of the appropriate volumes of concentrated stock solutions. The final concentration of ribosomes was approx. 1.1mg of ribosomal RNA/ml. The suspension was incubated for 30min at 37°C and then clarified by centrifugation at 17 600g (r_{av} , 6.9cm) for 15min in the Sorvall SS34 rotor. Samples (5ml) of the supernatant were layered on to 30ml convex gradients between 10 and 25% sucrose in medium C [20mM-tris – HCl buffer (pH 7.8) – 300mM-KCl – 3mM-magnesium acetate (Falvey & Staehelin, 1970) containing 2mM-dithiothreitol], and centrifuged for 15h at 42000g (r_{av} , 11.7cm) in the Beckman SW27 rotor. Peaks containing subunits were collected manually by following u.v. absorption at 254nm with the IScO density-gradient fractionator. The ribosomal subunits were precipitated with 0.7 vol. of ethanol in the presence of 8mM-magnesium acetate (Falvey & Staehelin, 1970) and stored as pellets at –20°C. Subunits were suspended in medium B containing 2mM-dithiothreitol and debris was removed by centrifugation at 17 600g (r_{av} , 6.9cm) for 10min in the Sorvall SS34 rotor.

Smooth and rough endoplasmic reticulum and free ribosomes were prepared from the post-mitochondrial supernatant by centrifugation on discontinuous sucrose gradients in medium A. Tubes contained 8.5ml of post-mitochondrial supernatant over 3.5ml and 2.0ml respectively of 1.3M- and

2.0M-sucrose in medium A and were centrifuged in the SW40 rotor at 130000g (r_{av} , 11.1cm) for 15h. Material at the interfaces between 0.25M- and 1.3M-sucrose (smooth endoplasmic reticulum) and between 1.3M- and 2.0M-sucrose (rough endoplasmic reticulum) was collected by aspiration, diluted with 2vol. of medium A and centrifuged at 165000g (r_{av} , 5.9cm) for 20min in the Beckman 50Ti rotor. The pelleted membranes were suspended with the tight-fitting pestle of a 15ml Dounce homogenizer (Kontes Glass Co., Vineland, N.J., U.S.A.) in medium B containing 0.25M-sucrose to a concentration of approx. 40mg/ml. Free ribosomes were collected by combining the 2.0M-sucrose layer (after aspiration of the interface) with the pellet, dilution with 3vol. of medium A and centrifugation at 165000g (r_{av} , 5.9cm) for 2h in the Beckman 50Ti rotor.

Ribosomal subunits were separated from rough endoplasmic reticulum as described above for the preparation of subunits. Membranes were generally freed of ribosomes by the same incubation procedure as used for preparation of ribosomal subunits, but after incubation 6-ml samples were layered onto a discontinuous gradient of 3ml of 0.5M-sucrose in medium B containing 0.5M-KCl over 3ml of 0.75M-sucrose in medium B and centrifuged at 165000g (r_{av} , 5.9cm) for 20min in the Beckman 50Ti rotor. This KCl-puromycin method removed 85–90% of 28S ribosomal RNA and 90–95% of the 18S ribosomal RNA as determined by density-gradient centrifugation in sodium dodecyl sulphate as described below, applied directly to the membrane preparation. Membranes free of ribosomes were also prepared from rough endoplasmic reticulum by the pyrophosphate-citrate method (Suss *et al.*, 1966) and with 2M-LiCl (Scott-Burden & Hawtrey, 1969). Both methods gave complete removal of ribosomal RNA from the membranes.

Density-gradient analysis of ribosome particles and ribosomal RNA. Ribosomal subunits were analysed and the reassociation to 80S monomers was measured by using linear 10–25% sucrose density gradients in medium B centrifuged in the Beckman SW50.1 rotor for 60–75 min at 234000g (r_{av} , 8.35 cm) at 0°C. Distribution of E_{254} was measured with an ISCo model UA2 ultraviolet analyser. Sucrose density-gradient centrifugation showed that the 40S-subunit preparations contained no 60S-subunit material, but that the 60S-subunit preparation contained an 80S peak approx. 10% of the major 60S peak, indicating some 3% contamination with 40S subunits (Fig. 1). These gradients also showed the presence of 90S particles, presumably dimers of the 60S subunit (Martin *et al.*, 1969).

RNA from the ribosomal subunits was analysed on 10–25% sucrose density gradients in 50mM-tris-HCl buffer (pH 7.8)–0.1% sodium dodecyl sulphate

centrifuged at 20°C for 2.5h at 243000g (r_{av} , 8.35cm) in the Beckman SW50.1 rotor. Subunits in medium B were treated with 10% sodium dodecyl sulphate (final concentration of 0.5%), incubated for 5 min at 37°C and layered on to the gradients at room temperature. Distribution of E_{254} was measured with an ISCo model UA2 ultraviolet analyser and fractions were collected (0.3 ml), treated with 15 ml of Aquasol and radioactivity was determined in a Unilux II liquid-scintillation spectrometer. Fig. 2 shows that virtually all the radioactivity on the gradient migrates with the ribosomal RNA, and confirms the purity of the two subunit preparations seen in Fig. 1.

Preparation of cell sap. Cell sap was prepared from the supernatant after centrifugation of liver homogenates at 165000g (r_{av} , 5.9cm) for 2.5h in the Beckman 50Ti rotor followed by dialysis overnight against 20vol. of medium B containing 2mM-dithiothreitol.

Assays of binding of ribosomes to membranes. The general procedure used was as follows: incubation mixtures (0.6 ml) containing membranes (0.5–2mg of protein in medium B), cell sap (6mg of protein) and adjusting buffer to give the ionic composition needed were mixed with labelled ribosomes (40S, 60S or 80S ribosomal particles, 5–500 μ g of RNA as specified, in medium B) and were incubated at 37°C for 5 min. The entire sample was then layered with a disposable Pasteur pipette on to 5.4 ml of 20% sucrose in buffer of the same ionic composition as the incubation buffer and centrifuged at 114000g (r_{av} , 6.4cm) for 20min in the Beckman 40.3 rotor. The supernatant fluid was aspirated and discarded. The pellet was rinsed by filling the tube once with the same buffer and aspirating, and the pellet was dissolved in 1 ml of 0.2% sodium dodecyl sulphate in the centrifuge tube. Dissolution was frequently aided by sonication with the microprobe of a Branson sonifier. The entire sample was then transferred to a scintillation-counting vial, treated with 15 ml of Aquasol and the radioactivity determined in a Unilux II scintillation spectrometer.

Assays of protein synthesis. Incorporation of [3 H]-phenylalanine from aminoacyl-tRNA into hot-trichloroacetic acid-insoluble material was measured as described by Martin *et al.* (1969), scaled down to incubation volumes of 0.2 ml.

Electron microscopy. Membrane fractions were suspended in 3% (v/v) glutaraldehyde in medium B for 2h at 0°C, centrifuged and the pellets rinsed with water. Post-fixation was with 2% (w/v) OsO_4 for 1.5h, after which the samples were dehydrated by increasing the concentration of ethanol to 100% and embedded in Epon. Sections of 7 μ m were stained with uranyl acetate and lead hydroxide and photographed in an RCA 3G electron microscope at 32000 times magnification.

Chemical analyses. Protein was determined by the

Fig. 1. Density-gradient analysis of ribosomal subunits

Density gradients were centrifuged in medium B and analysed as described in the Experimental section. (a) 40S ribosomal subunit preparation; (b) 60S ribosomal subunit preparation. The approximate S values of the peaks are indicated on the abscissa. The values in parentheses are the distances from the rotor centre.

biuret reaction (Gornall *et al.*, 1949), with crystalline bovine serum albumin (Pentex Inc., Kankakee, Ill., U.S.A.) as standard in the presence of 0.1% sodium deoxycholate to dissolve membranes. RNA of free ribosomes was determined by the method of Fleck & Munro (1962) as described by Wool & Cavicchi (1967) and RNA in endoplasmic reticulum fractions by the orcinol method (Schneider, 1945).

Results

Binding assay

The validity of the assay of binding of ribosomal subunits to rough endoplasmic reticulum membranes was tested under the conditions reported below in this paper. The centrifugation method described in the Experimental section allowed recovery of 83–86% of the membrane protein, and this recovery was not affected by the ranges of ionic concentrations (50–350 mM- NH_4Cl and 1–20 mM- MgCl_2) used in

these studies. Further, labelled ribosomes bound to membranes in medium B could not be washed from the membranes by resuspension in fresh binding mixture of the same ionic composition, reincubation and recentrifugation; the specific radioactivity (c.p.m./mg of protein) of the ribosome-membrane complex remained constant. Electron microscopy showed the ribosome-membrane complexes isolated after a typical binding assay in medium B to be very similar to native rough endoplasmic reticulum (Plate 1). Finally, it was also shown that sequential addition of 60S and 40S subunits to rough endoplasmic reticulum membranes restored their ability to synthesize polyphenylalanine in the presence of polyuridylic acid.

Effect of temperature of incubation. Previous workers have used incubation at 0°C and 37°C for studies of binding of ribosomes to membranes (e.g. Suss *et al.*, 1966; Ragland *et al.*, 1971; Burka & Schickling, 1970; Shires *et al.*, 1971b; Scott-Burden & Hawtrey, 1971). Comparison of these two temperatures showed

EXPLANATION OF PLATE I

Electron micrographs of rough endoplasmic reticulum and its complexes with ribosomal subunits

Electron micrographs of (a) rough endoplasmic reticulum as isolated, (b) rough endoplasmic reticulum membranes stripped of ribosomes by the KCl-puromycin method, (c) rough endoplasmic reticulum membranes from (b) treated with 60S ribosomal subunits (0.3 mg of ribosomal RNA/mg of membrane protein) and re-isolated by centrifugation, (d) membrane-60S subunit complexes from (c) treated with 40S ribosomal subunits (0.1 mg of ribosomal RNA/mg of membrane protein) and re-isolated by centrifugation. The scale marker indicates 1 μ m.

Fig. 2. Density-gradient analysis of ribosomal RNA

Density gradients were centrifuged in sodium dodecyl sulphate and analysed as described in the Experimental section. (a) 40S ribosomal subunit preparation, (b) 60S ribosomal subunit preparation. —, E_{254} ; ---, c.p.m./fraction. The approximate S values of the peaks are indicated on the abscissa. The values in parentheses are the distances from the rotor centre.

the higher to allow up to twofold increases in binding of 60S subunits to membranes, both by increasing the total radioactivity recovered in the membrane pellet and by decreasing radioactivity sedimenting in the absence of membranes in blank tubes (results not shown). The effects on binding of the 40S subunits were much less marked. Incubations were therefore generally performed at 37°C. At this temperature, binding was maximal in 5 min.

Effect of cell sap. Preliminary attempts to recover intact ^3H -labelled ribosomal RNA after binding of labelled ribosomes to membranes in the absence of cell sap showed extensive degradation of the ribosomal RNA. This could be prevented by the addition of ribonuclease inhibitor purified from rat liver by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography (Gribnau *et al.*, 1969) or by cell sap. No differences between these two additives were observed with respect to binding of ribosomes to membranes, and dialysis of the cell sap also had no effect (results

not shown). Dialysed cell sap was therefore generally included in incubation mixtures.

Ribosome concentration curve. Experiments in which various concentrations of ribosomes were incubated with constant amounts of membranes established the relative proportion of ribosomes and membranes needed in subsequent binding assays. By using 80S monomers and incubating in 100mM- NH_4Cl and 5mM- MgCl_2 (medium B) membranes became saturated with a ratio of ribosomal RNA/membrane protein above 0.5, whereas at values of this ratio below 0.06 maximal percentages of added ribosomes were bound to membranes (Fig. 3). Subsequent experiments on binding of 80S monomers therefore used ratios of ribosomal RNA/membrane protein above 0.5 or below 0.06 to determine respectively the ability of membranes to bind ribosomes and the ability of ribosomes to bind to membranes. Ratios in proportion to molecular weights of ribosomal RNA were used for 60S and 40S ribosomal subunits.

Binding of ribosomes to different preparations of membrane

Assays of various preparations of membranes to bind ribosomes confirmed reports (Suss *et al.*, 1966; Burka & Schickling, 1970; Ragland *et al.*, 1971) that removal of ribosomes from rough endoplasmic reticulum membranes increased their capacity for ribosomal subunits, and that smooth endoplasmic reticulum, either untreated or after treatment to remove ribosomes, was less active (Table 1).

Comparison of 60S and 40S subunits in binding to membranes

Comparison between 60S and 40S subunits in binding to rough endoplasmic reticulum membranes depended on the concentration of inorganic ions used in the assay and on the methods used to remove ribosomes from the membranes. Ion-concentration effects were tested by using membranes stripped by the KCl-puromycin and the pyrophosphate-citrate methods. These were incubated with 40S and 60S subunits in equimolar amounts in separate binding assays (Fig. 4). By using membranes stripped with pyrophosphate and citrate, the binding of the 40S subunit was less than that of the 60S subunit between 100 and 250 mM-NH₄Cl (Fig. 4), but 40S subunits bound as efficiently as 60S subunits at 50 mM-NH₄Cl. Varying the concentration of MgCl₂ from 1 to 15 mM had little effect on binding over this range of NH₄Cl concentrations (results not shown).

Membranes freed of ribosomes with KCl and puromycin were generally 10–30% less active in binding 60S subunits than membranes stripped with pyrophosphate and citrate (Fig. 4). The KCl-puromycin-treated membranes also showed greater specificity for 60S subunits, the binding of 40S subunits being generally much lower to these membrane preparations than to those prepared with pyrophosphate and

citrate. Binding of subunits to membranes prepared by the LiCl technique (Scott-Burden & Hawtrey, 1969) was similar to that with KCl-puromycin-treated membranes.

Fig. 3. Saturation of membranes by ribosomes

Membranes (0.6 mg of membrane protein) were incubated at 37°C for 5 min with ribosomes (up to 0.39 mg of ribosomal RNA) in the presence of cell sap (6 mg of protein) in medium B. The ribosomes were 80S monomers derived from total liver polyribosomes by incubation in the presence of puromycin, ATP, GTP, and cell sap and re-isolation by centrifugation through 1.0 M-sucrose. The specific radioactivity of ³H-labelled ribosomes was 8300 c.p.m./mg of RNA. Δ, Rough endoplasmic reticulum membranes prepared by the pyrophosphate-citrate method; ○, untreated rough endoplasmic reticulum.

Table 1. Effect of various membrane treatments on binding of subunits to membranes

Incubations contained membranes (2.1 mg of protein), 40S and 60S ribosomal subunits (53 or 125 μg of RNA), cell sap (5.8 mg of protein), 100 mM-NH₄Cl and 5 mM-MgCl₂ in 0.6 ml, and were for 5 min at 37°C. The ribosome specific radioactivity was 90000 c.p.m./mg of ribosomal RNA.

Membrane	Treatment	Radioactivity bound (c.p.m.)	
		40S subunit	60S subunit
Rough endoplasmic reticulum	None	468	690
	KCl-puromycin	980	4360
	Pyrophosphate-citrate	1899	4950
Smooth endoplasmic reticulum	None	525	600
	KCl-puromycin	460	570
	Pyrophosphate-citrate	387	620

Fig. 4. Effect of different methods for removing ribosomes from membranes and of NH_4Cl concentration on the binding of 60S and 40S subunits

Membranes were stripped of ribosomes by (a) the pyrophosphate-citrate method and (b) the KCl-puromycin method. Incubations contained 1.15 mg of membrane protein, ribosomes (15 μg of rRNA of 40S subunit and 38 μg of rRNA of 60S subunit), cell sap (6.5 mg of protein), 5 mM- MgCl_2 and the concentration of NH_4Cl indicated. ○, 60S subunit; △, 40S subunit.

Binding of 40S subunits to rough endoplasmic reticulum membranes in the presence of 60S subunits

The addition of unlabelled 60S subunits to rough endoplasmic reticulum membranes prepared by the KCl-puromycin method greatly increased the binding of 40S subunits. This was shown in two types of experiments. In one, membranes were mixed with an excess of unlabelled 60S subunits, and the resulting membrane-60S subunit complex was isolated and used for assays of binding of labelled 40S subunits. In the other approach, membranes and labelled or unlabelled 60S and 40S subunits were added sequentially to one incubation mixture.

Binding of 40S subunits to membrane-60S subunit complexes was dependent on the amount of 40S subunits added. An excess of 40S subunits caused saturation of binding sites, whereas with limiting amounts of 40S subunits, maximal percentages were bound (results not shown). Analysis of the effects of MgCl_2 and NH_4Cl (Fig. 5) showed that there was a tendency for higher percentage binding of 40S subunits to membrane-60S subunit complexes at higher MgCl_2 concentrations, that 100 mM- NH_4Cl gave optimum binding, and that increasing the concentration of NH_4Cl to 200 mM approximately halved the binding of labelled 40S subunits attributable to the presence of unlabelled 60S subunits.

Efficient binding of 40S subunits to membranes dependent on the presence of 60S subunits was also

seen when the three components were mixed sequentially in one incubation. This approach allowed the use of equimolar concentrations of subunits and determination of possible effects of 40S addition on binding of 60S subunits. With this method, it was shown that, in the presence of 60S subunits, the percentage binding of 40S subunits was comparable with that of the 60S subunit, and that addition of unlabelled 40S to labelled 60S subunits did not influence the binding of the latter (Table 2). Also binding of 40S subunits was not dependent on whether the 60S subunit was allowed to bind to the 40S subunit before addition of membranes, or whether the 60S subunit was bound to the membrane before addition of the 40S subunit.

Polyphenylalanine synthesis by reconstructed rough endoplasmic reticulum

Complexes containing membranes from rough endoplasmic reticulum and 60S and 40S subunits were shown to be active in synthesis of peptide bonds by assays of translation of polyuridylic acid (Table 3). Membranes stripped of ribosomes by the KCl-puromycin technique had some activity in polyphenylalanine synthesis owing to the residual 60S and 40S ribosomal subunits; the pyrophosphate-citrate-prepared membranes had no residual activity. Addition of excess of 60S subunits followed by isolation of the membrane-60S subunit complex increased

Fig. 5. Effects of $MgCl_2$ and NH_4Cl on binding of labelled 40S subunits to membranes pretreated with unlabelled 60S subunits

Rough endoplasmic reticulum stripped by the KCl-puromycin technique (30mg of protein) was incubated with unlabelled 60S subunits (10mg of RNA) at 37°C for 10min in 15ml of medium B containing cell sap (71mg of protein). The mixture was layered in 5ml portions over 7ml of 0.75M-sucrose in medium B and centrifuged at 165000g (r_{av} , 5.9cm) for 20min in the Beckman 50Ti rotor. The membranes were washed by suspending in fresh incubation medium with cell sap, incubating and recentrifuging as above. Control membranes were treated identically, with the omission only of the 60S subunits. For the assay of 40S subunit binding, membranes (0.75mg of protein) were incubated for 5min at 37°C in 0.6ml of 20mM-tris-HCl buffer, pH7.8, containing 2.8mg of cell-sap protein, 40S subunits (31µg of RNA containing 4650c.p.m.), the concentration of NH_4Cl shown and 2.5mM- (○), 5mM- (■) or 10mM- (△) $MgCl_2$. Parallel incubations of the pretreated and non-pretreated membranes with labelled 60S subunits (27µg of RNA) at 100mM- NH_4Cl and 5mM- $MgCl_2$ gave 17 and 66% binding respectively.

the synthesis of polyphenylalanine by the membrane preparation owing to the presence of some 40S subunits in the 60S preparation. Further addition of 40S subunits caused very marked stimulation in synthesis of polyphenylalanine by the re-isolated membrane preparations.

It should be noted that assays of polyphenylalanine synthesis were done in 15mM- $MgCl_2$, and therefore test only the ability of the reconstituted rough endo-

plasmic reticulum to synthesize peptide bonds in the presence of soluble cytoplasmic proteins.

Comparison between bound and free 60S and 40S subunits

No differences were seen in these assays between 60S and 40S subunits obtained from rough endoplasmic reticulum and from free ribosomes. 60S subunits from these two groups of ribosomes were compared under a range of conditions. Membranes were stripped of ribosomes by all three available techniques, pyrophosphate-citrate, KCl-puromycin, and LiCl. These membranes were used at ranges of $MgCl_2$ (1–5mM) and of NH_4Cl (100–250mM) and at 0°C and 37°C. Also two different means of preparing ribosomal subunits free of membranes were used: dissolving the membranes with deoxycholate, and removal of ribosomes by treatment with KCl and puromycin. Many combinations of these variations on the basic binding assay were tested and none revealed specificity of interaction of 60S ribosomal subunits with rough endoplasmic reticulum membranes. Similarly, 40S subunit interaction with membranes in the presence and absence of unlabelled 60S subunits was tested under a range of conditions or by using sequential addition of the two subunits and membranes to the incubations. No differences in binding between free and bound 40S subunits were seen. Further, no differences were seen when 80S monomers from free and membrane-bound fractions were compared. Monomers prepared by deoxycholate treatment of membranes bound to the same extent as untreated or detergent-treated monomers from free ribosomes.

Discussion

The results in this paper are relevant to two major points. First, they provide direct evidence from binding studies *in vitro* that 60S ribosomal subunits bind efficiently to membranes prepared from rough endoplasmic reticulum. This supports strongly the conclusions reached from sequential dissociation of ribosomal subunits from membranes with EDTA (Sabatini *et al.*, 1966) or KCl (Lawford & Kaiser, 1970) that ribosomes are attached to membranes through their 60S subunits, and also suggests that ribosomes do not attach to membranes in the form of polyribosomes (Baglioni *et al.*, 1971). Secondly, these results detect no differences between ribosomal subunits derived from rough endoplasmic reticulum and free ribosomal subunits with respect to binding to membranes, and thus argue strongly against the hypothesis that functional differences between the two groups of ribosomes are due to the existence of two fully distinct ribosomal classes.

Table 2. *Binding of 40S subunits to membranes in the presence of 60S subunits and vice versa*

Incubations were done in medium B and contained membranes (1.13 mg of protein), 60S and 40S subunits (30.1 and 10.2 μ g of RNA respectively) and dialysed cell sap (4.8 mg of protein) in 0.6 ml. The specific radioactivity of the labelled 60S and 40S ribosomal subunits was 73000 c.p.m./mg of RNA. In the first column, subunits were incubated for 5 min at 37°C in the full binding assay mixture in 0.5 ml. Membranes were then added in 0.1 ml and the incubation continued for 5 min before centrifugation and analysis as described in the Experimental section. In the second column, membranes were incubated with 60S subunits for 5 min in 0.5 ml, 40S subunits were added in 0.1 ml and the incubation was continued for 5 min. The binding of labelled 40S subunits in the presence and absence of unlabelled 60S subunits and vice versa is tested with both experimental approaches.

Order of reaction ...	Labelled ribosomes bound to membranes (% of added)	
	Form 60S subunit-40S subunit complex Then add membranes	Form membrane-60S subunit complex Then add 40S subunit
40S subunit binding		
+60S subunit	56	49
-60S subunit	12	13
60S subunit binding		
+40S subunit	60	61
-40S subunit	60	58

Table 3. *Synthesis of polyphenylalanine by reconstituted membrane-ribosome complexes*

Rough endoplasmic reticulum membranes were stripped of ribosomes by either the pyrophosphate-citrate method or the KCl-puromycin method. Membrane-60S subunit complexes were prepared by incubating membranes (6.5 mg of protein), 60S subunits (2.1 mg of ribosomal RNA) and cell sap (10 mg of protein) in 3.5 ml of medium B at 37°C for 10 min, layering over 7 ml of 0.75 M-sucrose in medium B and centrifuging at 0°C for 20 min at 165000 g (r_{av} , 5.9 cm) in the Beckman 50Ti rotor. Membrane-60S subunit-40S subunit complexes were prepared from membrane-60S subunit complexes in a similar manner, but by using 3.0 mg of membrane-60S subunit protein and 0.4 mg of 40S ribosomal subunits. Assays for protein synthesis used 50 μ g of membrane protein per assay. Parallel incorporation assays on the ribosomal subunits used showed the 60S subunit to incorporate 240 c.p.m./2.5 μ g of ribosomal RNA, the 40S subunit 20 c.p.m./ μ g of ribosomal RNA and the mixture of these amounts 2680 c.p.m./3.5 μ g of ribosomal RNA.

Membrane preparation ...	Radioactivity incorporated (c.p.m./assay)	
	Pyrophosphate-citrate treatment	KCl-puromycin treatment
Membranes alone	40	427
Membrane-60S subunit complex	690	780
Membrane-60S subunit-40S subunit complex	2480	1950

Rough endoplasmic reticulum that has been treated to remove ribosomes binds freshly added ribosomes much more efficiently than untreated rough or treated or untreated smooth endoplasmic reticulum. This leads to the conclusion that the rough endoplasmic reticulum membrane carries ribosome binding sites (Shires *et al.*, 1971a). The present work establishes that these sites are specific for the 60S ribosomal subunits, but that they are not able to distinguish 60S ribosomal subunits derived from the rough endo-

plasmic reticulum from those of free ribosomes. It is possible to argue that factors influencing the binding of subunits were removed during their preparation, which involves high-salt concentrations known to remove proteins from ribosomes. The high efficiency of reattachment of both free and membrane-bound 60S ribosomal subunits would require postulation of a binding inhibitor on the free ribosomal subunits. However, 80S monomers prepared without high salt concentrations from free ribosomes by incubation

to complete nascent chains in the presence or absence of puromycin were no less efficient in binding to rough endoplasmic reticulum membranes than were 60S subunits. The observation that free and bound ribosomal subunits bind to the same extents might be expected from the very extensive exchange of ribosomal proteins with cell-sap proteins reported by Dice & Schimke (1972), but is perhaps not consistent with reports of differences in the protein components of free and membrane-bound ribosomes (Fridlender & Wettstein, 1970; Burka & Bulova, 1971).

Scott-Burden & Hawtrey (1971) have reported that polyribosomes obtained from rough endoplasmic reticulum bound three times more efficiently than free polyribosomes to rough endoplasmic reticulum stripped with 2M-LiCl and incubated at 5°C. Shires *et al.* (1971b) report that membrane-bound polyribosomes attached to smooth endoplasmic reticulum membranes fifteen times more efficiently than free polyribosomes when incubated at 3–4°C and twice as well when incubated at 37°C. Further, at 37°C, 63% of the added membrane-bound polyribosomes were bound to smooth endoplasmic reticulum giving RNA/protein ratios comparable with native rough endoplasmic reticulum. In contrast, in the present study, no selectivity of membrane-bound over free ribosomal subunits was seen with smooth endoplasmic reticulum or rough endoplasmic reticulum membranes stripped with LiCl, KCl-puromycin or pyrophosphate-citrate.

These differences between the present studies with subunits and earlier studies with polyribosomes can be discussed in terms both of the assay conditions used and of the physiological significance of the observed binding. The low-ionic-strength buffers used in both studies of polyribosome binding have been shown in the present work to allow the presumably non-specific binding of 40S subunits to rough endoplasmic reticulum membranes. Also, in neither study of the binding of free and membrane-bound polyribosomes to membranes were size distributions of the polyribosomes bound in the assay reported. If polyribosomes isolated from the rough endoplasmic reticulum were on average significantly larger than the free polyribosomes, and only one ribosome per polyribosome chain were bound to the membranes, it would appear that membrane-bound polyribosomes were more efficient in binding. Conversely, if the polyribosomes from rough endoplasmic reticulum were smaller than free polyribosomes, artifacts could be induced in the centrifugation used to analyse the binding mixtures. The isopycnic-sedimentation method used causes the membrane vesicles to sediment to an interface with dense sucrose, whereas the ribosomes are expected to pass through the interface. The relatively small ribosomal particles would arrive at the interface after the membrane vesicles and therefore would be remixed with the membranes at

high concentrations relative to the original binding-assay mixture, and for a time inversely related to the sedimentation rate of the ribosomal particle. There would therefore arise a second opportunity for binding under relatively uncontrolled conditions. These possible artifacts of centrifugation and of particle size do not apply to the present study because well-characterized subunit preparations were used (Fig. 1), and because binding mixtures were separated into bound and unbound components by a rate-sedimentation method. The possible influences of the ionic composition of the binding reaction were also fully investigated in the present study.

The very high efficiency with which 60S subunits bind to stripped rough endoplasmic reticulum membranes suggests strongly that factors on polyribosomes, but not on subunits, play little part in the process of attachment of ribosomes to membranes. This conclusion was also reached by Baglioni *et al.* (1971), who observed that in intact plasmacytoma cells newly synthesized 60S subunits bound to endoplasmic reticulum in the absence of 40S subunits and of protein synthesis. The binding of polyribosomes to membranes reported by many workers (Suss *et al.*, 1966; Khawaja & Raina, 1970; Ragland *et al.*, 1971; Khawaja, 1971; Scott-Burden & Hawtrey, 1971; Shires *et al.*, 1971a,b) could arise because the membrane-binding site on 60S subunits is not masked in polyribosomes. Alternatively, it could arise because certain proteins, and thus presumably their nascent peptides, can bind to membranes. In this context, it has been shown that serum glycoproteins but not albumin will bind to membranes (Redman & Cherian, 1972), and it may be assumed that nascent peptides for membrane proteins will also show affinity for membrane structures. However, since 60S subunits bind efficiently both *in vivo* (Baglioni *et al.*, 1971) and *in vitro* (the present work) to endoplasmic reticulum membranes in the absence of protein synthesis, it appears unlikely that factors added to ribosomes as a result of protein synthesis are involved in the functional distinction between bound and free ribosomes.

The question therefore remains of how specific messages are translated by either bound or free ribosomes. It is apparent that membranes contain ribosome-binding sites specific for the 60S subunit, and that messenger RNA attaches to the 40S subunit. Since there is apparently no selectivity by free or membrane-bound ribosomal subunits for attachment to membranes, it can be suggested that prior binding of the 60S subunit to the ribosome-binding site on membranes causes the 60S subunit to allow initiation with selected messenger RNA molecules only.

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