Reconstitution into liposomes of membrane proteins involved in ribosome binding on rough endoplasmic reticulum

Ribosome-binding capacity

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A membrane protein fraction having a high affinity for polyribosomes was isolated from microsomal membranes of rat liver and was incorporated into liposomes made from microsomal lipids to evaluate the polyribosome-binding capacity of the reconstituted liposomes, with the following results. (1) The polyribosome binding to the reconstituted liposomes depended on the amount of polyribosomes added to the binding mixture. (2) Liposomes made from lipids alone did not bind any polyribosomes. (3) The polyribosome-binding capacity of the reconstituted liposomes was very sensitive to proteolytic enzyme and strongly inhibited by addition of 0.1 mM-aurintricarboxylic acid or by increasing KCl concentration. These results suggest that the binding mechanism of polyribosomes to the reconstituted liposomes is much like that for rough microsomal membranes stripped of endogenous polyribosomes.

It is now generally accepted that proteins destined to be secreted from a cell or for use as integral membrane proteins are synthesized on membrane-bound polyribosomes and are vectorially transported across the membrane (Palade, 1975; Sabatini & Kreibich, 1976). The mechanism for membrane recognition and the transfer process for the nascent polypeptides have been partially understood (Blobel & Doberstein, 1975; Heijne & Blomberg, 1979). However, the membrane components involved in polyribosome-membrane interaction remain to be characterized. Until the present time, many studies have been done to characterize the polyribosome-binding site on the membranes. Partial proteolysis with trypsin and chymotrypsin eliminated the polyribosome-binding capacity of stripped rough microsomal membranes, indicating that membrane proteins are involved (Shires *et al.*, 1971; Borgese *et al.*, 1974). Heat-lability of the binding capacity (Borgese *et al.*, 1974) and studies involving the modification of membrane proteins with succinic anhydride (Scott-Burden, 1976) have also shown a similar notion. It has been shown that cytochrome P-450 is involved in the polyribosome-binding site (Ohlsson & Jergil, 1977; Takagi, 1977), but phosphatidylcholine is not (Jothy *et al.*, 1973; Borgese *et al.*, 1974). Electrophoretic analysis of stripped rough microsomal membranes digested with trypsin and chymotrypsin has demonstrated that three mem-

brane proteins, with mol.wts. of 120 000, 93 000 and 36 000, are correlated with the binding capacity (Jothy *et al.*, 1975). Other workers have revealed that five membrane proteins are involved in the polyribosome binding (Aulinskas & Scott-Burden, 1979), one of which, of mol.wt. 65 000, may represent ribophorin I as described by Kreibich *et al.* (1978).

In our previous study (Fujita *et al.*, 1977), a protein fraction, termed R-fraction, which has a high affinity for polyribosomes was isolated from rough microsomal membranes of rat liver. The mode of polyribosome binding to the R-fraction was similar to that to the stripped rough microsomal membranes in heat-lability, sensitivity to proteolysis and suppression of polyribosome binding by increasing KCl concentration or by addition of aurintricarboxylic acid. In the present study, the R-fraction was reconstituted into liposomes, and the mode of polyribosome binding to these reconstituted liposomes was examined. With this system, it may be possible to study polyribosome binding *in vitro* in a more natural membrane environment than in our previous study (Fujita *et al.*, 1977).

Experimental

Materials

Sodium deoxycholate, aurintricarboxylic acid and

trypsin 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). Sephadex G-50 and Sepharose 4B were from Pharmacia (Uppsala, Sweden). [6-¹⁴C]-Orotic acid (61 mCi/mmol) and [Me-³H]choline (33 Ci/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.).

Methods

Isolation of subcellular fractions. Polyribosomes labelled for 18 h with [6-¹⁴C]orotic acid were prepared as previously described (Fujita *et al.*, 1977). The R-fraction was also prepared as previously described (Fujita *et al.*, 1977).

Lipid extraction from microsomal fraction

Total microsomal fractions were prepared by centrifugation at 105 000 *g* for 90 min of the post-mitochondrial supernatant of rat liver labelled *in vivo* for 60 min with 52 μ Ci of [Me-³H]choline/100 g body wt. (Ramsey & Steele, 1976). Total lipid was extracted from the total microsomal fraction with chloroform/methanol essentially by the method of Bligh & Dyer (1959). The lipid extract was finally dissolved in chloroform/methanol (2:1, v/v) and stored at -80°C under N₂ until use.

Preparation of liposomes

Liposomes were prepared by the method of Enoch & Strittmatter (1979), who described the formation and properties of 100 nm-diameter single-bilayer phospholipid vesicles. Total lipid in chloroform/methanol was dried under reduced pressure to a thin film in a 15 ml Pyrex tube. To 20 μ mol of phospholipid, assuming the mean molecular weight of phospholipid as 759 (Davison & Wills, 1974), 1 ml of TN buffer (20 mM-Tris/acetate/100 mM-NaCl, pH 8.1) containing 10 mM-deoxycholate was added and the tube was swirled to suspend the lipid. This suspension was sonicated at a frequency of 10 kHz for 4 min in a Tominaga model TI-100 sonicator. Most of the detergent was then removed by gel filtration through a Sephadex G-50 column (1.8 cm $\times$ 32 cm) equilibrated with TN buffer. The sample eluted with the void volume was concentrated by an Amicon apparatus equipped with PM-30 membrane and applied on a second gel-filtration column of Sepharose 4B (1 cm $\times$ 60 cm), followed by an elution with the same buffer.

Reconstitution procedure

For this, 1 ml of TN buffer containing 10 mM-deoxycholate and an indicated amount of R-fraction was added to 20 μ mol of phospholipid dried to a thin film in a tube, and treated by procedures similar to

those described above. The reconstituted liposomes thus prepared are termed the R-liposomes.

Analysis of polyribosome binding with R-liposomes

The binding mixture contained ³H-labelled R-liposomes and ¹⁴C-labelled polyribosomes in 0.5 ml of TKM buffer in quantities as indicated in the legend of each Figure. After the incubation for 30 min at 4°C, the mixture was layered over a linear 0.3–1.5 M-sucrose gradient (3 ml) in TKM buffer (50 mM-Tris/HCl/25 mM-KCl/5 mM-MgCl₂, pH 7.6) on top of 0.5 ml each of 1.7 M- and 2.4 M-sucrose in TKM buffer, and centrifuged at 102 500 *g* for 10 h in a Hitachi RPS 40T-2 rotor. After the centrifugation, the sucrose gradient was collected in about 20 equal fractions. Radioactivity was determined in a Aloka LSC-903 scintillation counter by using Aquasol scintillant.

Analysis

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (crystal-

Fig. 1. Sucrose-gradient centrifugation of the R-liposomes

The R-liposomes were prepared as described in the Experimental section by mixing the R-fraction and the ³H-labelled lipids at ratios of 3:10 (—) or 10:10 (----). The R-liposomes were centrifuged at 102 500 *g* for 10 h through a linear 0.2–1.2 M-sucrose gradient in TKM buffer. Values indicate the sucrose concentration (M) in the gradient.

lized, Sigma Chemical Co.) as standard. RNA was determined by the method of Fleck & Munro (1962) by using $A_{1\text{cm}}^{1\%} = 313$ at 260nm. Lipid P was determined by the method of Bartlett (1959).

Results

Sucrose-gradient centrifugation of the R-liposomes

Fig. 1 shows the sucrose-gradient centrifugation of the R-liposomes prepared as described in the Experimental section. A slow-sedimenting peak observed at 0.4M-sucrose was considered to be protein-deficient liposomes, as shown by determination of protein in this peak fraction. The R-liposome peak, which contains proteins, appeared

at 0.68M-sucrose at a low protein/lipid ratio (3:10, w/w). At a higher protein/lipid ratio (10:10, w/w), a major peak of the R-liposomes shifted to a higher sucrose concentration (0.91M) and, furthermore, a minor peak appeared at a higher sucrose concentration (1.1M). The protein-deficient liposome peak became small with increasing protein/lipid ratio. At a protein/lipid ratio of 6:10 (w/w), the sedimentation pattern revealed only two peaks, one with a slightly higher density than 0.68M-sucrose and the other with a density similar to that of protein-deficient liposomes (results not shown). The protein-deficient liposomes could be virtually eliminated from the R-liposomes by centrifugation at 22000g for 30min in a Hitachi RPR18-2 rotor. Approx.

Fig. 2. Binding of polyribosomes to the R-liposomes

The ^{14}C -labelled polyribosomes and the ^3H -labelled R-liposomes (protein/lipid ratio 3:10) were prepared as described in the Experimental section. The binding mixtures, containing 134 μg of proteins of R-liposomes and 132 μg (a), 82 μg (b) or 54 μg (c) of RNA of polyribosomes, were layered over a linear 0.3–1.5M-sucrose gradient on top of 1.7M- and 2.4M-sucrose in TKM buffer, and centrifuged at 102 500 g for 10h. Similar centrifugations were performed for the mixture (d) of polyribosomes (110 μg of RNA) and liposomes made from lipids alone containing the same quantity of ^3H radioactivity as in (a)–(c), the R-liposomes alone (e) (134 μg of proteins), and polyribosomes alone (f) (110 μg of RNA). —, ^3H radioactivity; ----, ^{14}C radioactivity.

84% of proteins and 54% of lipids of the R-liposomes were recovered as the pellet which was resuspended in TKM buffer.

Polyribosome binding to the R-liposomes

As shown in Fig. 2, after incubation of polyribosomes with the R-liposomes, a binding complex appeared on the sucrose gradient. The position of the binding complex on the sucrose gradient shifted to higher-density regions with increasing amounts of polyribosomes added (Figs. 2a–c). The $^{14}\text{C}/^3\text{H}$ ratio estimated from radioactivity localized in the binding-complex peak indicated that the amount of polyribosomes bound to the R-liposomes increased in proportion to the amount of polyribosomes added to the binding mixture (Fig. 3). The capacity of the R-liposomes to bind polyribosomes was not enhanced by incubation of the binding mixture at 37°C . Monoribosomes also attached to the R-liposomes, although the amount of monoribosomes bound to the R-liposomes was always lower than that of polyribosomes (results not shown). The liposomes prepared without addition of R-fraction had no capacity to bind polyribosomes (Fig. 2d).

Modes of polyribosome binding to the R-liposomes

Studies on the polyribosome–membrane association *in vitro* have so far been largely carried out with rough microsomal membranes stripped of endogenous polyribosomes (Shires *et al.*, 1971;

Fig. 3. Enhancement of polyribosome binding to the R-liposomes by increasing amount of polyribosomes added

The $^{14}\text{C}/^3\text{H}$ ratio in the binding complex was determined from the binding-complex peak in each of Figs. 2(a), 2(b) and 2(c).

Borgese *et al.*, 1974; McIntosh & O'Toole, 1976), so it was decided to compare the mode of polyribosome binding to the R-liposomes with that to these stripped rough microsomal membranes. 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. 4(b), addition of 0.1 mM-aurintricarboxylic acid to the binding mixture resulted in the disappearance of the binding complex between polyribosomes and the R-liposomes which was seen in Fig. 4(a).

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 0°C with a mixture of trypsin and chymotrypsin ($50\mu\text{g}/\text{ml}$ each). A similar result was obtained for the binding capacity of the R-liposomes. The capacity of the R-liposomes to bind polyribosomes virtually disappeared after treatment with trypsin ($3\mu\text{g}/\text{ml}$) at 0°C for 60 min (Fig. 4c).

Further, the effect of raising the KCl concentration on the binding of polyribosomes to the R-liposomes was examined. As shown in Figs. 4(d) and 4(e), the polyribosome-binding capacity of the R-liposomes decreased linearly with increasing KCl concentration such that the binding capacity fell to 36% at 100 mM-KCl and 12% at 200 mM-KCl of the control value observed at 25 mM-KCl. Borgese *et al.* (1974) also reported that increasing the KCl concentration from 25 mM to 200 mM caused an essentially linear decrease in the ribosome-binding capacity of stripped rough microsomal membranes. The inhibitory effect of raising the KCl concentration on the binding of polyribosomes to the R-liposomes seems virtually reversible, since centrifugation of the binding mixture containing high KCl concentrations on a sucrose gradient containing 25 mM-KCl resulted in the appearance of the binding complex to a similar extent to the control experiment containing 25 mM-KCl (results not shown). Such reversibility was seen even in a binding mixture containing a high concentration of KCl such as 500 mM.

Some part of the monoribosomes assembled in the polyribosomes bound to the R-liposomes may be linked to the R-liposomes merely through the mRNA. To examine this, the binding mixture was treated with ribonuclease ($3\mu\text{g}/\text{ml}$) at 0°C for 30 min after the incubation. After addition of ribonuclease inhibitor, which was prepared from rat liver cytosol by the method of Shortman (1961), the sample was centrifuged on a sucrose gradient. The results shown in Fig. 5 suggested that approx. 40% of monoribosomes assembled in the polyribosomes bound to the R-liposomes are directly attached to the R-liposomes.

Fig. 4. Inhibition of polyribosome binding to the R-liposomes by addition of aurintricarboxylic acid, by proteolysis with trypsin, and by increasing KCl concentration

The ^{14}C -labelled polyribosomes and the ^3H -labelled R-liposomes (protein/lipid ratio 3:10) were prepared as described in the Experimental section. In a control experiment (a), the binding mixture, containing $130\text{ }\mu\text{g}$ of protein of R-liposomes and $80\text{ }\mu\text{g}$ of RNA of polyribosomes, was layered over a linear $0.3\text{--}1.5\text{ M}$ -sucrose gradient on top of 1.7 M - and 2.4 M -sucrose in TKM buffer and centrifuged at $102\,500g$ for 10h. In (b), the binding mixture was incubated in the presence of 0.1 mM -aurintricarboxylic acid before centrifugation. In (c), before the binding reaction the R-liposomes were incubated at 0°C for 1h with $3\text{ }\mu\text{g}$ of trypsin/ml, followed by addition of soya-bean trypsin inhibitor giving a final concentration of $9\text{ }\mu\text{g/ml}$. In (d) and (e), the binding reaction was performed in the presence of 100 mM-KCl (d) or 200 mM-KCl (e), followed by centrifugation through the above gradient containing 100 mM-KCl or 200 mM-KCl respectively. —, ^3H radioactivity; ----, ^{14}C radioactivity.

Discussion

The present study shows that the R-fraction reconstituted into liposomes possesses the capacity to bind polyribosomes *in vitro*, whereas liposomes prepared from lipids alone do not. The mode of polyribosome binding to the R-liposomes was very similar to that to stripped rough microsomal membranes (Shires *et al.*, 1971; Borgese *et al.*, 1974) or to the R-fraction, which is a protein fraction isolated from rough microsomal membranes of rat liver, having a high affinity for polyribosomes (Fujita *et al.*, 1977). The polyribosome binding

depended on the amount of polyribosomes added to the binding mixture. The binding capacity of the R-liposomes was very sensitive to proteolytic enzyme and was strongly inhibited by addition of 0.1 mM -aurintricarboxylic acid. The suppression of polyribosome binding by raising KCl concentration was very similar for the R-liposomes and for the stripped rough microsomal membranes (Borgese *et al.*, 1974) or the R-fraction (Fujita *et al.*, 1977). These results suggest that the binding mechanism of polyribosomes to the R-liposomes is much like that for rough microsomal membranes stripped of endogenous polyribosomes.

Fig. 5. Effect of ribonuclease treatment on the binding complex between polyribosomes and the R-liposomes. The ^{14}C -labelled polyribosomes and the ^3H -labelled R-liposomes (protein/lipid ratio 3:10) were prepared as described in the Experimental section. In a control experiment (a), the binding mixture, containing $130\mu\text{g}$ of protein of R-liposomes and $80\mu\text{g}$ of RNA of polyribosomes, was incubated at 4°C for 30 min and layered over a linear 0.3–1.5 M-sucrose gradient on top of 1.7 M- and 2.4 M-sucrose in TKM buffer, and then centrifuged at $102\,500g$ for 10 h. In (b), after the incubation the binding mixture was treated with ribonuclease ($3\mu\text{g}/\text{ml}$) at 0°C for 30 min, followed by addition of ribonuclease inhibitor, and then centrifuged as described above. In (c), the ^{14}C -labelled polyribosomes ($80\mu\text{g}$ of RNA) were treated with ribonuclease and centrifuged as described above.

In recent years, electron-microscopy methods have been developed to show the ribosome-binding sites on freeze-fractured rough-endoplasmic-reticulum membranes. Ojakian *et al.* (1977) revealed the presence of homogeneously distributed 10.5 nm intramembrane particles in rough microsomal preparations of rat liver and showed that cleavage of the mRNA of bound polyribosomes by ribonuclease or removal of ribosomes by treatment with puromycin led to a redistribution of the particles into large aggregates on the cytoplasmic fracture face, leaving large particle-free regions. Giddings & Staehelin (1980) described that freeze-fracture micrographs of cells of the green alga *Microsterias denticulata* reveal imprints of polyribosomes on the rough-endoplasmic-reticulum membranes and that spiral rows of intramembrane particles associated with imprints of polyribosomes on the rough-endoplasmic-reticulum membranes can be recognized. They proposed that the 11 nm particles could be structural equivalents of ribosome-binding sites, consisting of a molecule each of ribophorin I and II (Kreibich *et al.*, 1978) and a nascent polypeptide chain.

In our previous paper (Fujita *et al.*, 1977), it was shown that the R-fraction was further fractionated into four subfractions (R_1 – R_4) by gel filtration in the presence of 0.4% (w/v) deoxycholate, and the high affinity for polyribosomes was localized only in the R_1 fraction. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the R_1 fraction revealed the existence of three major proteins characteristic of this fraction, one of which represented a molecular weight similar to that of ribophorin I. It is interesting that the sum of the molecular weights of fractions R_1 – R_4 (54×10^4) is comparable with the particle weights of the intramembrane particles found by Giddings & Staehelin (1980) or Ojakian *et al.* (1977). Assuming the density of the intramembrane particles to be identical with that of protein, particle weights are 56×10^4 daltons for the 11 nm particles described by Giddings & Staehelin (1980) and 49×10^4 for the 10.5 nm particles reported by Ojakian *et al.* (1977).

More recently, an electron-microscope study concerning the ribosome-binding sites has been reported by Nagata (1980). In his study, a protein fraction was prepared under conditions similar to our method (Fujita *et al.*, 1977) from 'Triton-polyribosomes'. The proteins were incorporated into a liposome-lipid bilayer (120–300 nm in diameter) to evaluate their ribosome-binding ability. After incubation with exogenous ribosomes, the liposomes were observed by thin-section electron microscopy, which revealed the morphology of the binding of the ribosomes to the membrane, which looked exactly like isolated rough microsomal membranes. Freeze-fracture micrographs of the reconstituted protein-

containing liposomes (corresponding to the R-liposomes in the present study) indicated that many discrete 8–10 nm particles similar to those in rough microsomal membranes were present on both the concave and convex fracture surfaces of the reconstituted liposomes. Although data were not shown, Nagata (1980) also observed electron-microscopically that: (1) the liposomes made from lipids alone did not accept any ribosomes; (2) the ribosomes attached to the reconstituted liposomes were readily released in a medium containing a high concentration of univalent ions, such as 0.5 M-KCl; (3) digestion of the reconstituted liposomes with trypsin resulted in the loss of their ability to bind ribosomes. These results are in fair agreement with those described in the present study.

The reconstituted liposome system described in the present study could be used for studies of the mechanism for membrane recognition and transfer process of the nascent polypeptides.

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