The Role of Membrane Phospholipids in the Interaction of Ribosomes with Endoplasmic-Reticulum Membrane

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(Received 11 December 1972)

It is shown that the ionic head groups of the membrane phospholipids cannot be solely responsible for the attachment of the ribosome and that other membrane components must also be involved in the binding process.

Eukaryotic cells contain two classes of ribosomes, namely those free in the cytoplasm and those associated with the intracellular membranes of the rough endoplasmic reticulum (Palade & Siekevitz, 1956). Each class has been reported to synthesize different types of protein (Siekevitz & Palade, 1960; Hicks *et al.*, 1969) and to have different functional properties (Shafritz & Isselbacher, 1972).

It has been suggested that the binding between the ribosome and the endoplasmic-reticulum membrane could result from an ionic interaction mediated by Mg^{2+} and/or spermidine (Schlessinger *et al.*, 1965; Khawaja & Raina, 1970), but there is increasing evidence that other factors are important, such as the presence of the nascent protein chains and mRNA (Redman & Sabatini, 1966). Nevertheless, as pointed out by Shires *et al.* (1971), these other mechanisms do not rule out the possibility that an ionic interaction could be predominant in the membrane-ribosome interaction.

The parameters of the binding process can be determined by reassociating labelled ribosomes with degranulated endoplasmic-reticulum membranes. Degranulation has been produced by pyrophosphate and citrate buffers (Suss *et al.*, 1966), 2M-LiCl (Scott-Burden & Hawtrey 1969) and ribonuclease-EDTA treatment (Shires *et al.*, 1971). The last method yields a degranulated membrane that will reattach approximately as many ribosomes as found associated with the original membrane. It has also been shown that the reattachment process is not due to entrapment of the ribosomes in the membrane vesicles (Ragland *et al.*, 1971).

To test the importance of the phospholipid ionic head groups in the binding process, the effect of phospholipase C treatment on the reassociation of ribosomes with rough endoplasmic-reticulum membranes degranulated by ribonuclease-EDTA treatment was studied.

Materials and methods

The general procedure for isolation of the endoplasmic-reticulum membranes from rat liver was that

described by Shires *et al.* (1971), with the following modification: the TKM buffer was 25mM-KCl – 10mM- $MgCl_2$ – 50mM-Tris-HCl, pH7.6 (TKM buffer). The post-mitochondrial fraction was prepared from 200g male Sprague-Dawley rats in 0.25M-sucrose-TKM buffer.

Labelled polyribosomes were obtained from rats fed until decapitation in order to avoid a decreased RNA synthesis induced by starvation (Blobel & Potter, 1967) and which had been injected intraperitoneally with [3H]orotic acid (23Ci/mmol), 200 μ Ci/100g body wt., 12–14h previously. After deoxycholate treatment [1.25% (w/v) for 60min] of the post-mitochondrial fraction, the labelled ribosomes were sedimented into the 2.0M-sucrose-TKM buffer layer of a two-step barrier (7ml of sample in 0.25M-sucrose-TKM buffer layered over 4ml of 1.3M-sucrose-TKM buffer resting on 3ml of 2.0M-sucrose-TKM buffer) by centrifugation at 170000g (for 14h) in a Spinco SW40 rotor. The average E_{260}/E_{280} ratio was 1.68 (s.d. ± 0.08) and the average d.p.m./ μ g of RNA was 350 (s.d. ± 57).

The methods employed to prepare 'heavy' rough endoplasmic-reticulum membranes were those described by Andrews & Tata (1968) except that 0.25M-sucrose was employed in the homogenizing medium and a 1.3M-sucrose barrier as described by Tata & Williams-Ashman (1967) was used in the first stage of fractionation.

Degranulation of membranes was carried out with pancreatic ribonuclease A and EDTA as described by Shires *et al.* (1971). Degranulated membranes were kept at 4°C no more than 2 days before phospholipase C treatment and reassociation. Phospholipase C (2.5 units/mg; Sigma Chemical Co., St. Louis, Mo., U.S.A.) from *Clostridium welchii* was prepared as described by Simpkins *et al.* (1971a), in 100mM-KCl – 2mM- $CaCl_2$ – 5mM-imidazole – HCl buffer, pH7.6. The degranulated endoplasmic-reticulum membranes were washed twice, then resuspended in the imidazole buffer. Samples (3mg) of membrane protein were incubated for 30min at 37°C with 0–800 μ g of phospholipase C in a final volume of 3.5ml. The reaction was stopped by cooling the tube

to 4°C, addition of 5.5 ml of imidazole buffer at 4°C and centrifugation of the membranes at 105000g for 50 min. Portions of the supernatants were taken for determinations of protein (Lowry *et al.*, 1951), with crystalline bovine serum albumin (Pentax) as standard, and total phosphorus (Bartlett, 1959). The membranes were finally resuspended in the TKM buffer.

Phospholipids were extracted by the procedure of Folch *et al.* (1957) and analysed by one-dimensional t.l.c. (Skipski *et al.*, 1962). Quantitative measurement of the spots was performed as described by Kahovcova & Odavic (1969).

The RNA associated with the membranes was determined as described by Schneider (1957) and that of ribosomes by the absorbance at 260 nm (Munro & Fleck, 1966).

The reassociation was performed by agitating a mixture of endoplasmic-reticulum membranes (500 µg of membrane protein) and labelled ribosomes (25 µg of RNA) in a total volume of 3 ml of 0.44 M-sucrose-TKM buffer for 20 s at 4°C. No post-microsomal fraction was added; the mixture was layered on 1 ml of 2.0 M-sucrose-TKM buffer and centrifuged at 290000g for 10 h in a Spinco SW 56 rotor. The non-sedimented material, the membrane fraction and the unbound ribosomes in the 2.0 M-sucrose layer were aspirated separately by a Pasteur pipette connected to a peristaltic pump, digested by NCS solubilizer (Amersham-Searle Inc., Des Plaines, Ill., U.S.A.) and their radioactivities counted with Bray's (1960) scintillation liquid. A paper by Rolleston (1972) suggests that problems can arise if a barrier method is used to analyse ribosomal binding, and he therefore employed a method by which the membranes are sedimented through a sucrose barrier, the non-bound ribosomes being washed off by the passage of the mixture through the sucrose. We employed this technique and obtained similar results to those observed by the technique described above.

Results and discussion

The ratio of endoplasmic-reticulum-membrane protein to RNA was kept constant at 20:1, which is in the saturation region of the binding curve (Shires *et al.*, 1971; S. Jothy, unpublished work). It was assumed that the phospholipase treatment was homogeneous over the whole membrane population.

Table 1 shows only a 6% decrease in the reassociation process, although there was liberation of 46–69% of the total lipid P. All phospholipids were appreciably hydrolysed, phosphatidylinositol and phosphatidylserine apparently being the most resistant, although there is only a relatively small amount of phosphatidylserine present and thus the values obtained for this phospholipid are subject to large error.

Table 1. *Effect of treating degranulated endoplasmic-reticulum membranes with increasing concentrations of phospholipase C*
The degree of reassociation of the untreated membrane was arbitrarily set at 100%. The values in parentheses refer to the phospholipid composition of the membrane expressed as percentages of the total lipid. Errors are expressed as $\pm$ S.E.M.

Phospholipase C treatment (µg of enzyme/ mg of membrane protein)	Reassociation (%)	Membrane lipid P (expressed as % of control membrane)	Phospholipid composition (expressed as % of control membrane)					
			Lysophosphatidyl- choline	Sphingomyelin	Phosphatidyl- choline	Phosphatidyl- inositol	Phosphatidyl- serine	Phosphatidyl- ethanolamine
0	100	100	100 (9.5 $\pm$ 1.2%)	100 (7.7 $\pm$ 1.2%)	100 (49.2 $\pm$ 7.3%)	100 (10.7 $\pm$ 1.7%)	100 (2.6 $\pm$ 0.4%)	100 (8.5 $\pm$ 1.4%)
20	94	54	45	24	24	80	76	64
200	94	31	40	19	20	62	62	50

It also appeared important to evaluate whether perturbation of the phospholipid regions of the endoplasmic-reticulum membrane with its native ribosomes bound *in situ* would produce degranulation. Table 2 indicates that phospholipase C treatment of the granulated membrane in an Mg^{2+} -free medium did not significantly alter the RNA/protein ratio, although 80% of the membrane phospholipids were hydrolysed.

To determine whether removal of the ribosomes from the membrane removes phospholipids perhaps essential to the binding process, the phospholipid pattern after ribonuclease-EDTA treatment was compared with that of the granulated membrane: no significant alteration in phospholipid composition was apparent (S. Jothy, unpublished work).

From the above results the involvement of the phospholipid polar head groups of the endoplasmic-reticulum membrane in the binding process appears minor and concepts such as Mg^{2+} bridges between the RNA phosphate groups and the charged phosphate groups of the phospholipids appear unlikely to be true. This observation does not imply that phospholipids cannot play any role at all in the binding process, as the phospholipid hydrocarbon chains could be involved in some form of hydrophobic bonding with the ribosomal protein. This, however, appears unlikely for the following reasons: (a) the architecture of the ribosome seems to feature an rRNA 'coat' masking the protein core (Cotter *et al.*, 1967); (b) the binding process is sensitive to cations (Khawaja & Raina, 1970); (c) phospholipase C treatment of erythrocyte and axonal plasma membranes (Glaser *et al.*, 1970; Simpkins *et al.*, 1971a,b) has shown that the membrane hydrocarbon chains became very mobile after enzyme treatment, and it therefore is unlikely that a hydrophobic interaction involving the membrane phospholipids would remain unaffected after phospholipase C treatment.

One remaining problem is that approx. 20% of the membrane phospholipids were not hydrolysed by this phospholipase C preparation, and thus the possibility must be considered that the non-hydrolysed phospholipids are those responsible for the attachment of the ribosomes to the endoplasmic-reticulum membrane. This idea is a little difficult to reconcile with two facts: (a) the size of the 60S ribosomal subunit is relatively large (20nm diameter) and therefore would be expected to interact with a relatively large area of the membrane surface; (b) the rRNA/membrane phospholipid ratio (w/w) is 0.6–0.9:1 [assuming a 1:1 ratio for both rRNA/ribosomal protein in the ribosome (Peterman, 1964) and phospholipid/protein in the endoplasmic-reticulum membrane (Andrews & Tata, 1971)]; thus the likelihood that only 20% of the total phospholipid could be the sole means of attachment for this relatively large concentration of ribosomes is small if it is assumed that no other membrane components are involved.

One possible approach to this problem is to employ another phospholipase whose specificity for the endoplasmic-reticulum-membrane phospholipids is different from that of phospholipase C. Attempts were therefore made to employ phospholipase A_2 , which has been shown to possess a different phospholipid specificity with erythrocyte phospholipids (Kahlenberg & Banjo, 1972). The experiments gave ambiguous results, probably due to the surface-active properties of the fatty acids and lysophosphatides (Mavis *et al.*, 1972), 10% of the membrane-associated protein and 60% of the total phosphorus being released into solution. These results were contrary to those obtained with phospholipase C treatment, where no loss of membrane protein was apparent.

The other possibility involves the protein moiety of the endoplasmic-reticulum membrane; the drastic decrease in the reassociation process after treatment with trypsin (Shires *et al.*, 1971) suggests that this

Table 2. RNA content of rough endoplasmic-reticulum membranes after phospholipase C treatment

The rough-microsomal fraction was prepared as described in the text under 'Materials and methods' but was not subjected to degranulation by ribonuclease-EDTA treatment. Total membrane protein (i.e. intrinsic membrane protein plus ribosomal protein) (3mg) was washed twice with the imidazole buffer in a 50Ti rotor at 105000g for 1 h, treated with phospholipase C (0–80 μ g of enzyme/mg of membrane protein) for 30 min at 37°C, washed and layered on a 2M-sucrose barrier as described in the text under 'Materials and methods' for the degranulated membrane. The RNA content of the membrane at the interface was determined by the orcinol method (Schneider, 1957). The results are given as means $\pm$ S.E.M.

Phospholipase C treatment (μ g of enzyme/mg of membrane protein)	Phospholipid P released (μ g/mg of membrane protein)	% of membrane phospholipid P liberated	RNA/membrane protein ratio
Control	4.1 $\pm$ 0.5	13.7 $\pm$ 1.6	0.34 $\pm$ 0.03
4	14.2 $\pm$ 2.0	47.3 $\pm$ 6.7	0.34 $\pm$ 0.05
80	24.0 $\pm$ 1.8	80.0 $\pm$ 6.0	0.33 $\pm$ 0.03

membrane component is important in the attachment process, although the amount of protein hydrolysed was so great (35%) that both the lipid and protein moieties were probably affected.

Note added in proof (received 15 January 1973)

A recent paper by Hochberg *et al.* (1972) showed that acid-denatured endoplasmic-reticulum membranes still bind a similar number of polyribosomes. These authors suggested therefore that reassociation data may not be biologically significant. We, for the following reasons, disagree with this idea: (a) Rolleston (1972) has shown that 'stripped' membranes show a specificity for the 60S subunit; (b) Hochberg *et al.* (1972) presented no evidence showing that the attachment of the polyribosomes to the denatured membrane vesicles was not a non-specific entrapment; (c) the biological activity of these bound polyribosomes was not assayed; (d) Shires *et al.* (1971) and the present authors (unpublished work) find marked inhibition of reassociation subsequent to treatment of the membranes with protolytic enzymes.

We thank the Medical Research Council of Canada for financial support of this work. S. J. is indebted to the France-Quebec Scientific Agreement for a Post-Doctoral Fellowship and H. S. is a Canadian Medical Research Council Scholar.

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