Preparation and properties of a complex from rat liver of polyribosomes with components of the cytoskeleton

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(Received 31 May 1983/Accepted 8 July 1983)

1. Gel filtration with 1% agarose (Bio-Gel A-150m) separates polyribosomes bound to microsomal membranes from 'free' polyribosomes when these fractions are prepared by standard centrifugal techniques. However, when polyribosomes contained in an unfractionated postmitochondrial supernatant are run on an identical column, over 90% of the total polyribosomes are present as aggregates, designated 'membrane–cytomatrix', which are eluted in the column void volume. Polyribosomes are not released from these aggregates on removal of microsomal phospholipids by treatment of postmitochondrial supernatant with 1% Triton X-100, a neutral detergent. The aggregates are disrupted by the usual ultracentrifugation techniques used in subcellular fractionation. 2. After treatment of membrane–cytomatrix with Triton X-100 to remove phospholipids and membrane proteins, 58% of the polyribosomes still remain associated with protein-containing complexes in the form of a cytomatrix and are not 'free'. 3. Preparations of both membrane–cytomatrix and cytomatrix are capable of sustained protein synthesis. 4. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis revealed that the cytoskeletal proteins actin and myosin are present in the cytomatrix. 5. Incubation of cytomatrix preparations with the actin-depolymerizing agent deoxyribonuclease I caused release of the polyribosomes. 6. Polyribosome release by deoxyribonuclease I was prevented by prior incubation with phalloidin, which is known to stabilize F-actin. 7. Thus polyribosomes are associated with cytoskeletal elements in rat liver, and this association is dependent on polymeric forms of actin.

Eukaryotic polyribosomes, when prepared by standard techniques of cellular homogenization and discontinuous-sucrose-gradient centrifugation (Blobel & Potter, 1967), are obtained in either 'free' or membrane-bound form (Lewis & Tata, 1973; Adelman *et al.*, 1973; Zähringer *et al.*, 1977; Ramsey & Steele, 1977). This operational separation of polyribosomes is thought to reflect a functional compartmentation of polyribosomes; membrane-bound polyribosomes have been shown to be enriched in mRNA species coding for integral membrane proteins and proteins destined for export from the cell, whereas 'free' polyribosomes synthesize proteins which ultimately function in their 'soluble'

cytoplasmic roles (Palade, 1975; Shore & Tata, 1977; Harwood, 1980). The view that the cytoplasm is organized into membranous and soluble domains has evolved largely from studies using homogenization and ultracentrifugation (see Penman *et al.*, 1982).

However, work based on the extraction of whole cells with non-ionic detergents has suggested that this view of polyribosomal organization is naive.

Lenk *et al.* (1977) demonstrated that an association appears to exist between polyribosomes and microfilaments in cytoskeletal preparations from whole cells prepared by an extraction procedure that removes most of the cellular phospholipid and most of the soluble cytoplasmic proteins (Brown *et al.*, 1976; Webster *et al.*, 1978).

Fulton *et al.* (1980) proposed that polyribosomes are attached to the cytoskeleton via mRNA. Wolosewick & Porter (1975) and Lenk *et al.* (1977) have observed that it is the 'free' (non-membrane-bound) polyribosomes which appear to be asso-

Abbreviations used: DNAase, deoxyribonuclease; SDS, sodium dodecyl sulphate; PMS, postmitochondrial supernatant.

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ciated with filaments, although the results that we present below indicate that the membrane-bound-polyribosomes population is similarly attached to filaments.

The two apparently conflicting views of polyribosome organization have evolved from two radically different methods of cellular fractionation. Homogenization and differential sucrose-density-gradient centrifugation are highly destructive techniques which disrupt many aspects of cellular architecture and have been shown to damage both membranous (Wattiaux *et al.*, 1971; Bronfman & Beaufay, 1973; Collot *et al.*, 1975) and filamentous structures (Marsland & Brown, 1942; Ikkai & Ooi, 1966; Josephs & Harrington, 1967), as well as to remove some of the polyribosomes from microsomal membranes (Bont *et al.*, 1972; Zauderer *et al.*, 1973; Palmer *et al.*, 1978). The extraction of whole cells with non-ionic detergents allows retention of filamentous networks and apparent overall morphological integrity while removing intracellular phospholipid membranes.

Gel filtration of the postmitochondrial supernatant from rat liver has been shown previously to separate the microsomal vesicles, eluted in the void volume of the agarose column, from most of the soluble protein and RNA (Bronfman & Beaufay, 1973; Tangen *et al.*, 1973; McCole *et al.*, 1979; Oliveira Filgueiras *et al.*, 1980). This mild fractionation does not avoid the homogenization step, but it does allow the separation of membrane vesicles and polyribosomes in the absence of ultracentrifugation.

Evidence that actin is involved in polyribosome-filament interactions comes mainly from studies using DNAase I. Muscle G-actin (monomeric actin) has been shown to inhibit DNAase I by forming a 1:1 complex with the enzyme (Hitchcock *et al.*, 1976), an interaction that also prevents the polymerization of G-actin. Filamentous actin (F-actin) also interacts with DNAase I (Blikstad & Carlsson, 1978) in a process that leads to the formation of 1:1 stable complexes between the enzyme and each actin monomer. Ramaekers *et al.* (1980) demonstrated that DNAase I released polyribosomes from calf lens plasma membranes, suggesting that the interaction is mediated by actin. Sheetz (1979) showed that the erythrocyte cytoskeleton is disrupted by DNAase I, and it thus appears that the integrity of F-actin is necessary for the attachment of polyribosomes to cytoskeletal elements.

The ability of DNAase I to depolymerize F-actin has yet to be assigned any biological role. Interestingly, actin is present in the G form at the high concentrations in various eukaryotic cells (Lazarides & Weber, 1974; Lengsfeld *et al.*, 1974) and the equilibrium between G- and F-actin could be important in polyribosomes organization.

The G-F-actin equilibrium can be modified by phalloidin, a toxin bicyclic heptapeptide from the green death-cap mushroom (*Amanita phalloides*). Three major effects of phalloidin on its target organ, the liver, have been reported. These are vacuolization of parenchymal cells, K⁺ efflux from the isolated organ, and an increase in filamentous structures associated with isolated membranes (Weiland & Weiland, 1972). Grovindan *et al.* (1973), using a phalloidin derivative, ³H-labelled phallo-toxin, showed that the toxin binds mainly to a fraction enriched in filaments, which Lengsfeld *et al.* (1974) identified as containing actin, by their reaction with heavy meromyosin to form arrow-head-like structures. Phalloidin binds exclusively with high affinity to polymeric actin, and the resulting complex cannot readily depolymerize to actin monomers. It also increases the rate of polymerization of G- to F-actin (Dancker *et al.*, 1975).

In the present paper we report the methodology to obtain a cytoskeletal preparation from rat liver that is rich in polyribosomes, active in protein synthesis and virtually free of membrane components. The association between polyribosomes and actin in this cytomatrix has been investigated.

Materials and methods

Chemicals

[6-¹⁴C]Orotic acid (61 Ci/mol) and [1-³H]-ethanolamine (8.8 Ci/mmol) were purchased from Amersham International (Amersham, Bucks., U.K.); [³⁵S]methionine (1195.3 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.); Bio-Gel A-150m (50–100 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Phalloidin, diethyl pyrocarbonate, bovine serum albumin, bovine pancreatic DNAase I (DN-100) and ribonuclease were from Sigma (St. Louis, MO, U.S.A.), as were all chemicals used in protein-synthesis experiments. Agarose-5'-(*p*-aminophenyl-phosphoryl)-uridine 2'(3')-phosphate was obtained from Miles Laboratories (Slough, Bucks., U.K.). Sephadex G-25 and G-10 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Radiolabelling

rRNA was uniformly labelled by the method of Ragland *et al.* (1971) by intraperitoneal injections of [6-¹⁴C]orotic acid (50 µCi/rat) in 0.9% NaCl 24 h before the rats were killed. [1-³H]Ethanolamine (100 µCi/rat) in 0.9% NaCl was injected to label phospholipid 20 h before death.

Preparation of postmitochondrial supernatant

Rats were killed by cervical dislocation and the livers perfused *in situ* via the portal vein with

30–50 ml of ice-cold 0.25 M-sucrose/TKME [0.05 M-Tris (pH 7.5 at 25°C)/0.025 M-KCl/0.005 M-MgCl₂/0.001 M-EDTA]. Livers were removed, chopped, rinsed in 0.25 M-sucrose/TKME and homogenized in ice-cold 0.25 M-sucrose/TKME (2.25 mg/g of liver) with three strokes of a Teflon/glass Potter–Elvehjem homogenizer (0.5 mm clearance) at 700 rev./min. Mitochondria and nuclei were removed by centrifugation for 15 min at 8000 $g_{av.}$, giving the PMS.

Fractionation of postmitochondrial supernatant by gel filtration

Analytical gel filtration. Analytical fractionation of PMS samples was performed by running 10 to 14 'mini-columns' (0.3 cm × 20 cm Bio-Rad Econo-columns) of Bio-Gel A-150m (50–100 mesh) simultaneously, and the effluents were collected and radioactivity was counted as described by Fey *et al.* (1981). Briefly, the Bio-Gel was poured as a thick slurry in vacuum-degassed 0.25 M-sucrose/TKME to form a bed of 18 cm length. The columns were pumped simultaneously at a rate of 88 μ l/min with Watson-Marlow MHRE 22 pumps with 10-channel Delta units. Samples (100 μ l) were run into the bed and the columns were eluted with 0.25 M-sucrose/TKME. When the visible membrane band reached the lower part of the column, eluate collection was commenced on strips (2.7 m × 2 cm) of Whatman Benchkote paper drawn past the collection points by a chart recorder (3 cm/min). The strips were dried, cut into 6 cm lengths and counted for radioactivity. When samples were solubilized in 1% (w/v) Triton X-100, the Bio-Gel A-150m columns were equilibrated and eluted with 0.25 M-sucrose/TKME containing 1% Triton X-100. This addition of Triton X-100 to the elution buffer has no effect on the fractionation characteristics of the column. Recoveries from the columns were in the range 95–102%. Average variation in ¹⁴C c.p.m. in the void-volume peak between ten control pairs was $2.2\% \pm 1.8\%$.

Preparative gel filtration. Preparative fractionation of PMS was done on glass columns (2.5 cm × 20 cm) of Bio-Gel A-150m. PMS (3 ml) was loaded on the column and eluted with 0.25 M-sucrose/TKME. The column eluate was monitored at 260 nm by using a flow cell fitted to a Beckman 35 spectrophotometer. The column microsomal fraction appeared in the void volume as a symmetrical peak, which was eluted in 17 ml of buffer. This void-volume peak was taken to be the gel-filtration equivalent to the centrifugally prepared mixed microsomal fraction. The eluate after this peak was denoted the 'included volume' and consisted of the equivalent to the centrifugally prepared 'free' polyribosomes, followed by a peak of soluble material denoted 'cell sap'.

Fractionation of postmitochondrial supernatant by ultracentrifugation

Mixed microsomal fraction and 'free' polyribosomes were prepared by a modification of the discontinuous-sucrose-density-gradient technique (Blobel & Potter, 1967).

PMS (diluted 1:10; about 30 ml) was layered over the sucrose gradient, consisting of a 5 ml cushion of 2 M-sucrose/TKME with a 5 ml overlay of 1 M-sucrose/TKME. The gradients were centrifuged in a fixed angle rotor at 103 000 $g_{av.}$ for 3.5 h. Mixed microsomal fractions were recovered from the 1 M/2 M interface, and 'free' polyribosomes from the pellet (resuspended in 0.25 M-sucrose/TKME).

Purification and quantitative analysis of polyribosomes

Polyribosome-containing fractions (from 3 ml samples of PMS) were prepared by gel filtration or ultracentrifugation as described above. All fractions were solubilized by adjustment to final concentrations of 1% (w/v) Triton X-100, 1% (w/v) deoxycholate and 0.5 M-KCl in 0.25 M-sucrose/TKME. Samples were layered over a cushion of 2 M-sucrose/TKME and centrifuged in a swing-out rotor for 48 h at 135 000 $g_{av.}$ to pellet all the polyribosomal material (including monoribosomes). The total ribosome pellets were resuspended in 0.25 M-sucrose/TKME, and total RNA was determined by the method of Fleck & Begg (1954).

Polyribosome profiles were determined by centrifugation in continuous sucrose density gradients (in TKME buffer) by the method of Lenk *et al.* (1977). Gradients (15–30%, w/w) were centrifuged for 30 min at 83 000 $g_{av.}$

Preparation of membrane-cytomatrix and cytomatrix by gel filtration

PMS (5 ml) was applied to a glass column (2.5 cm × 20 cm) containing Bio-Gel A-150m. The column was eluted with 0.25 M-sucrose/TKME at a rate of 0.5 ml/min. The void volume of the column, containing complexes of polyribosomes, cytoskeletal components and membranes, was collected in 5 ml fractions. The fraction with the highest A_{280} was used as the membrane-cytomatrix preparation. To prepare cytomatrix it was incubated with the non-ionic detergent Triton X-100 (1%, w/v) for 10 min on ice to solubilize the membrane. This preparation was used without further fractionation in all experiments except protein synthesis as the cytomatrix.

The cytomatrix was separated from all solubilized microsomal components (designated separated cytomatrix) for protein-synthesis studies. PMS (5 ml) was treated with Triton X-100 (1%) for 10 min on ice to solubilize the membrane before gel filtration. The Bio-Gel A-150m column (2.5 cm ×

20 cm) was eluted with 'polyribosome buffer' [50 mM-Tris (pH 7.6)/75 mM-KCl/5 mM-MgCl₂/3 mM-dithiothreitol]. Polyribosome buffer replaced sucrose/TKME as the elution buffer in the preparation of the membrane-cytomatrix, the cytomatrix and the intermediate complex when these preparations were to be used for protein-synthesis studies.

Protein synthesis

Cell-free protein synthesis was by the method of Hishino *et al.* (1977), with several modifications. The total volume of the assay mixture was decreased to 20 μ l and contained 19 amino acids (each 0.025 mM), 35 mM-Tris/HCl, pH 7.5, 1 mM-ATP, 0.25 mM-GTP, 7.4 mM-phosphocreatine, 10 μ g of creatine kinase/ml, 75 mM-NH₄Cl, 4 mM-magnesium acetate, 1 mM-dithiothreitol, 25.7 μ Ci of [³⁵S]-methionine, 10 μ l of cell sap and 5 μ l of the polyribosome-containing preparation under study.

Cell sap was prepared by passing 5 ml of PMS through a column (2.5 cm $\times$ 20 cm) of Bio-Gel A-150m, equilibrated with polyribosome buffer. The included volume was collected in 5 ml fractions. The fraction with the highest A_{280} was applied to a Sephadex G-25 column (1.5 cm $\times$ 15 cm), followed by elution with polyribosome buffer to remove amino acids.

Protein synthesis was performed at 25°C for 3 h or such other times as stated. Samples (2 μ l in triplicate) were then removed from the incubations and added to 0.5 ml of 0.1 M-NaOH/1.5% (v/v) H₂O₂ solution and incubated for 10 min at 37°C. They were cooled on ice and 3 ml of ice-cold 25% (w/v) trichloroacetic acid [containing 2% (w/v) casein hydrolysate] was added. Acid-insoluble material was collected on filters (GF/C; Whatman), washed with 2 $\times$ 5 ml of 8% (w/v) trichloroacetic acid and then with 5 ml of ice-cold ethanol. The filters were dried and counted for radioactivity in 2,5-diphenyloxazole/toluene (4 g/l solution).

Phalloidin and DNAase I

All buffers and glassware were autoclaved and plastic columns were treated with 0.005% (v/v) diethyl pyrocarbonate to eliminate ribonuclease contamination. DNAase I was freed of contaminating ribonuclease by affinity chromatography on agarose-coupled 5'-(*p*-aminophenyl-phosphoryl)-uridine 2'/(3')-phosphate (Maxwell *et al.*, 1976). The purified DNAase I was eluted with 0.02 M-sodium acetate, pH 5.2, then passed through a Sephadex G-10 column (2.5 cm $\times$ 10 cm), equilibrated with Ca²⁺-containing buffer, pH 7.5. The eluate was freeze-dried overnight and the product resuspended in sterile water to give a solution containing 50 mg of DNAase I/ml. The ionic conditions of the Ca²⁺-containing buffer were determined such that a final DNAase I solution was 50 mM in Tris, pH 7.5,

0.01 mM in phenylmethane-sulphonyl fluoride and 0.25 mM in CaCl₂. The ribonuclease activity of DNAase I and phalloidin (dissolved in sterile water at 2 mg/ml) was assayed by the method of Blackburn *et al.* (1977) and both had activities of ribonuclease less than 7 ng/ml. Incubations with phalloidin (40 μ g/ml) were performed at 25°C for 30 min before treatment with Triton X-100 or DNAase I. Incubations with DNAase I (2.5 mg/ml) were performed at 25°C for 45 min.

Monitoring the effects of phalloidin and DNAase I by gel filtration

Multiple analytical columns (Bio-Rad Econocolumns; 0.3 cm $\times$ 18 cm) were run simultaneously, and effluents were collected and radioactivity was counted by the methods reported by Fey *et al.* (1981), with the following modifications: 300 μ l samples were loaded on the columns and 1% Triton X-100 was omitted from the running buffer.

Polyacrylamide-gel electrophoresis

SDS/10%-polyacrylamide gels were run by the method of Laemmli (1970).

RNA and protein determinations

RNA was measured by a modification (Fey *et al.*, 1981) of the method of Fleck & Begg (1954). Protein was measured with bovine serum albumin as a standard by the method of Lowry *et al.* (1951), or by that of Wang & Smith (1975) when samples contained Triton X-100.

Results

Gel filtration of [1-³H]ethanolamine-labelled post-mitochondrial supernatant

Postmitochondrial supernatant prepared from rats injected with [³H]ethanolamine gave the elution profile shown in Fig. 1. Most of the labelled material (75%) ran in the column void volume. This is in agreement with the findings of McCole *et al.* (1979), who showed that 87% of the total phospholipid of the PMS was excluded from the agarose column and appeared in the void volume. Solubilization in 1% Triton X-100 shifted 94% of the label to the lower-molecular-weight region of the elution profile.

Gel filtration of centrifugally prepared polyribosomes

Membrane-bound polyribosomes (mixed microsomal fractions), 'free' polyribosomes and total polyribosomes were prepared by fractionation of PMS on discontinuous sucrose gradients. Free polyribosomes and total polyribosomes were purified as described in the Materials and methods section by solubilization in 1% Triton X-100 and 1% deoxycholate in 0.5 M-KCl and pelleting through a

Fig. 1. Gel-filtration elution profiles of $[1\text{-}^3\text{H}]\text{ethanolamine}$ -labelled postmitochondrial supernatant

Samples ($100\mu\text{l}$) of PMS prepared from livers of rats injected with $[1\text{-}^3\text{H}]\text{ethanolamine}$ were run on columns ($0.3\text{ cm} \times 20\text{ cm}$) of Bio-Gel A-150m. ■, Eluted with 0.25 M -sucrose/TKME (control); □, sample solubilized with Triton X-100 and eluted with 0.25 M -sucrose/TKME/1% (w/v) Triton X-100. Triton X-100 solubilization displaces 94% of the label from the void-volume peak.

2 M -sucrose cushion. Passage of these purified polyribosome fractions through a column of Bio-Gel A-150m produced the RNA profiles depicted in Fig. 2. For both the free and total polyribosome profiles 85–90% of the applied material was eluted in fractions 9–29, the included volume of the column. When PMS or mixed microsomal fractions were fractionated on identical columns, a large peak of polyribosomal material was eluted in the void volume of the columns (fractions 1–8, Fig. 2). Although the void-volume peak in Fig. 2(a) contained only 52% of the total $[^{14}\text{C}]\text{orotate}$ label in the PMS, it contained more than 90% of the total polyribosomes applied to the column (Table 1). This anomaly arises because a large percentage of the $[^{14}\text{C}]\text{orotate}$ label present in the included volume is due to the presence of unincorporated material. For the mixed microsomal fraction, the presence of such a high percentage of unincorporated label is probably due to entrapment of soluble components in the 1 M -sucrose layer during preparation.

The data in Fig. 3 show that the material labelled by $[^{14}\text{C}]\text{orotate}$ in these elution profiles is composed of polyribosomes. These conventional linear 15–30%-sucrose gradients clearly indicate that the labelled RNA in Fig. 2 is representative of essentially undegraded polyribosomes, as well as a peak of monomeric ribosomes (m).

Polyribosome distribution after centrifugation and gel filtration

Quantitative studies of polyribosome distribution were performed with 3 ml samples of PMS,

Fig. 2. Gel-filtration elution profiles: comparison of centrifugally derived fractions with postmitochondrial supernatant and total polyribosomes isolated from post-mitochondrial supernatant

Samples ($100\mu\text{l}$) of $[^{14}\text{C}]\text{orotic acid}$ -labelled material were run on columns ($0.3\text{ cm} \times 20\text{ cm}$) of Bio-Gel A-150m. Samples were incubated for 30 min at 25°C before loading. (a) ■, PMS; □, total polyribosomes from PMS purified as described and resuspended in 0.25 M -sucrose/TKME before being run on the column; Δ, total polyribosomes from $[^{14}\text{C}]\text{orotic acid}$ -labelled PMS mixed with unlabelled PMS. In both cases over 80% of the isolated polyribosomes are eluted in the included volume (fractions 9–29). (b) ●, Mixed microsomal fractions prepared from PMS by centrifugation; ○, 'free' polyribosomes prepared from PMS by centrifugation.

which were fractionated by either the centrifugal or gel-filtration protocols. All fractions were solubilized with Triton X-100 and deoxycholate and centrifuged through a 2 M -sucrose cushion as described in the Materials and methods section. Total RNA was determined for all fractions. The experimental data obtained are given in Table 1. The first clear difference between the two techniques is that

Fig. 3. Continuous-sucrose-density-gradient profiles of [^{14}C]orotic acid-labelled polyribosomes isolated from total postmitochondrial supernatant, centrifugally prepared mixed microsomal fractions and 'free' polyribosomes

Polyribosomes were isolated by solubilization of the sample in 1% Triton X-100/1% deoxycholate/0.5 M-KCl in 0.25 M-sucrose/TKME and sedimentation through 2 M-sucrose/TKME. Ribosomal pellets were resuspended in 0.25 M-sucrose/TKME and re-solubilized before being layered over the gradients (15–30%, w/w); 'm' denotes the monoribosome peak. (a) Total polyribosomes isolated from PMS. (b) Polyribosomes isolated from centrifugally prepared mixed microsomal fractions. (c) Centrifugally prepared 'free' polyribosomes.

the microsomal fraction and polyribosomes derived by centrifugation were recovered with a much lower efficiency, and the recovery showed considerable variation between individual experiments. Despite the recovery problems, the distribution of polyribosomal RNA was remarkably constant between separate experiments. Two individual representative experiments are summarized in Table 1.

After gel filtration on Bio-Gel A-150m, more than 90% of the polyribosomes are consistently found in the membrane-containing void volume of the column (fractions 1–8). Purified free polyribosomes are found mainly in fractions 9–29 (Fig. 2b) and are thus operationally much smaller than the uncentrifuged polyribosomes. By the criterion of gel filtration, PMS contains very little material corresponding to the free polyribosomes obtained by centrifugation. An explanation of this apparent size change is that in the absence of centrifugal perturbation polyribosomes behave as if attached to larger complexes. These complexes are destroyed by centrifugation and are thus quite different from the aggregates that can be produced as artefacts by incubating PMS at higher temperatures (McIntosh *et al.*, 1975a,b). The polyribosome profiles in Fig. 4 were obtained from both the void-volume and included-volume peaks obtained with a preparative Bio-Gel A-150m column. This clearly demonstrates the polyribosomal nature of the RNA determined in the corresponding fractions in Table 1.

The results of these experiments demonstrate that large complexes of polyribosomes bound to components other than membrane must be present in PMS not exposed to ultracentrifugation.

Effects of Triton X-100 on polyribosome binding

Since more than 90% of the total polyribosomes in the PMS are found in the excluded fractions of the column, these fractions must contain polyribosomes from both the 'free' and membrane-bound categories as defined by centrifugation. The latter category of polyribosomes should be released by treatment with 1% Triton X-100 provided that they are not also complexed to structural elements other than membranes. Treatment of the PMS with 1% Triton X-100 results in the release of only 15% of the labelled RNA from the polyribosomal complexes excluded from the column (results not shown), implying that the complexes containing polyribosomes are stable in the absence of the phospholipid membrane.

Similar treatment of membrane-cytomatrix caused a somewhat greater loss of RNA, but 58% of ^{14}C -labelled polyribosomes still remained associated with a cytomatrix, despite the removal of 86% of the ^3H -labelled phospholipid from the membrane-cytomatrix by Triton X-100 (Fig. 5).

SDS/polyacrylamide-gel electrophoresis of the

Table 1. Quantitative distribution of polyribosomes: comparison of the fractions prepared by centrifugation and gel filtration

Samples (3ml) of PMS were fractionated by centrifugation or gel filtration. Polyribosomes were isolated and total RNA was determined as described in the Materials and methods section. Recovery was determined by using the total polyribosomal RNA from a PMS control as 100%. Results for RNA determinations are means $\pm$ s.d. for the numbers of samples shown in parentheses.

Expt.	Protocol	Fraction	Polyribosomal RNA distribution		
			Total RNA (mg)	Recovery (%)	Percentage free or bound
A	—	PMS	2.02 $\pm$ 0.08 (3)	100	—
		Gel filtration	1.74 $\pm$ 0.11 (3)		
	Centrifugation	Void volume	0.10 $\pm$ 0.02	91	95% bound
		Included volume	0.56 $\pm$ 0.05		5% free
B	—	PMS	2.05 $\pm$ 0.04 (3)	100	—
		Gel filtration	1.63 $\pm$ 0.14 (4)		
	Centrifugation	Void volume	0.16 $\pm$ 0.02 (4)	87	91% bound
		Included volume	0.28 $\pm$ 0.05 (4)		9% free
	Centrifugation	Mixed microsomal preparation	0.46 $\pm$ 0.01 (4)	36	38% bound
		Free polyribosomes			62% free

Fig. 4. Continuous-sucrose-density-gradient profiles of [¹⁴C]orotic acid-labelled polyribosomes isolated from the void-volume peak and included volume prepared by gel filtration of postmitochondrial supernatant

Polyribosomes were isolated by solubilization of the samples in 1% Triton X-100/1% deoxycholate/0.5 M-KCl in 0.25 M-sucrose/TKME and sedimentation through 2 M-sucrose/TKME. Ribosomal pellets were resuspended in 0.25 M-sucrose/TKME and re-solubilized before being layered over the gradients (15–30%, w/w); ‘m’ denotes the monoribosome peak. ▲, Polyribosomes isolated from the void-volume peak; △, polyribosomes isolated from the included volume.

Fig. 5. Gel-filtration elution profiles of [¹⁴C]orotic acid-labelled membrane-cytomatrix

Membrane-cytomatrix prepared from rats injected with [¹⁴C]orotic acid was treated with either 0.25 M-sucrose/TKME (●) or 1% (w/v) Triton X-100 (○) for 10 min at 0°C. Samples (300 μl) containing 4340 ¹⁴C c.p.m. were removed from the incubations, loaded on to analytical columns containing Bio-Gel A-150 m and eluted with 0.25 M-sucrose/TKME.

protein components of the separated cytomatrix showed the presence of the cytoskeletal proteins actin and myosin, as well as some 20 other proteins (Fig. 6).

Effects of pretreating PMS with high salt, deoxycholate and DNAase I on the formation of cytomatrix

The elution profiles in Fig. 7 focus on the void-volume region of the gel-filtration columns and

Fig. 6. *SDS/polyacrylamide-gel electrophoresis of the membrane-cytomatrix and the separated cytomatrix*. Protein bands were stained with Coomassie Blue. Gel 1: (a) chicken muscle actin (1.5 μ g); (b) membrane-cytomatrix (25 μ g); (c) protein molecular-weight markers. Gel 2: (a) chicken muscle actin (1.5 μ g); (b) separated cytomatrix (25 μ g); (c) protein molecular-weight markers. • and ★ represent the positions of actin and myosin (210 kDa) respectively.

show the effect of various agents on the integrity of the cytomatrix. Deoxycholate (1%) (Fig. 7b) and 0.5M-KCl (Fig. 7a) effectively destroy the complex. This observation is significant because deoxycholate is routinely used in the purification of polyribosomes. Kreibich *et al.* (1978) found that when polyribosomes were solubilized with a non-ionic detergent in the absence of deoxycholate, the resulting products were frequently very different. These 'pseudo-polyribosomes' were enriched in the proteins actin and myosin, were of abnormally high molecular weight and pelleted through standard polyribosome gradients. They were disaggregated by treatment with high concentrations of salt. Fig. 7(a) shows that 0.5M-KCl removes much of the RNA from the void-volume region. Similar high-salt treatments have been shown to break down actin filaments *in vitro* (Gard *et al.*, 1979). The polyribosome complex is labile to trypsin (Fig. 7c), which suggests that proteins are required for the maintenance of the structure. Most intriguing is the observation (Fig. 7d) that DNAase I, at a concentration (4 mg/ml) causing depolymerization of F-actin *in vitro*, dissociates the polyribosomes from the matrix. These preliminary experiments suggest, but do not rigorously prove, that one of the major components of the cytomatrix to which the polyribosomes are attached consists of actin filaments.

Effects of Ca^{2+} ions, DNAase I and phalloidin on cytomatrix

To obtain further insight into the role of actin in the formation of the cytomatrix, the effects of DNAase I and phalloidin in the cytomatrix were investigated.

Since DNAase I was added to incubations in a buffer containing Ca^{2+} ions, their effects on the cytomatrix were investigated. As shown in Fig. 8, the concentration of Ca^{2+} used in the incubations (12.5 μ M final concentration) changed the chromatographic characteristic of the cytomatrix consistent with a decrease in its size to produce a material that we call the intermediate complex. As also shown in Fig. 8, incubation with DNAase I, to depolymerize F actin, destroys the cytomatrix and results in the appearance of polyribosomes, which are eluted from the column in a position similar to that of the soluble proteins. This demonstrates that, although Ca^{2+} ions decrease the size of the cytoskeletal network, polyribosomes remain attached to structural entities in the intermediate complex. Comparison of Figs. 5 and 8 shows that this complex is comparable in size with that of the polyribosomes released from the membrane-cytomatrix by Triton X-100 treatment. This suggests that the polyribosomes released by neutral detergent are simultaneously associated with both membrane components and cytoskeletal elements in the cell. Possibly detergent treatment destabilizes areas of the cytoskeletal network in close proximity to the membrane, thus causing the release of this population of polyribosomes still associated with their cytoskeletal elements. As shown in Fig. 9, phalloidin, which stabilizes F-actin, protects the intermediate complex against degradation by DNAase I, but does not prevent the conversion of the cytomatrix by Ca^{2+} ions into the intermediate complex.

Protein synthesis

It was clearly of importance to investigate the capability for protein synthesis of the various fractions prepared. Membrane-cytomatrix incorporated 512 c.p.m. of 35 S/ng of RNA after a 3 h incubation, compared with 472 and 437 c.p.m./ng in separated cytomatrix and intermediate complex respectively.

Investigation of the kinetics of incorporation of radioactivity showed that the protein-synthetic activity of membrane-cytomatrix is comparable with that of PMS and greatly exceeds (on an RNA basis) that of a mixed microsomal fraction or polyribosomes isolated by centrifugation (results not shown).

Since there is evidence that PMS retains initiating capacity (Tsurgi & Ogata, 1976), this is likely to be

Fig. 7. Gel-filtration elution profiles of Triton X-100-solubilized postmitochondrial supernatant pretreated with 0.5M-KCl, 1% deoxycholate, trypsin (10µg/ml) or DNAase I (4mg/ml)

Samples (100µl) of [^{14}C]orotic acid-labelled PMS were run on columns (0.3 cm $\times$ 20 cm) of Bio-Gel A-150m after the following treatments: (a)–(d), ■, PMS solubilized with 1% Triton X-100 (10 min on ice); (a) □, PMS pretreated with 0.5M-KCl (45 min, 25°C) before solubilization; (b) ▽, PMS pretreated with 1% deoxycholate (45 min, 25°C) before solubilization; (c) ○, PMS pretreated with trypsin (10µg/ml) for 10 min before solubilization; (d) ◇, PMS pretreated with DNAase (4 mg/ml) for 45 min at 25°C before solubilization.

true also for membrane–cytomatrix. After removal of detergent, separated cytomatrix shows similar protein-synthetic capabilities to membrane–cytomatrix.

Discussion

Previous work from our laboratory established that 16% of the polyribosomes apparently bound to

microsomal membranes separated by centrifugation at 28000g were removed from the membrane and became ‘free’ polyribosomes when the centrifugal force was increased to 112000g (Palmer *et al.*, 1978). Thus the observed association of a population of polyribosomes with microsomal membranes appeared to be labile under the conditions obtaining at high centrifugal forces.

The gel-filtration protocol presented here was

Fig. 8. Gel-filtration elution profiles of the cytomatrix pretreated with Ca^{2+} and DNAase I

Cytomatrix prepared from rats injected with [^{14}C]orotic acid was treated with 0.25 M-sucrose/TKME (○), buffer containing $12.5\ \mu\text{M}$ - CaCl_2 (●), or DNAase I (2.5 mg/ml) in buffer containing $12.5\ \mu\text{M}$ - CaCl_2 (▲), for 45 min at 25°C . Samples ($300\ \mu\text{l}$) containing 4200 ^{14}C c.p.m. were removed from the incubations, loaded on to analytical columns containing Bio-Gel A-150m and eluted with 0.25 M-sucrose/TKME.

developed to examine the extent of polyribosome-membrane interaction in the absence of ultracentrifugation. It is demonstrated to be capable of differentiating between 'free' polyribosomes prepared by centrifugation and polyribosomes present as aggregates in association with membranes or protein structures. When labelled 'free' polyribosomes were mixed with unlabelled PMS before gel filtration, there was no change in the elution characteristics of the labelled RNA. Thus purified polyribosomes do not bind to membrane vesicles or protein aggregates in a non-specific manner.

The finding that about 90% of the polyribosomal material in PMS ran in the void volume of the columns shows that only a very small proportion of the total polyribosomes are 'free' in the sense of being not associated with other structures.

It is clearly established in this paper that most of the polyribosomes in rat liver PMS can be isolated in association with a protein matrix by gel filtration. The same methodology demonstrated that most of the polyribosomes remain attached to a cytomatrix after treatment with Triton X-100, and this cytomatrix is labile to high-salt concentrations, ionic detergents, proteolytic enzymes and DNAase I.

Our results demonstrate an association between components of the cytoskeleton and polyribosomes in rat liver. Even the polyribosomes apparently released by treatment with Triton X-100 appear to

Fig. 9. Gel-filtration elution profiles of the cytomatrix pretreated with phalloidin and Ca^{2+} or phalloidin and DNAase I

Membrane-cytomatrix prepared from rats injected with [^{14}C]orotic acid was treated with phalloidin ($40\ \mu\text{g}/\text{ml}$) for 30 min at 25°C before preparation of cytomatrix (as described in the Materials and methods section). Phalloidin-treated cytomatrix was then incubated with buffer containing $12.5\ \mu\text{M}$ - CaCl_2 (●) or DNAase I (2.5 mg/ml) in buffer containing $12.5\ \mu\text{M}$ - CaCl_2 (○) for 45 min at 25°C . Samples ($300\ \mu\text{l}$) containing 4150 ^{14}C c.p.m. were removed from the incubations, loaded on to analytical columns containing Bio-Gel A-150m and eluted with 0.25 M-sucrose/TKME.

be associated with actin filaments. This is consistent with the observation that treatment of PMS with tumour-promoting phorbol esters causes a shift of RNA from the void volume to the included volume of the column (Rabin *et al.*, 1981; Fey *et al.*, 1981), in the light of the subsequent findings that phorbol esters disorganize the cytoskeleton in whole-cell preparations (Penman *et al.*, 1982). The observed loss of ribosomes *in vitro* would thus be due to the release of polyribosomes from cytoskeletal elements rather than from membranes.

The results demonstrate that the presence of actin in a polymeric form is a necessary condition for polyribosome attachment to the cytomatrix. The possibility that the disruption of the cytomatrix by DNAase I with concomitant release of polyribosomes is due to ribonuclease contamination has virtually been eliminated by using affinity-purified DNAase I and by our demonstration that dissociation is eliminated by prior treatment of the preparation with phalloidin.

Gentle preparation of the cytomatrix by gel filtration and extraction with neutral detergent leaves active mRNA molecules associated with cytoskeletal elements. High-molecular-weight proteins are synthesized both before and after detergent

treatment, and these preparations are greatly superior for protein synthesis to the centrifugally prepared proteins usually used. Retention of protein synthesis has also been demonstrated by Lenk *et al.* (1977) and Cervera *et al.* (1981) after extraction of cells with detergent.

The effects of Ca^{2+} ions on the cytomatrix in rat liver remain to be evaluated in detail. Our results show that Ca^{2+} decreases the size of the cytomatrix to produce an intermediate complex. The formation of the intermediate complex is probably not due to the depolymerization of F-actin, since phalloidin does not block the effects of Ca^{2+} ions. Proteins associated with the cross-linking of actin and the regulation of the formation of the intermediate complex may be associated with effects on these proteins. The intermediate complex retains active mRNA molecules and is disrupted by treatment with DNAase I, an effect that is prevented by prior treatment with phalloidin.

Our results confirm that the polyribosomes in postmitochondrial supernatants are associated with elements of the cytoskeleton in the form of a cytomatrix. The chemical nature of this association and its biological *raison d'être* are clearly matters of considerable importance. The poly(A) tail of the mRNA molecules is one possible candidate for involvement in interactions with actin filaments, since each actin monomer contains an ATP/ADP-binding site (Craig & Pollard, 1982). Another possibility is involvement of the messenger cap, and further studies on the cytomatrix are required to enable the detailed nature of the binding to be evaluated.

This work has been supported by grants from the Medical Research Council and Cancer Research Campaign.

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