LOCALIZATION OF PHOSPHOPROTEINS IN RIBOSOMAL SUBUNITS AND IN FREE AND BOUND POLYSOMES OF RAT LIVER

M. PIERRE, C. CREUZET and J. E. LOEB
Institut de Recherches Scientifiques sur le Cancer B.P. no. 8, 94800 – Villejuif, France

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1. Introduction

In vivo phosphorylation of eucaryotic ribosomal proteins has been previously demonstrated in a number of different tissues: rat liver [1,2], rabbit reticulocytes [3], sarcoma [4], bovine anterior pituitary gland [5], and mouse mammary gland [6]. It has also been found in the plant *Lemna minor* [7]. In rat liver, ribosomal phosphorylation is stimulated by glucagon [8] whose effects are mediated by cyclic AMP (cAMP).

The functions of this phosphorylation remain unknown but its stimulation by cAMP could be related to the induction of the synthesis of some enzymes by this nucleotide. For example some authors [9,10] think that in rat liver, tyrosine transaminase synthesis is regulated at the translational level by cAMP.

In the present study, we investigated the localization of in vivo phosphorylated ribosomal proteins. We found that 80% of the ^{32}P-bound ribosomal proteins were not extractable by high salt concentrations and that they were localized in the small subunit. A strongly labelled band 'M' and a weaker one 'L' were observed after one-dimensional electrophoresis. We also compared the in vivo phosphorylation of the two major classes of polyribosomes: membrane-bound polyribosomes and free ribosomes. It is generally admitted that these two classes synthesize different types of proteins: the free ones synthesize intracellular proteins and the bound ones, proteins for extracellular purposes. Free polysomes presented an autoradiographic pattern different from that of bound polysomes. The band 'M' was more labelled in bound polysomes but the band 'L' was more labelled in free polysomes.

2. Materials and methods

After an overnight fast Sprague–Dawley rats weighing 200–300 g were injected intraperitoneally with 1 mCi of carrier-free [^{32}P] orthophosphate and 100 μg of glucagon per 100 g body weight to obtain a general increase of the labelling [8]. Animals were sacrificed by decapitation 30 min later. The livers were pooled and homogenized in 0.44 M sucrose containing 0.001 M sodium phosphate, pH 6.7. Crude ribosomes were isolated according to Littlefield et al. [11]. They were further purified by Mg^{2+} precipitation as previously described [8]. The subunits were prepared according to Martin and Wool [12]: ribosomes were dissociated by 0.88 M KCl at 37°C in the presence of 0.0001 M puromycin and the subunits were separated on a linear 10–30% sucrose gradient by using a Spinco SW27 rotor for 4 hr at 28°C and 27 000 rpm. After dialysis, the subunits and the fraction obtained at the top of the gradient were precipitated by adding 0.4 vol of ethanol.

To separate free and bound polyribosomes, we modified the method of Leskes et al. [13]. The post-mitochondrial supernatant (8 ml) was layered onto two layers of sucrose (7 ml of 2 M and 9 ml of 1.32 M). The tubes were then filled with 0.01 M cacodylate buffer, pH 6.6, and centrifuged for 36 hr at 27 000 rpm in a Spinco 30 rotor at 4°C.

Free and bound polyribosomes were collected with an Isco gradient collector and pooled. These fractions were then centrifuged for 2 hr at 140 000 *g*. Pellets of both free and bound ribosomes were suspended in 0.44 M sucrose and desoxycholate was added to give a 0.2% and 0.5% solution respectively. The polysomes

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were suspended in 0.44 M sucrose and precipitated by increasing the $MgCl_2$ concentration to 0.1 M. In all cases, the ribosomal proteins were extracted with 67% acetic acid [14] containing 0.033 M $MgCl_2$ [15] and dialyzed for 18 hr against 6 M urea with two changes of this medium.

Proteins were estimated spectrophotometrically at 230 and 260 nm, nucleic acids at 260 nm.

Determinations of protein-bound radioactive phosphate, after extraction of lipids and acido-labile phosphate, were made on ribosomes and isolated ribosomal proteins. Samples were spotted on Whatman 3 MM filters. The proteins were fixed with 10% TCA* and heated at 95°C for 15 min in 5% PCA. The filters were washed with cold 5% PCA, twice with chloroform–ethanol (1:1) at room temperature for 20 min, finally with acetone and dried. The radioactivity was measured in a Packard Scintillation Counter.

Electrophoresis in 7.5% acrylamide gel was performed at pH 4.5 in 6 M urea [16] using a Yeda flat plate apparatus (Yeda corp. Israel). The gels were stained with 0.2% Amido black in 7.5% acetic acid. Autoradiography of the gels was made by applying a Kodirex film to the gel after it had been dehydrated with 75% ethanol and dried at room temperature between cellophane sheets. The film was exposed for about one week. Densitometry was performed with a Vernon densitometer (Vernon, Paris).

Carrier-free [^{32}P] orthophosphate was purchased from C.E.A. (Saclay, France) and glucagon was a gift of Novo laboratories (Paris).

3. Results and discussion

3.1. *Localization of phosphoproteins in ribosomal subunits*

Most of the ^{32}P bound to ribosomal proteins appeared to be strongly associated with ribosomes: 80% remained attached to ribosomal particles after treatment at high ionic strength (table 1). Rat liver phosphorylated proteins are therefore mostly 'true' ribosomal proteins although the definition of ribosomal

* The following abbreviations are used in the text: TCA, trichoroacetic acid; PCA, perchloric acid; EDTA, ethylene Diamino tetra-acetic acid; mRNP, messenger RNA protein particles.

Table 1
Treatment of ribosomes at high ionic strength

Experiment	A (cpm)	B (cpm)
^{32}P bound to ribosomal proteins before treatment	24 453	24 561
^{32}P bound to ribosomal proteins after treatment	20 251	20 581
% ^{32}P extracted	17.2%	15.5%

Ribosomes purified by Mg^{2+} precipitation were obtained as described in Materials and methods and extracted by 0.05 M Tris, pH 7.4, containing 0.88 M KCl for 45 min. Ribosomal particles were collected by ultracentrifugation. Radioactivity was measured in aliquots, as indicated in Materials and methods. Counts correspond to total protein i.e. 30 mg.

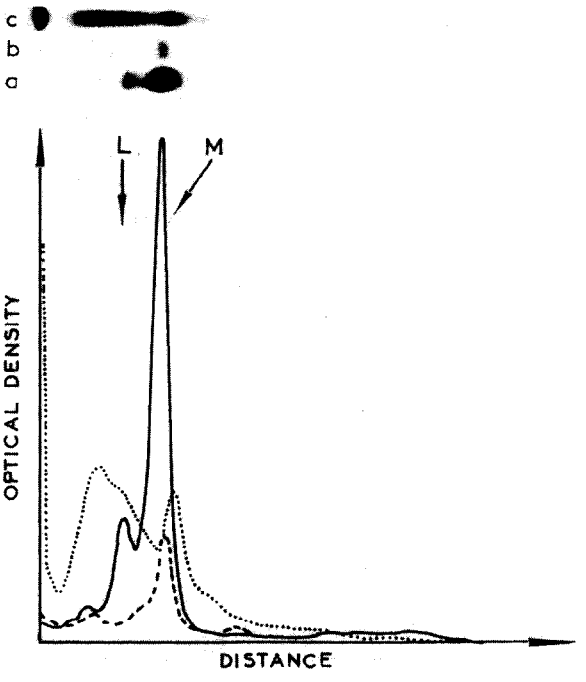

Fig. 1. Autoradiographic patterns of proteins from ribosomal subunits and supernatant. About 250 μg of each fraction was subjected to electrophoresis under the conditions described in Materials and methods. Densitometry of the autoradiograms (a, ———) small subunit (b, ...) large subunit (c, ...) supernatant.

proteins is still controversial. They were mainly localized on the small subunit (fig. 1). Most of the labelling was in band M which seems to be composed of two components; two-dimensional electrophoresis would elucidate this point. A slower migrating band L was always visible. Three or four weakly-labelled bands were observed in the large subunit. Those corresponding to band M may be a contamination from the small subunit. The preferential in vivo phosphorylation in the small subunit of ribosomes of rat liver may be compared with the results of Kabat [3] who found one major labelled protein in the small subunit from reticulocytes after dissociation with EDTA. Band M is probably identical to the protein II of Kabat though they are derived from different tissues. Kabat [4] described the same patterns of in vivo phosphorylation in reticulocytes and sarcomas. In the plant *Lemna minor,* Trewawas [7] has also found one ribosomal protein strongly labelled in vivo and located in the small subunit.

Three to four radioactive components were found in the supernatant of the gradient used to separate the subunits; they correspond to proteins extracted at high ionic strength. Their electrophoretic behaviour was different from those of the small subunit. These components may be ribosome-associated factors or proteins from bound mRNP which are at least partially dissociated from the mRNA at high ionic strength. Phosphoproteins in the 'mRNP' particles have been found in ascites cells by Egly et al. [17] and in duck reticulocytes by Grander et al. [18].

Since in vivo phosphorylation is essentially observed in the small subunit, to which initiation factors and mRNA appear to bind, it is tempting to postulate that phosphorylation plays a part in the initiation of eucaryotic protein synthesis. Eil and Wool [19] however did not find significant differences in the ability of phosphorylated and non phosphorylated subunits to translate poly U and encephalomyocarditis virus RNA. Translation of specific eucaryotic mRNA had not been tested by these authors.

3.2. *Localization of phosphoproteins in free and bound polysomes*

Although the total relative alkalinolabile phosphorus labelling of proteins was not very different in the two classes of polysomes (a little higher in the free ribosomes whereas L was the predominantly labelled band in free polysomes (fig. 2).

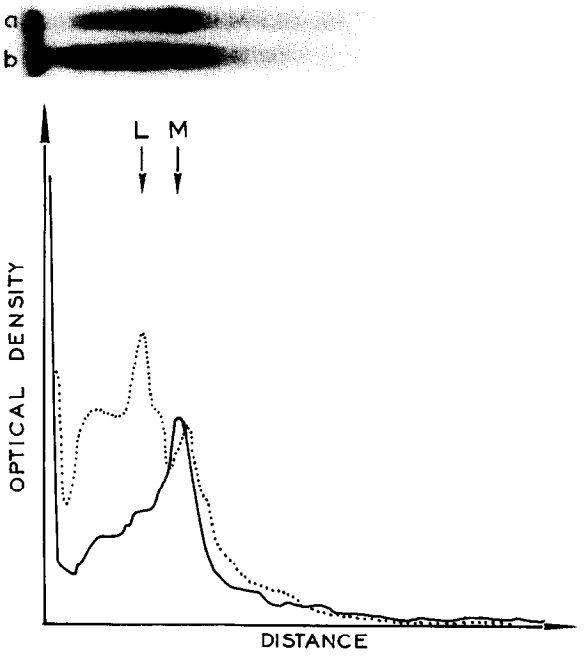

Fig. 2. Autoradiographic patterns of proteins from bound and free polysomes. About 300 μg of proteins were subjected to electrophoresis under the conditions described in Materials and methods. Densitometry of the autoradiograms (a,———) bound polysomes (b, ...)free polysomes.

one), the autoradiographic patterns presented striking reproducible differences.

The relative intensity of labelling of M and L bands was inversed in free and bound polyribosomes. M was the more strongly labelled band in the bound poly-

The other labelled bands (with slower mobilities) found especially on the free ribosomes could correspond to proteins found at the top of the gradient, when ribosomes were dissociated at high ionic strength to obtain subunits (see above). It is now difficult to give an interpretation for the different labelling intensities of phosphoproteins in free and bound polysomes. Accessibility to kinase and phosphatase can of course be different for these two classes of polysomes. Recently, Pavlovic-Hournac et al. [20] have reported that free and bound thyroid polyribosomes contain

protein kinase activities which behave differently when compared to endogenous and exogenous substrates. Nwizu et al. (C. Nwizu, G. Yoshida and H. Mower, personal communication) also reported that free ribosomes of rat liver were more labelled than bound ribosomes; however they did not carry out an electrophoretic analysis of the proteins. Moreover the differential labelling of phosphoproteins can be related to different synthetic activities of the two groups of polysomes: all these in vivo experiments were performed with injections of glucagon to increase the labelling 2–3 times. Comparison with normal rats will indicate whether glucagon, whose effects in rat liver are mediated through cyclic AMP, preferentially increases the labelling of one class of polysomes.

It is known that the large subunit binds to microsomal membranes. Our results, showing that only the small subunit is phosphorylated in vivo, do not prove that phosphorylation of ribosomal proteins is involved in the binding of ribosomes to microsomal membranes. Of course one cannot exclude a phosphorylation of proteins from the large subunit which would not be detected under our conditions. Phosphorylation of the proteins of microsomal membranes might also take part in the binding of the large subunit to the membranes.

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