IN VITRO PHOSPHORYLATION OF PROTEINS FROM FREE AND MEMBRANE-BOUND RAT LIVER POLYSOMES

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1. Introduction

Protein phosphorylation catalyzed by protein kinases has been found to occur both in vivo and in vitro in ribosomes from several eukaryotic systems, e.g., pituitary and mammary glands, sarcoma cells, kidney fibroblasts, yeast, rat liver and rabbit reticulocytes (for reviews see [1–3]). This reaction results from the transfer of phosphate from either ATP or GTP to the serine and threonine residues of specific proteins.

In the particular case of rat liver, various reports have shown that several proteins of either monosomes or free ribosomal subunits can be phosphorylated in vitro by cyclic AMP-activated cytosolic or ribosome-bound protein kinases [4–8]. However, to our knowledge, no data have been so far reported about protein phosphorylation in polysomes.

The present study deals with in vitro ribosomal protein phosphorylation in the two major classes of rat liver polysomes: membrane-bound polysomes which are generally considered to be involved in the synthesis of exportable proteins and free polysomes which seem rather specialized in the formation of intracellular proteins [9–12]. Phosphorylated proteins were found to be qualitatively and quantitatively different in these two populations of polysomes. In addition, proteins present in either kind of polysomes appeared much less accessible to phosphorylation than proteins in free subunits.

2. Materials and methods

2.1. *Preparation of free and bound polysomes*

The method used for preparing polysomes is a modification by Madjar [13] of that described by Blobel and Sabatini [14]. Briefly, Sprague-Dawley male rats weighing 200–220 g were fasted for 24 h and sacrificed by decapitation. The livers were pooled and homogenized in 2 vol. 0.25 M sucrose in TKM buffer (50 mM triethanolamine–HCl, at pH 7.4, 25 mM KCl, 5 mM $MgCl_2$). The post-mitochondrial supernatant was prepared by centrifuging the homogenate for 10 min at 10 000 × *g*. It was then layered onto three layers of sucrose (9 ml 2 M, 5 ml 1.6 M and 4 ml 1.35 M) and centrifuged for 24 h at 303 000 × *g*. Free polysomes were thus obtained as a pellet while bound polysomes remained at the 1.6–2.0 M sucrose interface. The latter were finally pelleted by a 24 h centrifugation at 198 000 × *g* through a 1.6 M sucrose solution in TKM buffer.

2.2. *Preparation of ribosomal subunits*

Subunits were prepared according to Blobel and Sabatini [15]. Free polysomes were suspended in 50 mM triethanolamine–HCl, at pH 7.4, 500 mM KCl, 1.5 mM $MgCl_2$ and 0.1 mM puromycin and incubated for 15 min at 0°C then for 10 min at 37°C. Subunits were separated by centrifugation for 4 h in a Ti 14 Beckman zonal rotor at 48 000 rev/min through a 10–45% sucrose gradient prepared in 50 mM

ethanolamine–HCl, at pH 7.4, 500 mM KCl, 5 mM $MgCl_2$ and 20 mM mercaptoethanol. Fractions containing 40 S and 60 S particles were pooled and dialyzed against 50 mM triethanolamine–HCl, at pH 7.4, 80 mM KCl, 3 mM $MgCl_2$ and 20 mM mercaptoethanol. They were finally concentrated and stored in liquid nitrogen.

2.3. *In vitro phosphorylation*

Polysomes or subunits were incubated in 4 ml 30 mM Tris–HCl, at pH 7.4, 0.3 mM ethylene glycol–bis-(amino-2 ethyl ether) –*N,N'* tetracetic acid, 3.5 mM theophyllin, 20 mM NaF, 12 mM $MgCl_2$ and 10 μM cyclic-AMP. The amounts of protein kinase (extracted from beef heart, spec. act. 12.5 units/μg, Sigma Chemical Co.) added to limiting amounts of either polysomes (80 A_{260} units) or 40 S particles (18.2 A_{260} units) or 60 S particles (36 A_{260} units) were, respectively, 0.9 mg, 0.29 mg and 0.57 mg. At zero time radioactive [γ-^{32}P]ATP (spec. act. 100–250 cpm/pmol, Amersham Center) was added at the final concentration of 40 μM. Incubation was carried out for 80 min at 30°C followed by an additional 30 min period at 4°C in the presence of 0.8 mM non-radioactive ATP.

Polysomes were then treated with 0.5% sodium deoxycholate for 10 min at 4°C then centrifuged for 4 h at 130 000 × *g* through a 5–20% sucrose gradient in TKM buffer.

Ribosomal subunits were pelleted by a 2 h centrifugation at 300 000 × *g*.

2.4. *Determination of the specific radioactivity of ribosomal proteins*

Ribosomal proteins were extracted from polysomes or subunits according to the procedure of Hardy et al. [16] modified by Sherton and Wool [17], then dialyzed for 15 h at 4°C against 1 M acetic acid and lyophilized. Two-dimensional polyacrylamide gel electrophoresis was achieved following the method of Howard and Traut [18] slightly modified: after migration in the first dimension, gels were dialyzed for 4 min against 0.3 M HCl [19] and migration in the second dimension was carried out under 105 V for 22 h at 20°C. Gel slabs were stained with Coomassie Blue R 250 as described by Subramanian [20]. They were then incubated for 2 h in 3% glycerol, dried under vacuum and autoradiographed for 1–5 days. Radioactive spots were visualized on the autoradiogram and the corresponding proteins, numbered according to Sherton and Wool [21], were selectively extracted from the gel slab by incubation in a 1% sodium dodecylsulfate–6 M urea mixture [22]. The radioactivity of the extract was counted and the amount of protein was determined from the absorbancy at 605 nm of the stain [23]. The specific radioactivity of ribosomal proteins was expressed in each case as the ratio of the number of picomoles of radioactive phosphate to the absorbancy at 605 nm. Control experiments have shown that an amount as low as 0.3 pmol radioactive phosphate could be detected under our experimental conditions.

Although phosphorylation induced a slight change in the electrophoretic mobility of proteins [1], the localization and the identification of labeled proteins were done however with great accuracy since no overlapping occured between phosphorylated proteins and any other neighbouring protein.

3. Results

3.1. *Effects of phosphorylation on polysome pattern*

In order to check first whether or not polysomes were maintained during in vitro phosphorylation, free and membrane-bound polysomes were analyzed by sucrose gradient centrifugation before and after incubation in the reaction mixture. Figure 1 shows that an 80 min incubation induces a reduction of the average size of free polysomes which is observed as a shift towards smaller polysomes. However the proportion of total persisting polysomes is only slightly reduced since no more than 5–8% are converted to monosomes. A similar observation can be made in the case of membrane-bound polysomes (not shown here). In any case only dimers and heavier polysomes were kept apart and later used for protein analysis. They were indeed selectively pelleted by a 240 min centrifugation at 130 000 × *g* through a 5–20% sucrose gradient in TKM buffer, the monosomes and ribosomal subunits being discarded under these conditions.

The same kind of analysis was performed with isolated ribosomal subunits since they were used as a control in the phosphorylation experiments further described. Neither the sedimentation properties of

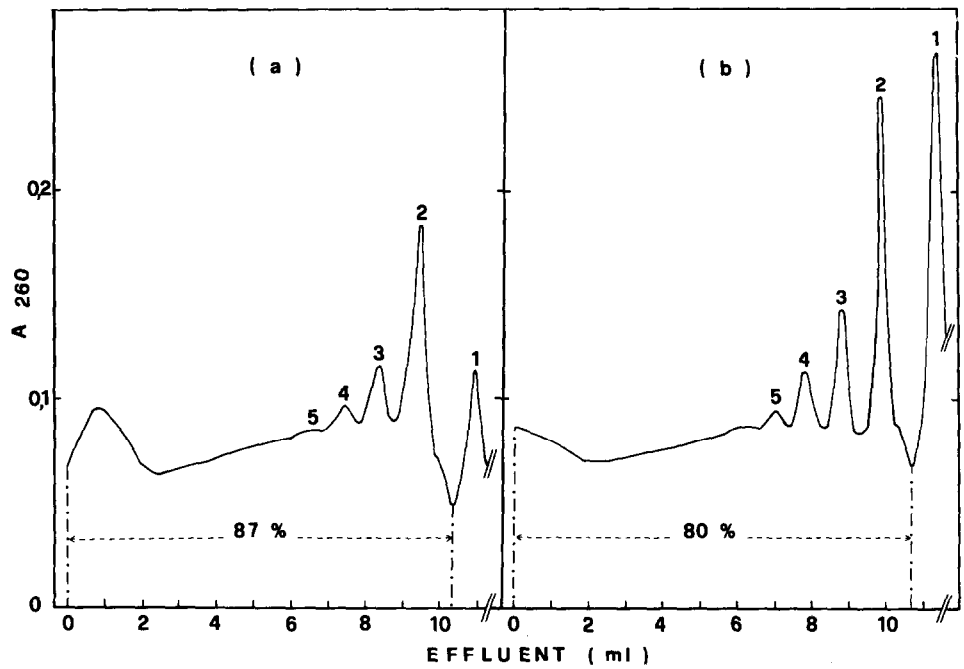

Fig.1. Effects of phosphorylation on the pattern of free polysomes. Ribosomal material obtained in the free polysome fraction (see Materials and methods) was centrifuged though a 15–40% sucrose gradient in TKM buffer for 75 min at 40 000 rev/min in the SW 41 Beckman rotor (a) before incubation and (b) after incubation for 80 min in the phosphorylation medium. Absorbance at 260 nm was then monitored by pumping the gradients though a continuous flow cell. Number 1 indicates the position of 80 S monosomes and numbers 2–5 indicate the position respectively of dimers, trimers, tetramers and pentamers. The amount of polysomes is expressed as % total ribosomal material (polysomes + monosomes + ribosomal subunits).

40 S subunits nor those of 60 S subunits appeared to be significantly modified after an 80 min incubation. It must be noted however that our starting subunit preparations contained a large proportion of polymers as previously reported [24–26]. More precisely, only 13% of the total 40 S appeared as separated subunits whereas 58% were present as dimers and 29% as trimers. In the case of the 60 S particles, 53% of the total preparation were single subunits and 36% were dimers, the 11% left being attributable to contaminating 80 S monosomes. Of importance however is the fact that all these proportions were rigorously maintained after incubation in the phosphorylation medium. In addition, the activity of phosphorylated subunits was tested by measuring their ability to stimulate in vitro poly(U)-directed polyphenylalanine synthesis and was compared to that of non-phosphorylated particles. No appreciable difference was found in the case of the 40 S subunits as previously reported by other authors [27] and a decrease of only about 20% was observed in the activity of 60 S subunits.

3.2. *Phosphoprotein phosphatase activity*

It was essential to make sure that in our experiments no phosphoprotein phosphatase activity occured which could dephosphorylate the ribosomal proteins. This activity indeed could be brought into the reaction mixture either by the exogenous protein kinase used to catalyze phosphorylation or by the polysomes themselves [1,2]. Therefore an estimate of the phosphatase activity in the complete incubation medium was made by measuring the appearance of paranitrophenol released from nitro-4-phenyl phosphate [28]. Table 1 shows that neither free polysomes nor protein kinase exhibit such activity whereas membrane-bound polysomes do. However when the inhibitor sodium fluoride is added to the medium at the final concentration of 20 mM, the activity of the enzyme is nearly

Table 1
Phosphatase activity in free and membrane-bound polysomes

	Bound polysomes		Free polysomes	
	– FNa	+ FNa	– FNa	+ FNa
Absorbance	29.1	1.1	0	0
% Activity	100	3.8	0	0

Incubation was carred out in 250 μl standard phosphorylation medium containing 2 A_{260} units polysomes, 22 μg protein kinase, 20 mM FNa when added and 14 mM nitro-4-phenyl-phosphate. The amount of released para-nitrophenol was measured by the absorbancy at 410 nm.

totally blocked since it is reduced by more than 96%. Consequently this inhibitor was later used throughout all phosphorylation experiments.

3.3. *Phosphorylation of ribosomal proteins*

Polysomes were incubated as described in Materials and methods, ribosomal proteins were isolated by two-dimensional gel electrophoresis and their specific radioactivity was determined after autoradiography. As shown in table 2, in bound polysomes one single protein, S6, from the small subunit was found radioactive and none from the large subunit. In free polysomes two proteins were labeled: the same S6 protein as in bound polysomes with however a higher specific radioactivity, and protein L18 from the 60 S particles.

The same determination was made with isolated 40 S particles as a control. Nine proteins appeared to be phosphorylated even though these particles were largely present as dimers in the incubation medium (see above): one major radioactive protein was again S6, five other proteins (S7, S9, S10, S13, S14) were significantly labeled and three more (S3, S5, S25) gave light spots on the autoradiogram, the specific radioactivity of which could not be calculated with enough accuracy (table 3).

Since ribosomal proteins from isolated 40 S subunits appeared more accessible to phosphorylation than polysomal proteins, the same kind of experiment was performed with the other class of isolated subunits, i.e., the 60 S particles. There again a much larger number of proteins were phosphorylated as compared to proteins present in polysomes. Indeed nine proteins were found radioactively labeled to some extent: L3, L6, L18, L24, L28, L29, L34, L35 and L36. In this case however no accurate determination of specific radioactivity could be made since, as reported above, our 60 S ribosome preparation obtained from zonal centrifugation was slightly contaminated by 80 S monosomes.

4. Discussion

The qualitative and quantitative differences which

Table 3
Protein phosphorylation in isolated 40 S ribosomal subunits

Protein	S6	S7	S9	S10	S13	S14
Specific radioactivity	14	0.5	2.4	5.1	2.2	1.3

Isolated subunits were incubated and specific radioactivity of proteins was determined as described in Materials and methods. All expressed values are relative to specific radioactivity of protein S6 in bound polysomes and can thus be directly compared to values reported in table 2.

Table 2
Protein phosphorylation in free and membrane-bound polysomes

	Specific radioactivity		
	Bound polysomes	Free polysomes	40 S Subunits
Protein S6	1	6.3	14
Protein L18	0	0.9	–

Polysomes were incubated in the presence of exogenous protein kinase and phosphorylated ribosomal proteins were isolated as indicated in Materials and methods. All expressed values are relative to the specific radioactivity of protein S6 in bound polysomes.

are observed in the ability for rat liver ribosomal proteins to be phosphorylated in free and membrane-bound polysomes appear to be related to the different environment of these two classes of polysomes. Indeed, it seems unlikely that these differences could be due to the very low residual phosphatase activity found in the membrane-bound polysome fraction which is not inhibited by sodium fluoride, although this possibility cannot be completely excluded. Our data suggest that protein S6 is more exposed in free polysomes than in bound polysomes. Moreover the selective phosphorylation of protein L18 in free polysomes might indicate that this protein would be involved in one way or the other in the attachment of the 60 S subunits to the membranes.

Another interesting finding lies in the fact that the number of phosphorylated proteins is much more limited in polysomes than in free subunits. Connectively it is worth noticing that the ribosomal protein phosphorylation pattern previously observed in vivo where only S6 is phosphorylated [29,30], is very similar to the pattern we have obtained in vitro with polysomes but not with free subunits. It is therefore likely that the selective phosphorylation observed in vivo would be related to the accessibility of ribosomal proteins in polysomes rather than to the specificity of the intracellular protein kinase activity.

We are currently checking the accessibility to phosphorylation of proteins from various populations of monosomes (run-off, reassembled from separated subunits and monosomes derived from ribonuclease-treated polysomes).

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