ENDOGENOUS PHOSPHORYLATION OF RIBOSOMAL PROTEINS FROM MEMBRANE-FREE RAT LIVER POLYSOMES

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1. Introduction

Endogenous phosphorylation of ribosomal proteins catalyzed by ribosome-associated protein kinases has been reported to occur in various eucaryotic systems [1–11]. In most cases, such protein kinases have been shown to be rather loosely bound to ribosomes since they can be totally solubilized by in vitro treatment with high ionic strength buffers, e.g., containing 0.5–1.0 M potassium chloride. This property has led several authors (reviews [12,13]) to ask whether those enzymes are specific components of the ribosomes or, instead, are cytosol contaminants casually attached to the particles, like some other intracellular enzymes [14–16].

In the particular case of rat liver, the occurrence of an endogenous kinase activity in ribosomes has been described [17,18]. However, the effects in vitro of various salt concentrations on the behaviour of the enzyme(s) have not been studied.

Such effects were therefore investigated in the present work. For this, the enzymatic activity remaining in ribosomes treated with increasing concentrations of potassium chloride was estimated, in each case, through its ability to still phosphorylate ribosomal proteins. In order to avoid any possible interference in phosphorylation, due to protein kinases bound to cellular membranes, only those ribosomes present in membrane-free polysomes were examined. It is mainly shown, in this report, that hepatic polysomes harbor a firmly-attached enzyme fraction not removable by 1.5 M KCl.

2. Materials and methods

2.1. *Preparation of membrane-free polysomes*

Polysomes were prepared as previously described [19]. The postmitochondrial supernatant prepared from a rat liver homogenate was centrifuged through three successive layers of sucrose (1.35 M, 1.6 M and 2 M) for 24 h at 303 000 × *g*. Free polysomes were obtained as a pellet while membrane-bound polysomes remained at the 1.6–2.0 M sucrose interface and were discarded.

2.2. *Salt treatment of polysomes*

After suspending free polysomes at 4°C in TKM buffer (50 mM triethanolamine–HCl, at pH 7.4, 25 mM KCl, 5 mM $MgCl_2$), the KCl concentration was either maintained at 25 mM or raised to 0.5 M, 1.0 M or 1.5 M. In each case, the final concentration of polysomes was 50 A_{260} units/ml. The suspension (1 ml) was heated for 1 h at 37°C then layered onto 2.8 ml 30% sucrose in TKM buffer and centrifuged in a SW 60 Beckman rotor at 55 000 rev/min for 3 h at 4°C. Salt-treated polysomes were thus pelleted, then resuspended in TKM buffer and centrifuged once more under the same conditions. Sucrose gradient analysis (not reported here) of the material recovered

after such treatment showed, on the one hand, that no trivial ribosomal aggregates were formed, but, on the other hand, that the average polysome size was reduced.

2.3. *In vitro endogenous phosphorylation assay*

The endogenous kinase activity of untreated or salt-treated polysomes was estimated from the amount of radioactive orthophosphate incorporated from [γ-^{32}P]ATP into the total ribosomal proteins. Polysomes (1 A_{260} unit) were suspended in 250 μl incubation mixture similar to that already described [19]. This mixture contained 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 and 12 mM $MgCl_2$. After 150 min at 30°C, 50 μl aliquots were withdrawn then spotted on Whatman 3 MM paper discs as in [20] and their radioactivity was counted. In some experiments, incubation was carried out in the presence of 10 μM cyclic-AMP added to the medium.

2.4. *Analysis of phosphorylated ribosomal proteins*

Total ribosomal proteins were extracted from incubated polysomes by the acetic acid procedure in [21]. In each analysis, the same amount of protein (200 μg) was subjected to two-dimensional polyacrylamide gel electrophoresis. Only basic proteins were analyzed as in [22]. Gels were stained with Coomassie blue R 250, dried under vacuum and autoradiographed for 5–8 days [19]. Radioactive spots were visualized on the autoradiogram and the corresponding proteins were identified according to the nomenclature proposed [23].

3. Results and discussion

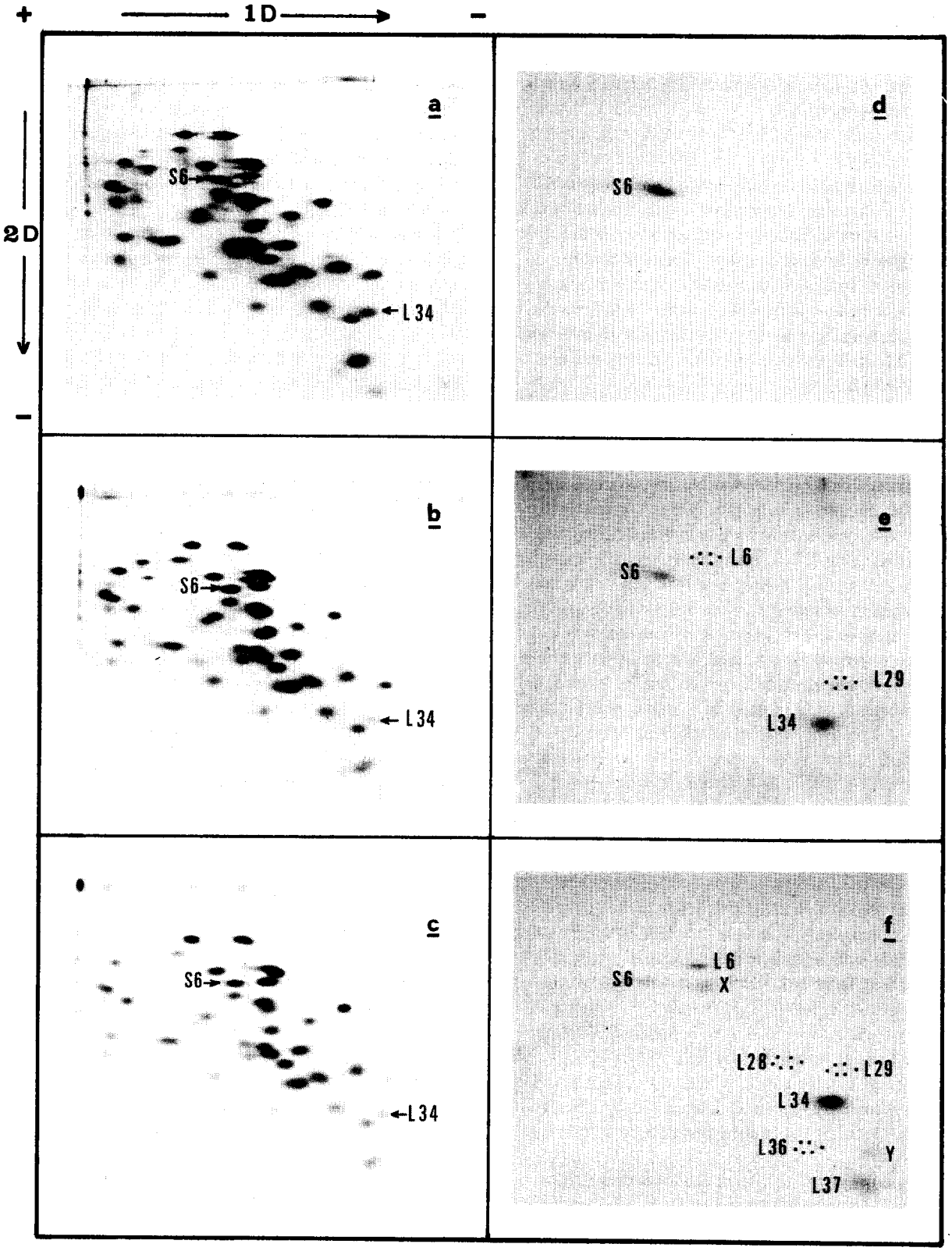
Endogenous phosphorylation of total ribosomal proteins was first measured in polysomes treated by various salt concentrations. It is shown, in table 1, that incubation in KCl at a concentration as low as 25 mM induces a decrease in kinase activity down to 61% control value. This result indicates that membrane-free polysomes contain a loosely-attached enzyme fraction extractable by low ionic strength buffers. An even more pronounced decrease (down to 27% control) is observed in polysomes treated by 0.5 M KCl, suggesting that an additional enzyme fraction is released under such treatment. However, it is of particular interest to note that a near-constant residual activity (26–28% control) is instead maintained in polysomes when KCl concentration is varying from 0.5–1.5 M.

Table 1
Endogenous kinase activity of salt-treated polysomes

	KCl concentration				
	Control	0.025 M	0.5 M	1.0 M	1.5 M
Kinase activity	38.1 (100)	23.1 (60.6)	10.4 (27.3)	10.2 (26.8)	10.6 (27.8)

The amount of radioactive orthophosphate present in total ribosomal proteins was measured (see section 2) in untreated membrane-free polysomes (control) or in polysomes previously treated for 1 h at 37°C by the indicated KCl concentration. Mean values from 3–6 experiments are expressed as pmol orthophosphate incorporated/polysome A_{260} unit. The % activity relative to control is given in brackets

Fig.1. Analysis of endogenously-phosphorylated ribosomal proteins from rat liver polysomes. Two-dimensional polyacrylamide gel electrophoresis was carried out on proteins extracted from untreated polysomes (a) or from polysomes previously treated with 0.5 M KCl (b) or 1.5 M KCl (c) and incubated in the presence of radioactive ATP as indicated in section 2. The corresponding autoradiograms (respectively d, e and f) were obtained after 8 days exposure. Faint spots are surrounded by a dotted line.


+
1D
-
2D
-
a
S6
L34
b
S6
L34
c
S6
L34
d
S6
e
S6
L6
L29
L34
f
S6
L6
X
L28
L29
L34
L36
Y
L37

It thus appears that polysomes also harbor a firmly-attached enzyme fraction not extractable by high ionic strength buffers.

In an attempt to characterize further the protein kinase(s) bound to polysomes, the effect of cyclic-AMP on endogenous phosphorylation was studied. None of the values reported in table 1, including the control, was significantly modified when radioactive orthophosphate incorporation was measured in presence of the cyclic nucleotide. Therefore, all of the ribosomal protein kinase activity found in vitro in hepatic polysomes seems to be cyclic-AMP independent, like some other mammalian kinase activities described [24–26].

In order to achieve a precise identification of phosphorylated compounds, proteins were extracted from control and salt-treated polysomes then analyzed by two-dimensional polyacrylamide gel electrophoresis followed by autoradiography. Three typical electrophoregrams and corresponding autoradiograms are presented in fig.1. No significant qualitative variation was found in the protein content of salt-treated polysomes as compared to the untreated control. In contrast, an increasing number of radioactively-labeled proteins was observed when polysomes were treated with a higher KCl concentration. Indeed, on the one hand, only protein S6 was phosphorylated in untreated polysomes. This result is qualitatively identical to that obtained from in vivo experiments [12], but it is difficult to determine whether this protein is phosphorylated to the same degree in both in vitro and in vivo situations. On the other hand, 3–6 additional ribosomal proteins were labeled to a variable extent after treatment of polysomes by, respectively, 0.5 M and 1.5 M KCl. In the latter case, 2 additional unidentified spots appeared on the autoradiogram and were numbered as X and Y. In polysomes treated with 1.0 M KCl (not shown), 4 ribosomal proteins (L6, L28, L29 and L34) were found phosphorylated in addition to protein S6. Whether this increasing number of labeled proteins as a function of salt concentration is due to a change in substrate accessibility or to a change in enzyme activity and/or specificity cannot be distinguished from our data.

In addition, it must be noted that, under the electrophoresis conditions used here [22], acidic ribosomal proteins could hardly be revealed and thus could not be analyzed with enough accuracy as reported [27]. Experiments were therefore undertaken to estimate the phosphorylation degree of those proteins by making use of a different gel system in which they could be more precisely detected [28]. This work is still in progress but preliminary results seem to indicate that an acidic protein, likely L40/L41, is also phosphorylated endogenously to a significant extent at least in the case of polysomes treated by 25 mM KCl. Connectively, we are also checking currently the stoichiometry of those acidic proteins in polysomes treated by high KCl concentrations since it is conceivable that their possible removal by salts might induce a higher accessibility of basic proteins to phosphorylation.

In the experiments described above, polysomes treated with a high salt concentration (0.5–1.5 M) were pelleted by centrifugation through 30% sucrose in TKM buffer (see section 2) prior to phosphorylation assay. Since KCl concentration in this buffer is only 25 mM, one may envisage that an enzyme fraction possibly removed from polysomes by a high ionic strength buffer could re-attach to them during centrifugation in a lower ionic strength buffer. To check this possibility, a new set of preparations was carried out by centrifuging polysomes previously treated by a given salt concentration (0.5 M, 1 M or 1.5 M) through a sucrose buffer containing the same salt concentration. Neither the total endogenous kinase activity of polysomes thus obtained nor the corresponding pattern of phosphorylated ribosomal proteins were significantly modified, ruling out the above-mentioned possibility.

One can therefore conclude that membrane-free polysomes from rat liver actually support a firmly-attached protein kinase activity. Whether or not this activity belongs to a structural ribosomal protein is currently under investigation.

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