DIFFERENTIAL PHOSPHORYLATION OF BASIC AND ACIDIC RIBOSOMAL PROTEINS BY PROTEIN KINASES BOUND TO MEMBRANE-FREE RAT LIVER POLYSOMES

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1. Introduction

Protein kinase activities that are able to phosphorylate specific ribosomal proteins have been found in a variety of eukaryotic systems (reviewed [1–3]). Such enzymes catalyze the incorporation of phosphoryl groups into the serine and threonine residues of those proteins [4–6]. They are active as well in the in vitro phosphorylation of exogenous protein substrates other than ribosomal proteins. According to their substrate specificity, protein kinases can thus be classified into two main groups: protein kinases that preferentially phosphorylate acidic proteins such as casein or phosvitin, and protein kinases which instead are rather specific for basic proteins such as histones or protamin [7]. These two groups of enzymes can be further differentiated by their degree of stimulation of phosphorylation in the presence of cyclic AMP, their ability to utilize ATP or GTP as a phosphoryl donor, and their relative activity in the presence of salts or or other effectors such as diamide [8].

Such distinct enzymes have been reported to be bound to ribosomes in several systems including horse thyroid glands [9], yeast [10], mouse plasma cell tumors [11], bovine corpus luteum [12] and rabbit reticulocytes [7]. Furthermore, experiments carried out with rat liver have shown that membrane-free polysomes harbor simultaneously at least two different protein kinase activities: a histone kinase-type enzyme and a casein kinase-type enzyme [13]. However, in neither of these cases, it has been described whether the substrate specificity of kinases found for exogenous protein substrates applies to the endogenous phosphorylation of ribosomal proteins.

In this work such a study was therefore undertaken in rat liver by estimating the extent of in vitro phosphorylation of specific ribosomal proteins catalyzed separately by each protein kinase activity associated with membrane-free polysomes. The results presented indicate mainly that basic and acidic ribosomal proteins are selectively phosphorylated by different protein kinases.

2. Materials and methods

The method used for preparing membrane-free rat liver polysomes and the experimental procedure followed to treat them by various KCl concentrations have been detailed in [14,15].

The protein kinase activity of polysomes on exogenous substrates (histone and casein) was determined as in [13,14] by estimating the amount of orthophosphate incorporated into protein at the expense of radioactive ATP.

Acidic ribosomal proteins were extracted according to [15] by suspending phosphorylated polysomes (200 A_{260} units) in 0.6 ml of a buffer containing 50 mM triethanolamine–HCl, at pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 20 μM β-mercaptoethanol and 0.6 ml absolute ethanol. The suspension was stirred for 15 min at 4°C then centrifuged for 10 min at 5000 × g. The procedure was repeated twice and the supernatant fractions containing the acidic proteins were dialyzed 3 times for 8 h each against 5 l of 1 M

acetic acid, then lyophilized. The ribosomal material insoluble in ethanol, was kept apart and treated according to [16] for the extraction of basic proteins.

Acidic and basic ribosomal proteins were subjected to bidimensional polyacrylamide gel electrophoresis as in [17] and [18], respectively. Gel slabs were stained with Coomassie blue R 250 and the radioactive spots were cut out and treated for protein extraction by incubation in a 1% sodium dodecylsulfate–6 M urea mixture [19]. The radioactivity of the extract was counted and the corresponding amount of protein was determined from the A_{605} of the stain [20]. The specific radioactivity of ribosomal proteins was expressed in each case as the ratio of the n. pmol radioactive phosphate to the A_{605}. Control experiments, not reported here, have shown that an amount as low as 0.3 pmol radioactive phosphate can be detected under our experimental conditions.

3. Results and discussion

In a first series of experiments, the endogenous in vitro phosphorylation of ribosomal proteins catalyzed by polysome-associated protein kinases was measured. After incubation of membrane-free polysomes in the presence of radioactive ATP, only two ribosomal proteins appear to be phosphorylated. The first one is a basic protein from the small ribosome subunit identified as S_6 according to the nomenclature proposed [16]. None of the other basic ribosomal proteins is radioactively labeled to a significant extent, as in [15]. The second one is an acidic protein selectively extractable by ethanol from the large ribosome subunit and identified as P_1 [21]. The extent of phosphorylation of protein P_1 and protein S_6 after incubation of polysomes in the minimum medium is indicated in table 1. It can also be seen from the same table that the degree of phosphorylation of each protein is not equally affected when incubation of polysomes is carried out in the presence of two different effectors: cyclic AMP and KCl. Indeed, on the one hand, the specific radioactivity of protein P_1 is not significantly changed in the presence of cyclic AMP whereas a 16-fold increase is observed in the case of protein S_6. On the other hand, the addition of KCl together with cyclic AMP to the incubation medium induces a 4–5-fold increase in the specific radioactivity of protein P_1 while, on the contrary, the value for protein S_6 is reduced 6–7-fold. It therefore seems clear that cyclic AMP and KCl have quite different effects on the phosphorylation of proteins S_6 and P_1.

Table 1
Phosphorylation of ribosomal proteins by endogenous kinase activities bound to polysomes

Assay system		Specific radioactivity	
Cyclic AMP (μM)	KCl (M)	Protein S_6	Protein P_1
0	0	0.2	4.7
10	0	3.2	5.0
10	0.2	0.5	23.9

Membrane-free polysomes were incubated for 80 min at 30°C in the standard phosphorylation assay mixture [13] with or without cyclic AMP or KCl as indicated. Ribosomal proteins were extracted and the specific radioactivity of proteins S_6 and P_1 was determined [14] and expressed, in each case, as the ratio of the n. pmol incorporated radioactive phosphate to A_{605} of the protein stain. Mean values from 3 experiments are given

This result must be related to the data in [13] concerning the in vitro action of the same two effectors on the protein kinase activities bound to polysomes. The histone kinase was indeed shown to be activated by cyclic AMP and inhibited by 0.2 M KCl; on the contrary, the casein kinase is cyclic AMP-independent but is activated by 0.2 M KCl. Taking into account this differential behavior of enzymes and their substrate specificity for basic (histone) or acidic (casein) proteins, it appears most likely that the histone kinase activity preferentially phosphorylates the basic ribosomal protein S_6 whereas the casein kinase activity catalyzes mainly the phosphorylation of the acidic ribosomal protein P_1.

Further experiments were performed to check this hypothesis. For that purpose, the specific in vitro phosphorylation of either ribosomal protein by each of the kinase activities was analyzed. In a first step, the histone kinase and casein kinase activities were extracted by taking advantage of their differential solubilization by salts. The data in [13] were confirmed by showing that treatment with 25 mM KCl already results in the release of >90% of the total

histone kinase activity present in polysomes; the same wash (W_{25}) contains, instead, only ~6% of the total casein kinase activity. By increasing KCl to 0.5 M, the remaining 94% of the casein kinase activity is then released into the wash fraction (W_{500}); the residual histone kinase activity still attached to polysomes treated with the low salt concentration also becomes soluble. Polysomes thus treated with 0.5 M KCl do not harbor any more detectable enzymatic activity able to phosphorylate histone or casein.

As indicated in table 2, when the low salt concentration wash (W_{25}) is incubated with polysomes pre-treated with 0.5 M KCl, only protein S_6 is phosphorylated. This result strongly suggests that the histone kinase which accounts for nearly the total kinase activity of W_{25} is specific for the phosphorylation of the basic ribosomal protein. In agreement with this interpretation, it must be noted that the specific radioactivity of protein S_6 is drastically diminished when the incubation is carried out in the presence of 0.2 M KCl, which could be expected from the data reported in table 1. When salt-treated polysomes are incubated with the high salt concentration wash (W_{500}), protein P_1 is phosphorylated. Its specific radioactivity is increased by a factor of ~4 if 0.2 M KCl is added to the reaction

Table 2
Phosphorylation of ribosomal proteins in salt-treated polysomes

Addition to washed polysomes	Specific radioactivity	
	Protein S_6	Protein P_1
none	1.1	0
0.2 M KCl	0.1	0
W_{25}	19.0	0
W_{25} + 0.2 M KCl	1.7	3.7
W_{500}	1.5	5.0
W_{500} + 0.2 M KCl	3.8	21.3

Membrane-free polysomes pre-treated with 0.5 M KCl for 1 h at 37°C were incubated either alone or in the presence of either W_{25} obtained from 25 mM KCl-treated polysomes or W_{500} obtained from 0.5 M KCl-treated polysomes. Incubation was carried out for 80 min at 30°C in the standard phosphorylation assay mixture [13] containing 10 μM cyclic AMP with or without 0.2 M KCl as indicated. Ribosomal proteins were extracted and the specific radioactivity of proteins S_6 and P_1 was determined and expressed as in table 1. Mean values from 2 experiments are given

medium. From what is known on the protein kinase content of W_{500} and on the activation by salts of the casein kinase activity (table 1), it seems very likely that this enzyme is indeed responsible for the selective phosphorylation of the acidic ribosomal protein. Accordingly, the low but significant degree of phosphorylation of protein P_1 observed after incubation of polysomes with W_{25} in the presence of 0.2 M KCl could be due to the relatively small proportion of casein kinase present in this wash, which would be activated by the salt but would not be detectable in its absence under our assay conditions.

The fact that the extent of phosphorylation of protein S_6 is much higher when incubated with W_{25} than with W_{500} (table 2) might a priori seem paradoxical since, as indicated above, >90% of the total soluble histone kinase activity is extracted by 25 mM KCl as compared to 100% if 0.5 M KCl is used. One probable explanation for this result is that, in the assay, the histone kinase is inhibited by the salt concentration present in W_{500} itself. Indeed, if the same W_{500} is dialyzed overnight against a buffer containing a relatively low KCl concentration (50 mM triethanolamine–HCl, at pH 7.4, 5 mM $MgCl_2$, 25 mM KCl) before being used for phosphorylation of protein S_6, the specific radioactivity value found for this protein is much higher than that obtained by incubation with non-dialyzed W_{500} since it reaches 9.9 units. Moreover, it is reduced to 1.2 units when dialyzed W_{500} is incubated in the presence of 0.2 M KCl.

One can also note that the specific radioactivity of protein S_6 after incubation of polysomes with W_{500} is surprisingly higher in the presence of 0.2 M KCl than in its absence (table 2). However, no satisfactory explanation for this result can be proposed so far.

Finally, of special interest is the fact that the estimation of the degree of phosphorylation of ribosomal proteins depends in some instances, on the procedure used to prepare polysomes. In particular, the specific radioactivity of protein S_6 measured in polysomes not treated by salts (table 1) is much lower than in the case of salt-treated polysomes incubated with either W_{25} or dialyzed W_{500} (table 2). This result might be interpretated in terms of protein topography in ribosomes by considering that protein S_6 would in the first case be less accessible to

phosphorylation because of its partially hidden position inside the polysomal structure. No such situation appears to occur for protein P_1 whose specific radioactivity is essentially the same in polysomes treated or not by 0.5 M KCl. It is therefore suggested that protein P_1 is already maximally accessible to phosphorylation even in polysomes not treated by salts because of its rather external position.

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