Role of phosphorylation on the maturation pathways of a 100 kDa nucleolar protein

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1. INTRODUCTION

Post-translational modification of proteins is a widely observed phenomenon which plays a key role in the control of many physiological functions. Prominent among such modifications are phosphorylation of amino acid residues [1,2] and specific proteolytic cleavage [3]. The effects of phosphorylation on the function of proteins is not completely understood although some data are available. For example, phosphorylation of histones appears to induce condensation of chromatin [4]; the level of ribosomal DNA transcription in *Physarum polycephalum* is regulated in vitro by the degree of phosphorylation of a protein [5]; and the level of phosphorylation of the S6 ribosomal protein may regulate polysome assembly and protein synthesis [6]. Maturation of proteins may also involve highly specific proteinases which catalyse only one or a few cleavages within a molecule [7]. Such a mechanism is observed in hormone maturation [8], excretion of protein [9], insertion of protein into membrane [10] and maturation of pro-enzymes [11].

In the nucleoli of CHO cells a 100 kDa protein transiently associated with pre-ribosomes, is first processed into a 95 kDa protein, then into several smaller proteins which either remain in the nucleolus or are translocated into the cytoplasm [12]. The thiol protease that specifically cleaves the precursor has been characterized [13]. Here, we establish that the 100 kDa protein is phosphorylated and that it can be processed 'in vitro' according to two different maturation pathways. The first pathway results in specific proteins which are unphosphorylated, while the second results in cleavage products of different size which are phosphorylated. The results suggest that the level of phosphorylation of the 95 kDa protein directly regulates the choice of the following processing pathway.

2. MATERIALS AND METHODS

Chinese hamster ovary (CHO) cells were grown in monolayer culture in Falcon flasks [14] and [^{32}P]orthophosphate labeled as in [15]. Cell fractionation was done as in [16]. Nucleoli were prepared from purified nuclei by two sonication cycles and differential centrifugation [17]. Partially purified 100 kDa protein was obtained by extraction from nucleoli in the presence of 1 mM EDTA (pH 7.4), for 15 min at 4°C followed by a centrifugation at 3400 × *g* for 5 min. Protein samples were analyzed by electrophoresis on SDS 10–16% polyacrylamide gradient slab gels [18]. Electrophoretic transfer of proteins separated on slab gel to nitrocellulose filters and immunological detection were done as in [12]. The in vitro maturation of the 100 kDa protein was carried out in a

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reaction mixture of 0.3 ml containing 30 μg protein in 10 mM Tris-acetate (pH 5.5), 10 mM DTT for 1 h at 37°C. To this mixture was added for medium: (1) low ionic strength, 1 mM EDTA; (2) high ionic strength, 0.5 M NaCl; (3) 10 mM $MgCl_2$. After precipitation 20% (v/v) trichloroacetic acid, proteins were analyzed by SDS–polyacrylamide slab gel electrophoresis.

3. RESULTS

3.1. *Characterization of the 100 kDa protein*

Nucleolar proteins were resolved into >50 bands by SDS–polyacrylamide gel electrophoresis (fig.1A). A major protein which represents ~20% of these preparations migrates with an app. M_r of 100000. Nucleolar phosphorylated proteins extracted from cells labeled for 1 h with [^{32}P]orthophosphate were similarly analyzed and detected by autoradiography. Seven proteins were highly phosphorylated and among them, the protein of 100 kDa (fig.1B). Since the complexity of such a nucleolar protein fraction is too high to permit direct observation of in vitro maturation of the 100 kDa protein, a partially purified nucleolar protein fraction was subsequently prepared by lysis of nucleoli with 1 mM EDTA. The 100 kDa protein is solubilized along with all the other highly phosphorylated proteins (fig.1C,D) and is the most abundant protein of this fraction. In vitro, this fraction (S_2) contained the protease activity which carries out the maturation of the 100 kDa and was used in all further experiments.

3.2. *In vitro maturation of the 100 kDa at low ionic strength*

Incubation of the S_2 fraction at a low ionic strength for 3 h at 37°C results in cleavage of 30% of the 100 kDa protein molecules and in the appearance or increase of protein species of 95 kDa, 68 kDa and 24 kDa (cf. fig.2A and 2C). The other major components of the S_2 fraction (histones and 40 kDa protein) were not degraded during the incubation demonstrating the specificity of the protease involved in this maturation of the 100 kDa protein. In a second set of experiments, the maturation products of the 100 kDa protein were detected by immunological staining after transfer onto nitrocellulose paper. Before in vitro incubation, the 100 kDa is strongly stained by the anti-

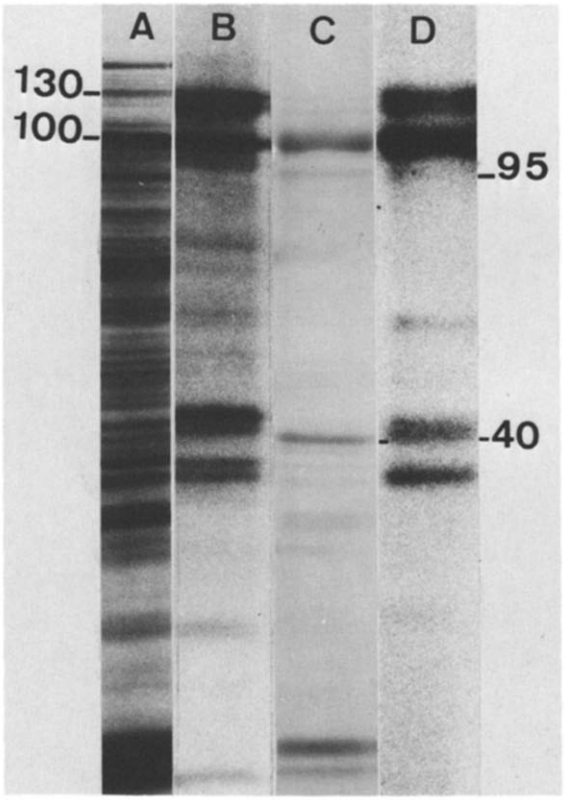

Fig.1. Proteins from nucleoli and S_2 fractions: Chinese hamster ovary cells were ^{32}P-labeled for 1 h at 37°C. Nucleoli were isolated and the S_2 fraction was prepared as in section 2. Proteins were analyzed by electrophoresis on SDS 10–16% polyacrylamide gradient slab gels. After electrophoresis gels were stained with Coomassie blue (A,C). Gels were dried and exposed for 24 h for autoradiography (B,D). Calibration was accomplished by running markers on the same slab gel. Proteins are indicated on the side in kDa. Proteins are from nucleolar (A,B) and S_2 (C,D) fractions.

serum and the 95 kDa, 68 kDa and 50 kDa proteins that are present in low amount stained less strongly (fig.2B). After incubation, all 4 proteins crossreact strongly (fig.2C) implying an increase in quantities of the 95 kDa, 68 kDa and 50 kDa species. This result indicates a precursor–product relationship between the 100 kDa and the smaller protein species. Simultaneously, the behaviour of the phosphorylated proteins was studied. The 130 kDa and 125 kDa phosphorylated proteins, which are minor component of the S_2 fraction, are completely dephosphorylated and other minor

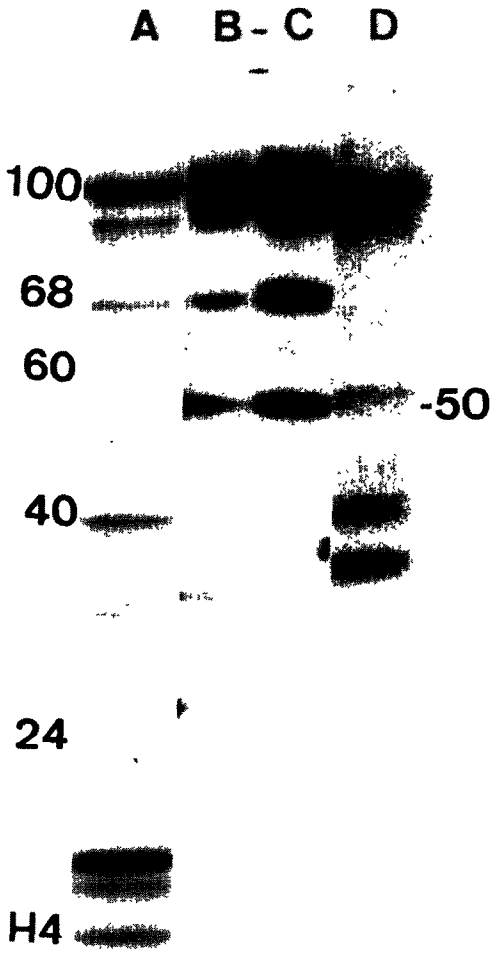

Fig.2. Incubation of S_2 fraction at low ionic strength. The S_2 fraction was incubated for 1 h at 37°C before protein analysis: (A) Coomassie blue staining; (B) immunodetection before in vitro maturation; (C) immunodetection after in vitro maturation; (D) autoradiography after in vitro maturation. Legend as in fig.1.

phosphorylated proteins of the fraction are not affected by the treatment (fig.2D). In contrast, the amount of label observed in the 100 kDa protein band decreases following the disappearance of this species but its specific activity remains largely unchanged (table 1). Among the maturation products of the 100 kDa protein, the 95 kDa is the only species phosphorylated, and its specific activity remains constant during the in vitro incubation.

3.3. *Effects of Mg^{2+} on the 100 kDa maturation*

Incubation of the S_2 fraction in the presence of 10 mM Mg^{2+} for 1 h at 37°C results in cleavage of 70% of the 100 kDa protein molecules. Several new protein species appear which, except for the 95 kDa protein, are different from the maturation products obtained at a low ionic strength. These species are 85 kDa, 76 kDa, 52 kDa, 32 kDa and 21 kDa. The 40 kDa protein is also slightly degraded while the histones are not affected (fig.3A). The 3 high-M_r maturation products crossreact with serum raised against the 100 kDa protein (fig.3B) as do the 95 kDa and the 68 kDa proteins which are present before the in vitro incubation (fig.2B). All of the maturation products observed under these conditions are phosphorylated (fig.3C). The specific activity of the 100 kDa protein which remains after incubation is 25% lower than before incubation while the specific activities of the 85 kDa, 76 kDa, 52 kDa proteins are the same as that of the 100 kDa precursor before incubation:

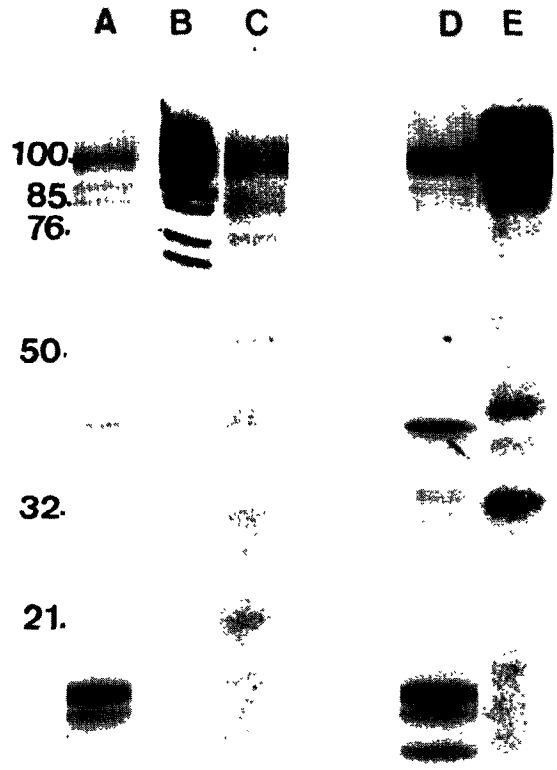

Fig.3. Incubation of S_2 fraction in presence of Mg^{2+} or high ionic strength: (A–C) incubation in presence of Mg^{2+}; (D,E) incubation in 0.5 M NaCl; (A–D) Coomassie blue staining; (C,E) autoradiography; (B) immunodetection. Legend as described in fig.1.

Table 1

Summary of 100 kDa protein maturation under different incubation conditions

Proteins	Amount			^{32}P specific activity		
	Control	EDTA	Mg^{2+}	Control	EDTA	Mg^{2+}
100 kDa	2	1.5	0.74	1	0.78	0.75
95 kDa	0.4	0.7	0.34	0.6	0.52	0.73
85 kDa	0	0	0.23	–	–	1.0
76 kDa	0	0	0.15	–	–	1.5
68 kDa	0.05	0.42	0.10	0	0	0
60 kDa	0.06	0.20	0.10	0	0	0
52 kDa	0.03	0.03	0.09	2	2	1
50 kDa	0.20	0.10	0.15	–	–	1.3

(–) Specific activity cannot be determined since no protein was detected for that species by densitometric scanning

Protein samples that were labeled with [^{32}P]orthophosphate, were either: (1) unincubated (control); (2) incubated at low ionic strength (EDTA); or (3) incubated in the presence of 10 mM Mg^{2+} (Mg^{2+}). Identical amounts of the proteins were electrophoresed on SDS–polyacrylamide gels and the gels were stained with Coomassie blue and subjected to autoradiography. The relative amount of each protein species in the sample was determined by densitometric scanning of the stained gel and measuring the area of each peak. The values listed for each protein species in the 'control', 'EDTA' and 'Mg^{2+}' columns are the relative amount of that species to the amount of the 100 kDa protein in the 'control' sample. The amount of the 100 kDa protein in the 'control' sample has been arbitrarily assigned a value of 1. The ^{32}P specific activity for each protein species was determined by densitometric scanning of the autoradiograms, measuring the area of each peak, and then dividing the ^{32}P peak area by the relative amount of each species. The values for each species shown are compared to the specific activity of the 100 kDa protein in the 'control' column which is arbitrarily assigned a value of 1

this result suggests a conservation of the parts of the molecule containing the phosphate groups and an inhibition of the phosphatase activity. Under these conditions, minor phosphorylated proteins of the S_2 fractions unrelated to the 100 kDa protein, are dephosphorylated during the in vitro incubation (cf. fig.3C and 1D).

3.4. *Lack of in vitro maturation of the 100 kDa protein at high ionic strength*

The 100 kDa protein remains phosphorylated and undegraded when the S_2 fraction is incubated at high ionic strength (0.5 M NaCl) (fig.3D,E). The 130 kDa and 125 kDa proteins also remained unmodified, while the 40 kDa and 35 kDa proteins appeared to be partially dephosphorylated (cf. fig.3E and 1D). The 32 kDa protein appears to be a degradation product of one of these latter species.

4. DISCUSSION

Phosphorylated proteins may be readily extracted together with proteinase and phosphatase activities from nucleoli of CHO cells. The proteinase activity is highly substrate specific for a 100 kDa protein which is transiently associated with pre-ribosomes [12]. The physicochemical characteristics of this 100 kDa protein suggest that it is similar to the protein C23 of Novikoff hepatoma nucleoli [19]. According to the incubation conditions used during in vitro maturation of this protein here, two cleavage pathways may be followed resulting in phosphorylated or dephosphorylated maturation products. Immunodetection is used to indicate the precursor–product relationship between the protein species. We have established tryptic peptide maps of these different proteins to show unambiguously their precursor–product relationship (in preparation). This work further indicates that the

130 kDa protein is the precursor of the 125 kDa protein which subsequently gives rise to the 100 kDa protein (in preparation). At least two phosphatase activities appear to be present in the S_2 nucleolar protein fraction [13]. The first activity is enhanced at low ionic strength in the presence of EDTA and is specific for the 100 kDa protein and its closely related species (130 kDa, 125 kDa, 95 kDa). It shows properties, similar to those of the nucleolar phosphoprotein phosphatase characterized in Novikoff hepatoma [20]. A second activity, which requires Mg^{2+} or high ionic strength does not use the 100 kDa as substrate but dephosphorylates the other nucleolar proteins (40 kDa and 35 kDa).

These results suggest that the phosphatase activity could induce the selection of the 100 kDa maturation pathways. As soon as a 95 kDa molecule is dephosphorylated, it is immediately processed according to the pathway obtained 'in vitro' at low ionic strength, while phosphorylated 95 kDa is slowly processed according to the 'in vitro' pathway obtained in presence of Mg^{2+}.

The two maturation pathways are used in vivo [12]. The major one gives rise to the proteins obtained by the in vitro incubation of the 100 kDa at a low ionic strength. Two of these maturation products are recovered as structural protein of the small ribosomal subunit (submitted). Proteins arising from the second maturation pathway are present only in small amounts in the cell nucleus and their function is still unknown. Thus, the 100 kDa protein may play a key role in both the maturation process of pre-ribosomes and as constituents of the functional ribosome.

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