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Phosphorylation of yeast ribosomal proteins by CKI and CKII in the presence of heparin*

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We have found that heparin has a different effect on *Trichosporon cutaneum* ribosomal protein phosphorylation by CKI and by CKII. In the presence of heparin, modification of 13 kDa, 19 kDa and 38 kDa proteins catalyzed by CKII was inhibited, while in the case of CKI, in addition to protein of 15 kDa, phosphorylation of 20 kDa and 35 kDa proteins was detected. It was also found that, in the presence of heparin, phosphorylation of P proteins (13 kDa and 38 kDa) by ribosome-bound protein kinases was inhibited. Moreover at the same conditions modification of 40 kDa protein was observed in all four yeast species tested.

Phosphorylation of ribosomal proteins is one of the mechanisms controlling mRNA translation in eukaryotic cells. Particularly relevant in this regard is the phosphorylation of acidic ribosomal proteins (P-proteins) which are the main phosphoproteins of eukaryotic ribosomes stimulating the rate of peptide chain elongation. Eukaryotic ribosomal P-proteins are phosphorylated by protein kinase CKII. In *Saccharomyces cerevisiae* two other protein kinases, namely PK60 [1, 2] and RAP [3] engaged in phosphorylation of P-proteins were reported. Several observations indicate that protein kinases responsible for ribosomal protein phosphorylation are tightly bound to ribosomes [2, 4]. In this paper we

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Abbreviations: CKI and CKII, protein kinase I and II, PMSF, phenylmethylsulphonyl fluoride; P-protein, ribosomal acidic proteins; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate.

present the studies on the effect of heparin on protein phosphorylation by ribosome-bound protein kinases CKI and CKII in yeast.

MATERIALS AND METHODS

Yeast species. *Saccharomyces cerevisiae*, strain SKQ2N (C.S.Mc Laughlin, California University, Irvine, U.S.A.), *Trichosporon cutaneum*, strain CCY30-5-4, and *Pichia stipitis*, strain CCY39-50-1P-5 (A. Kockova-Kratochvilova, Slovac Academy of Science, Bratislava, Slovak Republic), *Schizosaccharomyces pombe*, strain 972h$^-$ (P. Nurse, Department of Biochemistry, Oxford, U.K.). Yeast were cultivated under aerobic conditions in YPD medium (1% yeast extract, 2% peptone, 2% glucose) to the exponential growth phase.

Preparation of yeast cell free extract and ribosomes. The cell free extracts were prepared according to the described procedure [2]. Membrane-free 80S ribosomes were released from the endoplasmic reticulum by 1% Triton X-100 treatment of the microsomal fraction [5]. These ribosomes were used for phosphorylation of ribosomal proteins by ribosome-bound protein kinases.

For further purification, membrane free 80S ribosomes were washed twice with 0.5 M KCl and sedimented through 1 M sucrose. The concentration of ribosomes ($11 \times A_{260}$ = 1 mg/ml) was estimated according to Van der Zejist *et al.* [6].

Purification of CKI and CKII from* T. cutaneum *ribosomes. Membrane-free 80S ribosomes were washed with buffer A (50 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO)_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 1 mM phenylmethylsulphonyl fluoride (PMSF) containing 0.5 M KCl. Ribosomes were sedimented at 100000 $\times$ ***g*** and the supernatant was dialysed overnight against buffer B (50 mM Tris/HCl, pH 7.5, 6 mM β-mercaptoethanol, 0.5 mM EDTA, 1 mM PMSF, 10 mM β-glycerophosphate) and then applied on a DEAE-cellulose column. Protein kinase CKI which was recovered in flow-through fractions was subjected to P-cellulose column chromatography. It was eluted at about 0.5 M NaCl and after dialysis against buffer B was applied on a casein-Sepharose column. CKI was recovered at about 0.25 M NaCl. The final purification step was performed on heparin-Sepharose column. CKI activity was eluted at about 0.35 M NaCl.

CKII was eluted from DEAE-cellulose column at about 0.25 M NaCl, then it was purified on P-cellulose column. CKII activity was recovered at about 0.55 M NaCl.

The final CKI and CKII preparations were stored in buffer B containing 50% glycerol at -20°C.

Ribosomal protein phosphorylation. Membrane-free 80S ribosomes were used for studying ribosomal protein phosphorylation. The standard reaction mixture in a total volume of 50 μl contained: 50 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM dithiothreitol (DTT), 150 μg of ribosomes, 0.09 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP.

When purified ribosomes were phosphorylated using exogenous CKI or CKII, the reaction mixture contained: 20 mM Tris/HCl, pH 7.5, 6 mM $Mg(CH_3COO)_2$, 5 mM β-mercaptoethanol, 0.03 mM [γ-^{32}P]ATP, 0.3–0.5 μg of enzyme and 150 μg of ribosomes.

The mixture was incubated at 30°C for 20 min and the reaction was stopped by the addition of 25 μl of sample buffer [7]. The phosphorylation level of ribosomal proteins was analysed by electrophoresis in SDS containing 12% polyacrylamide (w/v) slab gels (SDS/PAGE) according to Laemmli [7] and subsequent autoradiography.

RESULTS AND DISCUSSION

CKI and CKII, usually referred to as casein kinases I and II, are highly conserved serine/threonine protein kinases that are ubiquitous in eukaryotic organisms. A distinctive characteristic feature of CKII (but not CKI) is

its ability to use GTP in addition to ATP as a phosphate donor, activation by polycations such as spermine and polylysine and inhibition by polyanions, particularly by heparin [8, 9, 10].

Acidic ribosomal proteins P1/P2-P0, components of the eukaryotic ribosomal stalk, were identified among more than hundred other substrates phosphorylated by CKII [9, 10, 11].

Taking advantage of the fact that both kinases, CKI and CKII, are tightly associated with ribosomes we have used this ribonucleoprotein complex for studies on protein phosphorylation in the presence of heparin. We found that, in ribosomes of different yeast species namely: *S. cerevisiae, Sch. pombe, P. stipitis and T. cutaneum,* proteins of 13 kDa and 38 kDa (P1/P2-P0 complex) were phosphorylated by ribosome-bound protein kinases. The phosphorylation level was decreased by low concentrations of heparin (1–4 μg/ml), confirming the participation of CKII in this modification (Fig. 1).

Interestingly, at higher heparin concentrations (10 μg/ml, 40 μg/ml) significant phosphorylation of a 40-kDa protein was observed. When GTP was used as a phosphate donor this protein was not phosphorylated (not shown), which suggested that an unidentified protein kinase other than CKII participated in its modification.

As can be seen in Fig. 1, in ribosomes of *T. cutaneum*, unlike in other yeast species, in addition to protein complex P1/P2-P0 of 60S ribosomal subunit, two proteins of 15 kDa and 19 kDa, constituents of small ribosomal subunit, were phosphorylated. As found previously these proteins were also phosphorylated *in vivo* [12]. When purified *T. cutaneum* ribosomes were incubated with [γ-^{32}P]ATP or [γ-^{32}P]GTP in the presence of exogenous CKII, selective phosphorylation of 13 kDa, 19 kDa, 38 kDa (but not 15 kDa) ribosomal proteins was detected. Inhibition of protein phosphorylation by low concentrations of heparin was also observed (Fig. 2). The radioactive protein band of 22 kDa, visible in Fig. 2, lanes e and j, reflects the position of β subunit phosphorylated by subunit α of CKII.

It is important to mention that the 15-kDa protein was modified by CKI [12].

For a long time heparin was considered a potent inhibitor of CKII but not of CKI activity. However, several recent data indicate that heparin can stimulate or inhibit CKI activity depending on the substrate used [13, 14]. Stimulation of casein and inhibition of phosvitin phosphorylation was often observed [14, 15].

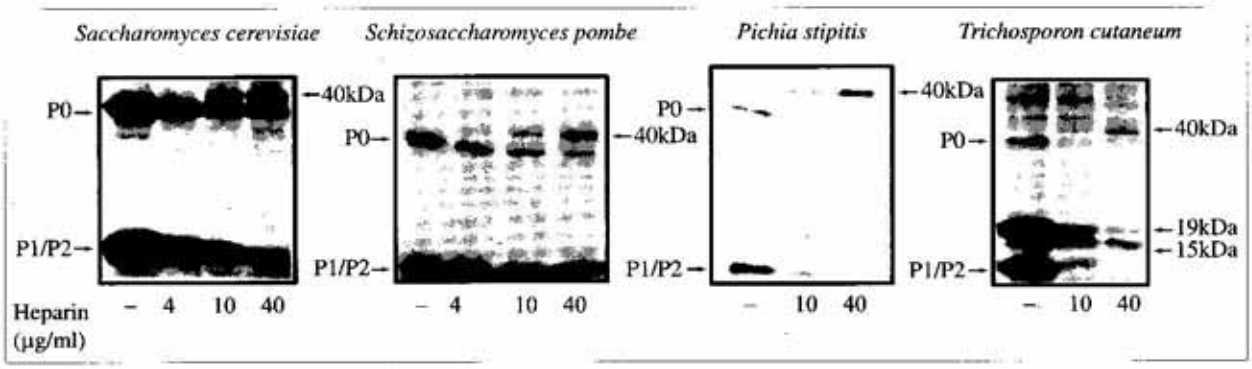

Figure 1. The effect of heparin on ribosomal protein phosphorylation by ribosome-bound protein kinases of four different yeast species.

Membrane-free 80S ribosomes were incubated with [γ-^{32}P]ATP under conditions described in the Materials and Methods section in the absence or presence of heparin. After the reaction ribosomes were subjected to SDS/PAGE and autoradiography.

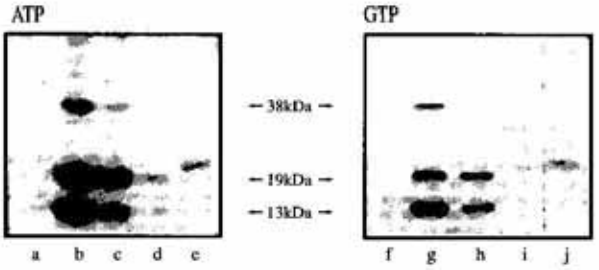

Figure 2. Phosphorylation of ribosomal proteins in purified *T. cutaneum* ribosomes by protein kinase CKII from *T. cutaneum*.

The reaction was performed in the presence of [γ-^{32}P] ATP – left, or [γ-^{32}P]GTP – right, under conditions described in the Materials and Methods. Lanes of the autoradiogram show: a, f, ribosomes alone (control); b, g, ribosomes phosphorylated by CKII; c, h, ribosomes phosphorylated by CKII in the presence of heparin (1 μg/ml); d, i, ribosomes phosphorylated by CKII in the presence of heparin (5 μg/ml); e, j, CKII alone (phosphorylated form of CKII β is visible).

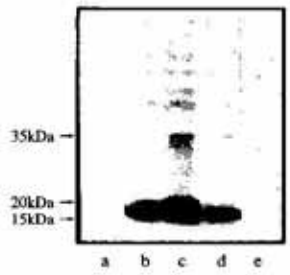

Figure 3. The effect of heparin on phosphorylation of *T. cutaneum* ribosomal proteins by CKI.

The reaction was performed as described in the Materials and Methods. Lanes of the autoradiogram show: a, purified ribosomes alone; b, phosphorylation of ribosomal proteins by CKI; c, phosphorylation of ribosomal proteins by CKI in the presence of heparin (40 μg/ml); d, phosphorylation of ribosomal proteins by CKI in the presence of heparin (40 μg/ml) and KCl (100 mM); e, ribosomes incubated with CKI and KCl (100 mM) – control.

According to Graves and Roach [14] the activity of CKI can be regulated by phosphorylation of its COOH-terminus, which may serve to create an autoinhibitory domain. Heparin, by the interaction with this part of the enzyme, could cause conformational change that mimics dephosphorylation.

Interestingly, if phosphorylation of purified *T. cutaneum* ribosomes by CKI was performed in the presence of heparin at the concentration of 40 μg/ml, in addition to the 15-kDa protein, two other ribosomal proteins of 20 kDa and 35 kDa were phosphorylated (Fig. 3). Phosphorylation of these proteins depended on ionic strength of the reaction mixture and was reversed by 100 mM KCl (Fig. 3d). The obtained results might suggest that heparin can affect the CKI substrate recognition and/or change its phosphorylation site(s). It was found recently that heparin could change specificity of the phosphorylation site of neuronal Cdc2-like kinase [16].

Heparin is not a physiological regulator of CKI activity in yeast. We, however, do not exclude the possibility that some other naturally occurring compound(s) can modulate substrate specificity of CKI in the cell. Heparin can mimic such natural ligand(s). It was found, for example, that the activity of the double stranded RNA dependent protein kinase (PKR) could also be mediated by an alternative activator – heparin [17]. In the light of this observation the question arises whether ribosome-associated CKI can be regulated by rRNA? Further studies are needed to answer this question.

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