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Protein kinases CKI and CKII are implicated in modification of ribosomal proteins of the yeast *Trichosporon cutaneum*

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Phosphorylation of acidic ribosomal proteins P1/P2–P0 is a common phenomenon in eukaryotic organisms. It was found previously that in *Trichosporon cutaneum*, unlike in other yeast species, in addition to the two acidic ribosomal proteins, two other proteins of 15 kDa and 19 kDa of the small ribosomal subunit were phosphorylated. Here we describe two protein kinases: CKI and CKII, which are engaged in the modification of *T. cutaneum* ribosomal proteins. The acidic ribosomal proteins and the protein of 19 kDa were modified by CKII associated with ribosomes, while the protein of 15 kDa was modified by CKI. Protein kinase CKI was purified from cell-free extract (CKIC) and from ribosomal fraction (CKIR). The molecular mass of CKIC was established at 33 kDa while that of CKIR at 35–37 kDa.

A protein of 40 kDa copurified with CKIR but not CKIC. Heparin significantly increased 40 kDa protein phosphorylation level by CKIR. Microsequencing analysis revealed the presence of CKI recognition motifs in the N-terminal fragment of the 40 kDa protein.

Eukaryotic ribosomes consist of four individual rRNA molecules and more than 80 proteins organized into the large and the small subunit. The majority of ribosomal proteins are basic (pI > 8.5). There is also a group of acidic ribosomal proteins (pI 3–5) which form a lateral stalk structure on the large ribosomal subunit. The stalk structure is present in the active site of the ribosome where interactions between mRNA, tRNA and translation factors occur during protein biosynthesis (Pestova & Hellen, 2000; Roll-Mecak *et al.,* 2001). The acidic ribosomal proteins are designated P-proteins due to their capacity for being

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Abbreviations: CKIC, protein kinase CKI isolated from *T. cutaneum* cytosol; CKIR, protein kinase CKI isolated from membrane-free *T. cutaneum* ribosomes.

phosphorylated. They form a pentameric complex that contains one molecule of P0 and two heterodimers of P1 and P2 ribosomal proteins [(P1/P_2)–P0] (Uchiumi *et al.,* 1987; Wool *et al.,* 1991). Phosphorylation of P-proteins plays an important role in the regulation of translational activity of ribosomes. So far, three protein kinases responsible for P-proteins phosphorylation *in vitro*, namely CKII (Pinna & Meggio, 1997; Glover, 1998), PK60S (Pilecki *et al.,* 1992; Jakubowicz *et al.,* 1993) and RAP (Ballesta & Remacha, 1996; Szyszka *et al.,* 1996) have been identified in yeast although only CKII has been proven to phosphorylate P-proteins *in vivo*. We have found that in *Trichosporon cutaneum*, unlike in other yeast species, besides the P-proteins, two additional ribosomal proteins of molecular mass 15 kDa and 19 kDa, were phosphorylated both *in vivo* and *in vitro*. These proteins do not belong to the acidic ribosomal protein family and were identified on the small ribosomal subunit (Wojda *et al.,* 1996).

It is known that CKI and CKII (previously known as casein kinase I and II) are multifunctional, second messenger independent, serine/threonine protein kinases that are ubiquitous in eukaryotic organisms (Tuazon & Traugh, 1991). A distinctive characteristic feature of CKII (but not CKI) is its heterotetrameric structure, ability to use GTP besides ATP as phosphate donor, activation by polycations such as spermine and polylysine, and inhibition by polyanions, particularly by heparin (for a review see Pinna, 1990; Allende & Allende, 1995; Pinna & Meggio, 1997; Glover, 1998). CKII is required for cell viability and for cell cycle progression (Hanna *et al.,* 1995). Expression of CKII is significantly elevated in proliferating normal and transformed tissues.

As for CKI, it is a monomeric enzyme which uses only ATP as phosphate donor. Several isoforms of CKI have been identified. In mammals six forms, encoded by six different genes, were described (Graves & Roach, 1995). In *Saccharomyces cerevisiae* four genes, namely *HRR25, YCK1, YCK2* and *YCK3*, were identified. In *Schizosaccharomyces pombe,* five genes *Cki1, Cki2, Cki3, Hhp1* and *Hhp2* were detected (for a review see Gross & Anderson, 1998).

CKI is implicated in the control of cytoplasmic and nuclear processes such as DNA repair, cell osmosensing, yeast bud growth and others (Fish *et al.,* 1995). It can phosphorylate many cytoskeletal and nuclear proteins, metabolic enzymes and proteins involved in mRNA translation (Grankowski & Issinger, 1990; Tuazon & Traugh, 1991; Vancura *et al.,* 1994). The protein of 15 kDa of the yeast *T. cutaneum* is the only known ribosomal protein substrate for CKI (Wojda *et al.,* 1996). In this paper we describe protein kinases: CKI and CKII, which are implicated in the modification of *T. cutaneum* ribosomal proteins. It is worth mentioning here that *T. cutaneum* is a strictly aerobic yeast species which is able to metabolize different organic compounds such as disaccharides and xylans, utilize lignine, cellulose, starch and also phenol derivatives.

MATERIALS AND METHODS

Yeast strains and growth conditions. Yeast species: *T. cutaneum*, strain CCY30-5-4 (A. Kockova-Kratochvilova, Slovak Academy of Sciences, Bratislava) and *S. cerevisiae*, strain SKQ2N (C.S. McLaughlin, University of California, Irvine, U.S.A.) 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. Cell-free extracts were prepared according to the described procedure (Jakubowicz *et al.,* 1993). Membrane-free 80S ribosomes were released from the endoplasmic reticulum by 1% Triton X-100 treatment of microsomal fraction (Schneider *et al.,* 1976). These ribosomes were used as a source of ribosome bound CKI and CKII and for the

studies of endogenous phosphorylation of ribosomal proteins. For obtaining purified ribosomes (ribosomes deprived of endogenous protein kinase activity), membrane free 80S ribosomes were washed twice with 0.5 M KCl and sedimented through 1 M sucrose. The concentration of ribosomes (A_{260} 11 = 1 mg/ml) was estimated according to Van der Zeijst *et al.* (1972). Protein concentration was determined by the Bradford (1976) method using protein determination reagent (Sigma) and bovine serum albumin (BSA) as a standard.

Purification of protein kinases CKI and CKII

Purification of CKI from cell-free extract. The procedure of CKI purification from cell-free extract (CKIC) of *T. cutaneum* included: ammonium sulphate precipitation, two ion-exchange chromatography and two affinity chromatography steps. Cell-free extract containing 4695 mg of protein was precipitated with ammonium sulphate at the final saturation 55%. The protein pellet (1800 mg) was dissolved in buffer A (50 mM Tris/HCl, pH 7.5, 6 mM β-mercaptoethanol, 0.5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM β-glycerophosphate) and after dialysis against the same buffer, loaded on a DEAE-cellulose column. The flow-through fraction of the column, containing CKI activity (120 mg protein) was then subjected to P-cellulose column chromatography. The protein was eluted with a linear gradient 0–0.8 M NaCl. Fractions containing CKI activity, eluted at about 0.5 M NaCl, were pooled, concentrated on polyethylene glycol 20000 (PEG 20000) and after dialysis against buffer A, applied on a casein-Sepharose column. Proteins were eluted with a linear gradient 0–0.5 M NaCl and CKI activity was recovered at about 0.25 M NaCl. The obtained CKI preparation (0.869 mg protein) was concentrated on PEG 20000 and after exhaustive dialysis loaded on a heparin-Sepharose (Heparin Sepharose CL6-B, Pharmacia) column. Linear gradient 0.1–0.5 M NaCl was applied. CKI activity was eluted at about 0.35 M NaCl. The final CKI preparation containing 0.461 mg protein was concentrated by dialysis in buffer A containing 50% glycerol and stored at –20°C. The specific activity of purified CKIC was estimated at 5000 U/mg. (1 U kinase activity was defined as the amount of the enzyme required for the incorporation of 1 pmol phosphate from [γ-^{32}P]ATP into protein substrate per minute, under optimal conditions).

Purification of CKI and CKII from membrane free* T. cutaneum *ribosomes. Membrane-free 80S ribosomes were washed with buffer B (50 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO^-)_2$, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 1 mM PMSF) containing 0.5 M KCl. The ribosomes were sedimented at 100 000 × ***g*** and the supernatant containing 27.5 mg protein was dialysed overnight against buffer A (see previous section) and then applied on a DEAE-cellulose column. CKI activity was recovered in flow-through fractions. The next purification steps were the same as in the case of CKIC. The final CKIR preparation contained 0.230 mg protein with specific activity of 500 U/mg.

CKII was eluted from the DEAE-cellulose column at about 0.25 M NaCl (linear gradient 0–0.6 M NaCl was used). Fractions containing CKII activity (8 mg protein) were pooled, concentrated on PEG 20000 and after dialysis against buffer A applied on a P-cellulose column. Proteins were eluted with a linear gradient 0–1 M NaCl. Fractions containing CKII activity, eluting at about 0.55 M NaCl, were pooled, concentrated by dialysis in buffer A containing 50% glycerol and stored at –20°C.

CKII from *S. cerevisiae* was a kind gift from R. Szyszka (Maria Skłodowska-Curie University, Lublin, Poland).

Electrophoretic methods and protein silver staining. Protein electrophoresis in 12.5% SDS/PAGE polyacrylamide slab gel was performed according to Laemmli (1970). The gel was silver stained according to Oakley *et al.* (1980).

Protein kinase assay. The standard reaction mixture, in a total volume of 50 μl, 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 or [γ-^{32}P]GTP, 0.1 U of enzyme and 150 μg of purified ribosomes. The mixture was incubated for 15 min at 30°C. The reaction was stopped by the addition of 25 μl sample buffer (Laemmli, 1970). The ribosomal protein phosphorylation level was analyzed by SDS/PAGE and autoradiography.

Autophosphorylation assay. Autophosphorylation reaction was performed in the standard reaction mixture, without an exogenous substrate in the presence of 0.09 mM [γ-^{32}P]ATP. The mixture was incubated for 1 h at 30°C. After that time the reaction was stopped by addition of 25 μl sample buffer and after SDS/PAGE radioactive bands were detected by autoradiography.

In gel assay. Samples of CKI (1–2 μg protein) were separated by SDS/PAGE on gel matrix containing 1 mg/ml casein in the polymerization solution. After the run, the excess of SDS was removed from the gel in a buffer containing: 50 mM Tris/HCl, pH 8.0, 20% 2-propanol. Next the gel was transferred for 1 h to a buffer containing 50 mM, Tris/HCl pH 7.5, 5 mM β-mercaptoethanol, 6 mM guanidine hydrochloride and left overnight at 4°C in 50 mM Tris/HCl, pH 8.0, 5 mM β-mercaptoethanol, 0.04% Tween 20. The gel was subsequently transferred to a buffer consisting of: 20 mM Tris/HCl, pH 7.5, 6 mM $Mg(CH_3COO^-)_2$, 5 mM β-mercaptoethanol for 30 min at 30°C and then to the same buffer containing 0.03 mM [γ-^{32}P]ATP and incubated for 2 h at the same temperature on a gently rocking platform. Excess of radioactivity was then removed from the gel by washing with 5% trichloroacetic acid. Detection of radioactivity was by autoradiography of dried gel.

Endogenous phosphorylation of ribosomal proteins. Membrane-free 80S ribosomes were used as both an enzyme and substrate source. The standard reaction mixture contained in a total volume of 50 μl: 50 mM Tris/HCl pH 7.5, 10 mM $Mg(CH_3COO^-)_2$, 1 mM dithiothreitol, 150 μg ribosomes, 0.09 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP. The mixture was incubated for 30 min at 30°C and the reaction was stopped by the addition of 25 μl sample buffer. The phosphorylation level of ribosomal proteins was analysed by polyacrylamide gel electrophoresis according to Laemmli (1970) and subsequent autoradiography.

Coupling of CNBr-Sepharose with casein. CNBR-Sepharose 4B (Pharmacia) was suspended in 1 mM HCl and washed with the same solution of HCl on a sintered glass filter. The gel was next washed with coupling buffer containing: 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0. The gel was mixed with casein dissolved in the coupling buffer and then left for 2.5 h on a gently rocking platform at room temperature. The excess of the ligand was washed away with the coupling buffer and the remaining active groups were blocked with 1 M ethanolamine, pH 8.0, for 2 h at room temperature. The gel was then washed with 0.1 M sodium acetate, pH 4.0, and next with 0.1 M sodium biborate, pH 8.0, and finally with buffer A (see section "Purification of protein kinases CKI and CKII").

Microsequencing of 40 kDa protein fragment. The CKI preparation purified by DEAE- and P-cellulose, casein-Sepharose and heparin-Sepharose chromatography was subjected to SDS/PAGE (6 μg protein). Then proteins were electroblotted onto Immobilon PVDF membrane (0.2 μm) (Millipore) for 60 min at 150 V. Proteins were visualized using Coomassie Brilliant Blue R-250 staining and the protein band of 40 kDa was excised from the membrane.

Amino-acid sequencing was performed by the Edman degradation method (Proseq, Boxford, MA, U.S.A.).

RESULTS

Phosphorylation of *Trichosporon cutaneum* ribosomal proteins by CKII

Two different strategies were used for the studies on *T. cutaneum* ribosomal protein phosphorylation. Supposing that protein kinase CKII engaged in phosphorylation of ribosomal proteins may be associated with membrane-free 80S ribosomes, we studied the phosphorylation *in situ* (endogenous phosphorylation of ribosomes) in the presence of the absence of exogenous protein kinases. Four ^{32}P-labelled ribosomal phosphoproteins of 13 kDa, 15 kDa, 19 kDa and 38 kDa were identified (Fig. 1 – left). If [γ-^{32}P]GTP was used as a phosphate donor only three ^{32}P-labelled protein bands (13 kDa, 19 kDa and 38 kDa) were detected (Fig. 1 – right). The phosphorylation level of these proteins was significantly decreased by low concentrations of heparin (1 μg/ml and 5 μg/ml) suggesting that CKII associated with *T. cutaneum* ribosomes was engaged in this modification (Fig.1 b, c, e, f).

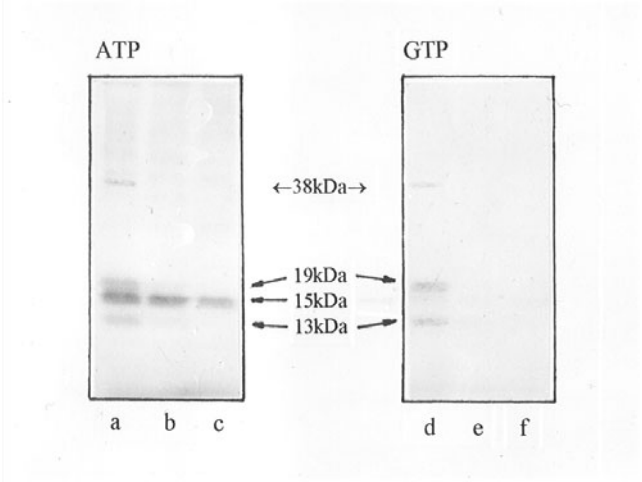

Figure 1. Endogenous phosphorylation of *T. cutaneum* ribosomal proteins (autoradiogram).

Membrane-free 80S ribosomes were incubated with [γ-^{32}P]ATP, left; or [γ-^{32}P]GTP, right, under conditions described in the Materials and methods section without (a, d) or in the presence of heparin: 1 μg/ml (b, e) and 5 μg/ml (c, f). Positions of phosphorylated proteins are indicated by arrows.

CKII effectors. Next, we used purified CKII from *T. cutaneum* and *S. cerevisiae* for *in vitro* phosphorylation of purified 80S ribosomes (deprived of endogenous protein kinase activity).

To study phosphorylation *in situ*, membrane-free 80S ribosomes of *T. cutaneum* were incubated with [γ-^{32}P]ATP or [γ-^{32}P]GTP in

The results presented above were confirmed by *in vitro* phosphorylation of ribosomal proteins by purified CKII. If CKII from *T. cutaneum* or *S. cerevisiae* was used for phosphorylation of *T. cutaneum* ribosomes, again the same picture of ^{32}P-labelled proteins was obtained (Fig. 2A, d, e). If, however, CKII from both sources was incubated with ribo-

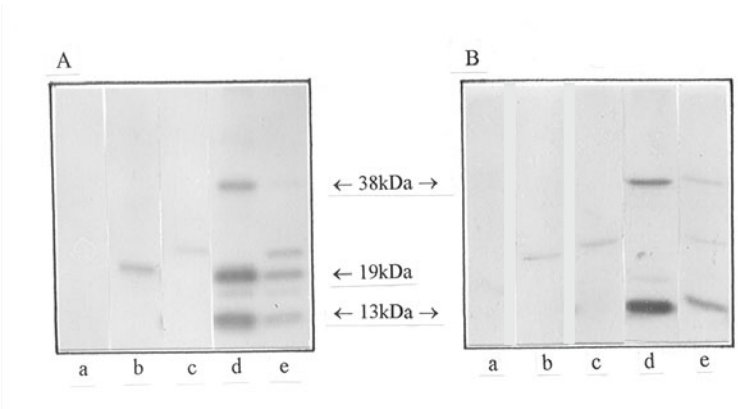

Figure 2. Phosphorylation of *T. cutaneum* (A) and *S. cerevisiae* (B) ribosomes by CKII.

Purified ribosomes from the exponential growth phase were incubated alone (a) and with CKII from *T. cutaneum* (d) or CKII from *S. cerevisiae* (e). When both enzymes were incubated alone (control) autophosphorylation of CKII from *T. cutaneum* (b) and *S. cerevisiae* (c) was visible. All lanes are from the same experiment.

somes of *S. cerevisiae* only two proteins of 13 kDa and 38 kDa were phosphorylated (Fig. 2B, d, e). Phosphorylation of 19 kDa was not detected in *S. cerevisiae* ribosomes showing again that its modification is specific for *T. cutaneum* ribosomes.

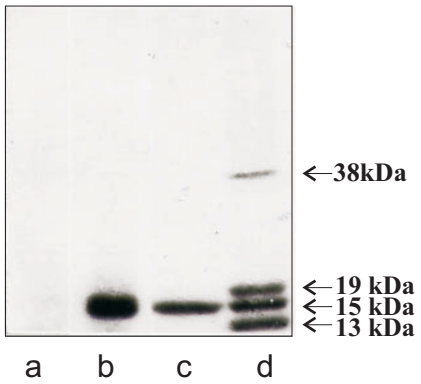

Figure 3. Phosphorylation of *T. cutaneum* ribosomal proteins by purified CKIC and CKIR.

Purified ribosomes were incubated alone (a) and with CKIR (b) or CKIC (c). Endogenous phosphorylation of *T. cutaneum* ribosomal proteins was performed to facilitate identification of proteins modified by CKI.

Purification and biochemical characterization of cytosolic and ribosomal CKI of *T. cutaneum*

The above results indicate that the ribosomal protein of 15 kDa is not phosphorylated by CKII. Our preliminary studies showed that CKI is responsible for the 15 kDa protein modification (Wojda *et al.,* 1996; 1999). In order to characterize closer the protein kinase engaged in the modification of the 15 kDa ribosomal protein, we performed CKI purification from two subcellular *T. cutaneum* sources: the cytosol (CKIC) and 0.5 M KCl ribosomal extracts (CKIR). The procedure described in the Material and Methods section comprised ion exchange column chromatography on DEAE-cellulose and P-cellulose and affinity chromatography on casein-Sepharose and heparin-Sepharose. By the use of such highly purified CKIC and CKIR we confirmed our previous observation that the 15 kDa ribosomal protein of *T. cutaneum* is modified by protein kinase CKI (Fig. 3).

SDS/PAGE analysis and subsequent silver staining of purified CKIC revealed the presence of a single protein band of 33 kDa (Fig. 4I, a). In the case of CKIR two protein bands: of 35 kDa and a predominant one of 40 kDa were observed in the stained gels (Fig. 4I, b).

The molecular mass of CKIC and CKIR was also estimated using protein kinase renaturation test on casein containing SDS/polyacrylamide gels (In gel assay – Materials and Methods). In the case of CKIC only one ^{32}P-labelled band, reflecting the position of casein phosphorylation by renatured enzyme was identified. Its molecular mass was estimated as 33 kDa. As for CKIR, a doublet of radioactive bands of 35 kDa and 37 kDa was detected (Fig. 4II, A). The above results suggested that CKIR can exist in differently phosphorylated forms. To test this hypothesis the ability of CKIC and CKIR to undergo autophosphorylation was examined. Only one protein band of 33 kDa was observed for CKIC after autophosphorylation reaction performed in the presence of [γ-^{32}P]ATP. In the case of CKIR a doublet of proteins 35 kDa and 37 kDa was detected (Fig. 4II, B). However, a significant shift toward the slower migrating form of 37 kDa was visible (Fig. 4IIB). As can be seen in Fig. 3IIB in addition to the 35 kDa and 37 kDa proteins, a slight amount of ^{32}P-labelled 40 kDa protein was detectable in the autoradiogram after CKIR autophosphorylation reaction. When the protein kinase renaturation test was done after autophosphorylation performed in the presence of unlabelled ATP, again in the case of CKIR two separate bands of 35 kDa and 37 kDa were detected with a significant increase in the amount of the higher molecular mass form and only one form of 33 kDa for CKIC (Fig. 4II, C). As it was shown in Fig. 4I, a protein of 40 kDa was present at a high concentration in purified CKIR preparation. Since this polypeptide did not phosphorylate casein in the polyacrylamide gels in the kinase renaturation test (Fig. 4IIA, C), it was sup-

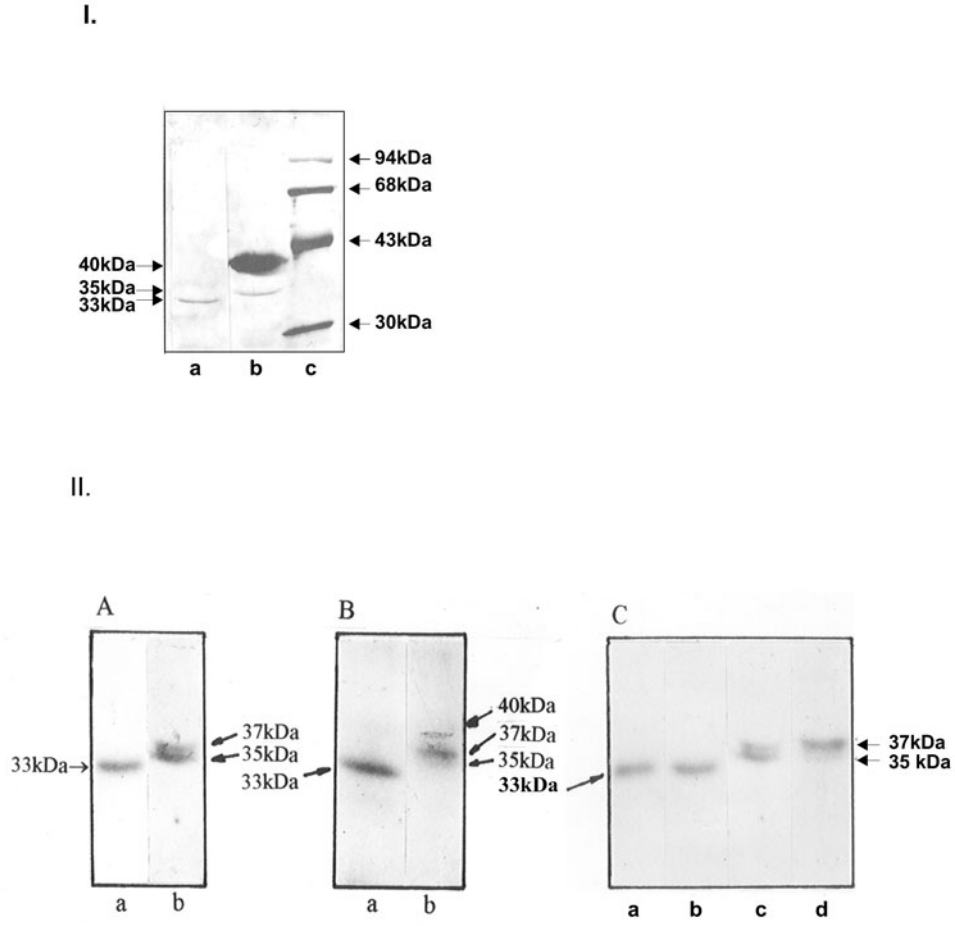

Figure 4. Estimation of molecular mass of CKIC and CKIR.

I. Electrophoretic analysis of purified CKIC and CKIR. Purified CKI from cytosol (a) and ribosome-associated fraction (b) were subjected to SDS/PAGE and silver stained as described in the Material and Methods section; (c) protein standards. The amount of protein containing 2.5 U of CKIC or CKIR was loaded on the gel. **II. A**. Samples of CKIC and CKIR (1 μg protein) were separated by SDS/PAGE on gel matrix containing 1 mg/ml casein. Renaturation experiment was as described in the Material and Methods section. Phosphorylation of casein by renatured CKIC (a) and CKIR (b) is indicated by arrows. **B**. Autophosphorylation reaction of protein kinase CKIC (a) and CKIR (b) in the presence of [γ-^{32}P]ATP was performed for 1 h at conditions described in the Material and Methods section. After reaction the enzymes were subjected to 15% SDS/PAGE and analysed by autoradiography. **C**. Renaturation test of CKIC (a, b) and CKIR (c, d) without (a, c) or after autophosphorylation performed in the presence of unlabelled ATP (b, d).

posed to be a CKIR substrate, which copurified throughout the multistep purification procedure.

In further studies the effect of heparin on the autophosphorylation of CKIC and CKIR was tested. As can be seen in Fig. 5 heparin did not affect the phosphorylation level of CKIC and the 35 Da–37 kDa forms of CKIR. However, in the case of CKIR, a significant increase in the phosphorylation level of the 40 kDa protein was clearly visible at 5 μg/ml concentration of heparin (Fig. 5).

Attempt to identify the 40 kDa protein

In order to identify the 40 kDa protein, CKIR preparation (purified as described in the Material and Methods section) containing high amount of the p40 protein was subjected to SDS/PAGE and electroblotted onto Immobilon membrane. The protein band of 40 kDa was cut out and amino-acid sequence analysis was performed by Edman degradation. The obtained 50 amino-acid sequence is presented in Fig. 6.

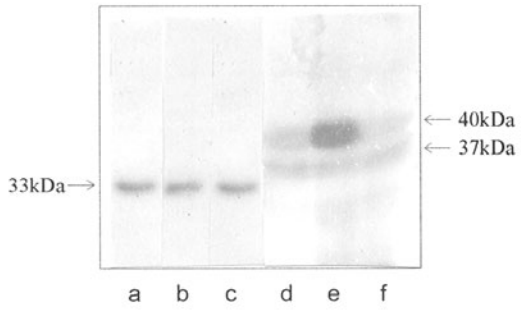

Figure 5. Autophosphorylation reaction of protein kinase CKIC (a–c) and CKIR (d–f) in the absence (a, d) and presence of 5 μg/ml (b, e) and 40 μg/ml (c, f) heparin.

After reaction performed as described in the Material and Methods section the enzymes were subjected to 15% SDS/PAGE and analysed by autoradiography.

Analysis of the obtained sequence revealed seven amino acids containing hydroxyl groups, which can be considered as phosphate acceptors. Those are: S^{19}, T^{24}, T^{27}, T^{28}, T^{45}, T^{49} and Y^{26}. It is noticeable that T^{24} is preceded by the sequence EVL forming a CKI recognition site. It is worth mentioning here that an important feature of CKI phosphorylation sites is the presence of a phosphorylated serine or threonine or an acidic amino acid at position –3 in relation to the serine or threonione which is the phosphate acceptor (Tuazon & Traugh, 1991). Phosphorylation of T^{24} in the p40 protein can subsequently create a new CKI recognition motif: T(P)XXT^{27} for T^{27} phosphorylation. On the other hand, T^{27} phosphorylation forms the potential recognition site T^{24}XXT(P)^{27} for T^{24} phosphorylation by CKII. In the identified sequence, the presence of a myristoylation signal (G^{47}KTV^{50}) is also remarkable. In addition the N-terminal amino-acid sequence presented in Fig. 6 showed 41% identity with the C-terminus of the Ssa4 protein of *S. cerevisiae,* a chaperone of the HSP70 family.

1 5 10 15 20 25 30 35 40 45 50
^{N}LKKPAPAAEEQKEEQKGLSNEVLTKYTTAGQALGVAIKNLVPQVTAGKTVL

Figure 6. Amino-acid sequence of the N-terminal end of the p40 protein associated with CKIR.

CKIR preparation containing a significant amount of the p40-protein was subjected to SDS/PAGE, transferred to PVDF membrane and the p40 protein was excised and sent for Edman degradation sequencing (Proseq, U.S.A.).

DISCUSSION

Our earlier studies (Cytryńska *et al.,* 1995; Wojda *et al.,* 1997; 1999) and the results presented in this paper indicate that in *T. cutaneum*, similarly to other yeast species, the complex of acidic ribosomal proteins P1/P2–P0 is modified by CKII. Additionaly, in *T. cutaneum* ribosomes, but not in other yeast species, a protein of 19 kDa is also phosphorylated by CKII. Modification of this protein, which is a member of the 40S ribosomal subunit, was shown to occur not only *in vitro* but also *in vivo* (Wojda *et al.,* 1996). Furthermore when CKII of *S. cerevisiae* was used for phosphorylation, the same proteins, namely the P1/P2–P0 complex and a protein of 19 kDa were modified. If, however, *S. cerevisiae* ribosomes were used as phosphorylation substrate for *T. cutaneum* CKII, only P-proteins modification was observed. These results cleary indicate that the difference in the pattern of ribosomal proteins phosphorylation between *T. cutaneum* and *S. cerevisiae* is caused by the specificity of ribosomal protein composition.

A significant difference between the ribosomes of *T. cutaneum* and other yeast species was also phosphorylation of a 15 kDa protein

which was identified on the small ribosomal subunit. We found that this protein is modified by CKI (Wojda *et al.,* 1999, this paper). Knowing that CKI of different organisms can be associated with particulate subcellular components, we purified CKI from ribosomal fraction (CKIR) and cytosol (CKIC) of *T. cutaneum.* The molecular mass of the obtained forms of CKI was estimated as 35 kDa–37 kDa and 33 kDa, respectively. It was found that CKI of *T. cutaneum,* like most protein kinases, can undergo autophosphorylation. Phosphorylation of the cytosolic form of the enzyme did not change its electrophoretic mobility while in the case of CKIR it resulted in a mobility shift from 35 kDa to 37 kDa. These data might indicate that CKI activity can be regulated by reversible phosphorylation. It is also possible that the phosphorylation can affect its subcellular localization or its ability to associate with other cellular components that regulate CKI activity. On the basis of the presented results we presume that the higher phosphorylated form of CKI is associated with ribosomes. We can not, however, exclude the possibility that the cytoplasmic form contains autophosphorylation sites which were already modified in the cells.

So far, not much is known about the control of CKI activity. Structural studies performed on CKI from other sources revealed that members of the CKI family contain a short N-terminal domain, a highly conserved kinase domain and variable C-terminus that ranges from 24 to more than 200 amino acids in length. The C-terminal domain is a potential region of enzyme phosphorylation. It has been suggested that this region plays autoinhibitory role in the regulation of substrate recognition, modulation of catalytic activity and/or determination of kinase cellular localization for different CKI homologs (Graves & Roach 1995; Cegielska *et al.,* 1998). Several observations indicate that polyanions such as heparin can affect the activity of CKI by interaction with the carboxyl domain, mimicking its dephosphorylation.

For a long time heparin had been considered a potent inhibitor of CKII, but not CKI activity. We showed before that the activity of CKI of *T. cutaneum* can be inhibited by heparin when phosvitin, but not casein, was used as the phosphorylation substrate (Wojda, 1997).

We also showed that heparin changed the substrate specificity of CKI. A ribosomal protein of about 20 kDa was phosphorylated only in the presence of this polyanion (Wojda *et al.,* 1997; 1999). In this paper we show that in the presence of heparin at 5 μg/ml (but not at 40 μg/ml) a significant increase in the phosphorylation level of the 40 kDa protein was visible. These observations suggest that heparin acting in a dosage-dependent manner may "mimic" a natural, not yet identified, regulator(s) of CKI.

The protein of 40 kDa whose phosphorylation is heparin-dependent copurified with CKIR (but not CKIC) through the multistep purification procedure. This protein similarly to CKIR is tightly associated with ribosomes. Analysis of the amino-acids composition of an N-terminal fragment of this protein revealed high content of threonine residues of which T^{24} and T^{27} are located in potential CKI phosphorylation sites. The observed significant increase of this protein phosphorylation in the presence of heparin can be relevant with changing CKI substrate recognition and as a consequence phosphorylation site(s). It was found earlier that the phosphorylation site specificity of a Cdc2-like kinase was changed by heparin (Qui *et al.,* 1998). The physiological role of the p40 protein is not known. We can not exclude the possibility that in addition to being a CKIR substrate it can also play a regulatory role for CKI and possibly CKII activity, or, conversely, be regulated by those kinases. The N-terminal amino-acid sequence analysis revealed a significant homology with the C-terminus of Ssa4 of *S. cerevisiae.* The sequence identity in the analysed fragment was 41% (20 out of 49) in the optimum alignement. The protein Ssa4 is a protein chaperone, a member of the

HSP70 family. It is a cytoplasmic protein associated with yeast polysomes and ribosomes as well as with CKII. It plays an important role in such processes as SRP-dependent protein folding and co-translational membrane targeting (Gavin *et al.,* 2002; Rassow *et al.,* 1997). It contains 15 potential phosphorylation sites for CKI and 19 for CKII. In the light of this observation the question arises whether the *T. cutaneum* p40 protein is also a stress response ribosome chaperone. Answering this question requires further studies.

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