Volume 309, number 1, 103–106 FEBS 11463 August 1992
© 1992 Federation of European Biochemical Societies 0014-5793/92/$5.00

Ca^{2+}/calmodulin-activated protein phosphatase (PP2B) of *Saccharomyces cerevisiae*

PP2B activity is not essential for growth

Taro Nakamura[a], Hiroko Tsutsumi[a], Hideyuki Mukai[b], Takayoshi Kuno[b] and Tokichi Miyakawa[a]

[a]*Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Higashi-Hiroshima 724, Japan* and [b]*Department of Pharmacology, Kobe University School of Medicine, Chuo-ku, Kobe 650, Japan*

Received 22 April 1992; revised version received 9 July 1992

Protein phosphatase (PP2B) whose activity is stimulated 12–20-fold by Ca^{2+}/calmodulin (CaM) was partially purified by CaM-Sepharose and heparin-agarose chromatographies from cell extract of the yeast *Saccharomyces cerevisiae*. PP2B activity was not detectable in a mutant in which two genes (*CMP1* and *CMP2*) encoding homologs of mammalian PP2B catalytic subunit were disrupted. We have previously shown that the double gene disruption has no significant effect on the growth of yeast [1991, Mol. Gen. Genet. 227, 52–59]. The results indicated that *CMP1* and *CMP2* are the only genes that encode the PP2B catalytic polypeptide in *S. cerevisiae*, and PP2B activity is not essential for the growth of the yeast under normal conditions.

Protein phosphatase type 2B; Gene disruption; *Saccharomyces cerevisiae*

1. INTRODUCTION

Protein phosphatases (PPases) that dephosphorylate phosphoserine/threonine are classified into four major types, PP1, PP2A, PP2B and PP2C, according to substrate specificity and mode of enzyme regulation (for review see [1]). It is recognized that PPases play important roles in various cellular processes, as the extent of phosphorylation of proteins depends on the relative activities of protein kinases and PPases that act on the protein (for review see [1–3]). From genetic and biochemical analyses of cell division cycle of eucaryotic microorganisms, it has become clear that PPases such as PP1 and PP2A have essential regulatory functions in vivo [4–7]. Enzymatic properties of yeast PP1, PP2A and PP2C have been demonstrated to be remarkably similar to those of mammalian enzymes [5,8].

Recently, we cloned and characterized two *S. cerevisiae* genes (*CMP1* and *CMP2*) that encode the catalytic subunit of PP2B [9]. The deduced amino acid sequences of *CMP1* and *CMP2* are 64% identical to each other, and each gene is 54% identical to the mammalian counterpart. Mammalian PP2B, also designated as calcineurin, consists of two polypeptides: the catalytic A subunit and the regulatory B subunit. The B subunit is a Ca^{2+}-binding protein homologous to calmodulin (CaM) (for review see [10]). In the presence of Ca^{2+}, CaM reversibly binds to the A subunit. As the activity of PP2B is regulated by Ca^{2+}, the phosphatase is thought to play important roles in various aspects of cellular processes that are regulated by the Ca^{2+} signal. Single or double disruption mutations of the *CMP* genes, however, had no effects on growth rates, heat shock responses, sensitivity to nitrogen starvation, mating or sporulation [9,11]. The lack of phenotype in the double null mutant can be explained by either of the following possibilities: PP2B may be dispensable for normal growth due to functional complementation of its loss by other classes of PPases; alternatively, PP2B is essential, but there is a gene(s) homologous to *CMP1/2*, and the function of the unidentified gene(s) is identical or overlapping with that of *CMP1/2*. Since the amino acid sequences of the catalytic domains of PP1, PP2A and PP2B are homologous with each other [3], it would be difficult to determine by genomic Southern analysis at low stringency if there are more homologous genes for *CMP1/2*.

To answer the question, we compared PP2B activity of wild type (*CMP1 CMP2*) *S. cerevisiae* and a mutant strain (*Δcmp1 Δcmp2*) that lacks the two Cmp proteins. The result shows that PPase activity that is stimulated 12–20-fold by Ca^{2+}/CaM is present in CaM binding protiens isolated from a cell extract of the wild-type strain, whereas the activity is not detectable in the double null mutant.

Correspondence address: T. Miyakawa, Department of Fermentation Technology, Hiroshima University, Higashi-Hiroshima 724, Japan. Fax: (81) (824) 22-7191.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine brain CaM purified as previously described [12] was used throughout the experiments.

2.2. *Yeast strains*

Saccharomyces cerevisiae strains HTT-8b (*MATα trp1 CMP1 CMP2 leu2 ura3 pep4-3*) and HHT-8c (*MATα trp1 Δcmp1::LEU2 Δcmp2::URA3 pep4-3*) were used for enzyme preparation. HTT-8b and HTT-8c were constructed by crossing with a haploid *pep4* strain THT1 (*α leu2 ura3 CMP1 CMP2 pep4-3*) with YLL-2b (*MATα leu2 ura3 his3 trp1 CMP1 CMP2*) [9] or YLL-2d (*MATα leu2 ura3 his3 trp1 cmp1::LEU2 cmp2::URA3*) [9], respectively. The strains with desired genetic markers were obtained from a tetrad of spores. The *pep4* phenotype was determined by staining with a chromogenic substrate as described [13].

2.3. *Partial purification of PP2B*

S. cerevisiae cells were grown at 30°C to mid-log phase in YEPD medium [9]. Yeast cells cultivated in 9 l medium (4×10^{11} cells, 1.7 g protein) were harvested by centrifugation, and washed in 20 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. The cells suspended in Buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol and a mixture of protease inhibitors that contain 0.5 mM PMSF and 5 μg/ml each of pepstatin and leupeptin) were disrupted by French press at 20,000 psi. Clear supernatant was obtained by centrifugation at 160,000 × *g* for 1 h, and the solution was then loaded onto a 20 ml column of DE52 cellulose equilibrated in Buffer A. After washing the column with 200 ml of Buffer A, protein was eluted with 100 ml of Buffer A containing 0.15 M NaCl. The eluted proteins were precipitated by adding solid ammonium sulfate to give 55% saturation. The precipitates were collected by centrifugation, dissolved in 5 ml of buffer A and precipitated again with ammonium sulfate. These procedures eliminate CaM present in the preparation. The precipitate was then dissolved in 1 ml of Buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM $CaCl_2$, 1 mM $Mg(Ac)_2$, 5 mM β-mercaptoethanol and protease inhibitors), and the solution was loaded onto a 6 ml column of CaM-Sepharose equilibrated in buffer B. The column was washed with 60 ml of Buffer B, and then Buffer B containing 0.2 M NaCl. Protein was eluted from the column with 20 ml of Buffer C (same composition as Buffer B, except $CaCl_2$ was replaced with 1 mM EGTA). The CaM-binding proteins were dialyzed against buffer D (20 mM Tris-HCl, pH 7.0, 1 mM imidazole, 1 mM $Mg(Ac)_2$, 0.1 mM EGTA and 5 mM β-mercaptoethanol). The CaM-binding proteins were loaded onto a 1 ml heparin-agarose (Pierce Chemical Co.) column equilibrated in Buffer D [14]. The column was washed with 10 ml of Buffer D and eluted with a 20-ml linear gradient of NaCl (0–0.6 M) in Buffer D. Each fraction was assayed for PPase activity.

2.4. *Assay of PPase*

PPase activity was measured using ^{32}P-labeled casein as substrate. Dephosphorylated casein (Sigma Chemical Co.) was labeled with [γ-^{32}P]ATP (Amersham, Japan) as described previously [12]. Specific radioactivity of labeled casein was about 8 μCi ^{32}P per mg protein. The reaction mixture for PP2B assay contained the following in 100 μl: 20 mM Tris-HCl, pH 7.5, 1 mg/ml bovine serum albumin, 0.3 mM $Mg(Ac)_2$, 2 mM $CaCl_2$, 10 μg CaM and ^{32}P casein (cas. 40,000 cpm). When required, 2 mM EGTA was added instead of Ca^{2+}/CaM. After an incubation at 30°C for 30 min, the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 10% (w/v). Free ^{32}P that remained in the supernatant fraction after centrifugation was determined by the Cerenkov counting in a liquid scintillation counter.

2.5. *Western blotting*

Antibodies against β-galactosidase-Cmp1 and β-galactosidase-Cmp2 fusion proteins described previously [9] were used for detection. Antisera were first passed through a column of β-galactosidase coupled to Sepharose 4B, and unadsorbed proteins were further purified through an affinity column made with respective fusion protein coupled to CNBr-activated Sepharose 4B. Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 10% polyacrylamide gel. Western blotting was performed as described [9]. For detection of the B subunit of PP2B, antiserum directed against bovine brain calcineurin B [15] was used. This antibody detects the B subunit of yeast PP2B [16].

3. RESULTS AND DISCUSSION

First, we investigated the subcellular distribution of yeast PP2B between soluble and particulate fractions of the cell extract of wild-type strain HTT-8b, using antibody against Cmp1 and Cmp2 proteins. Both Cmp1 and Cmp2 proteins were present in the soluble fraction as well as the particulate fraction (Fig. 1). Neither of these proteins were detected in the sample similarly prepared from a double null mutant HTT-8c (data not shown). The cell extract from wild type and the null mutant was applied to a DE52 column at pH 7.5, and

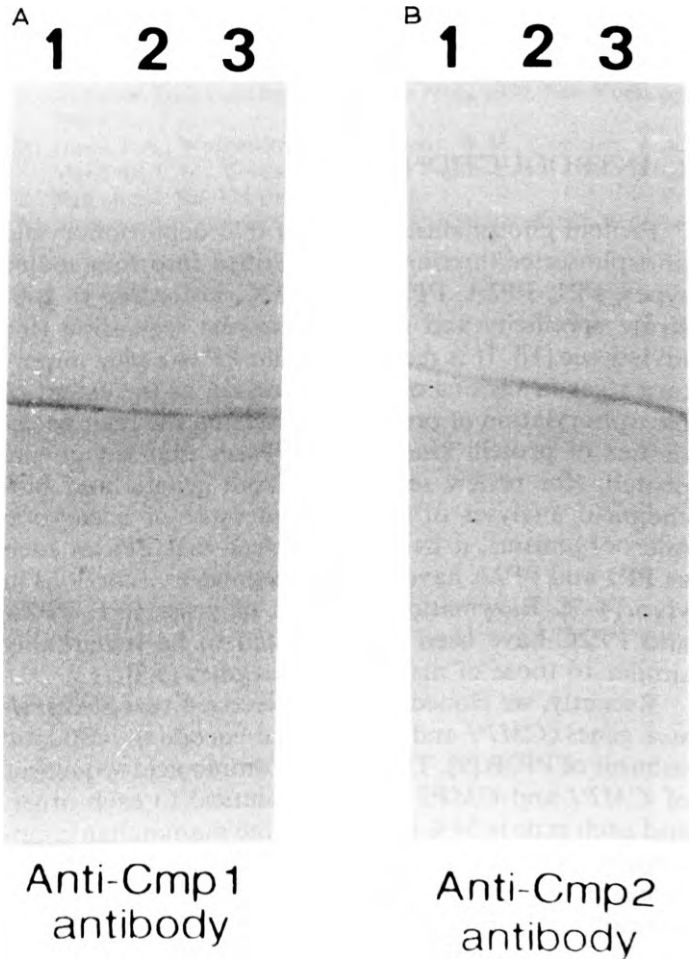

Fig. 1. Subcellular localization of yeast PP2B. About 20 μg proteins of subcellular fractions from *S. cerevisiae* (HTT-8b) cells were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel). Proteins were detected for Cmp1 (A) and Cmp2 (B) by anti-Cmp1 and anti-Cmp2 antibodies, respectively. 1, Total protein; 2, soluble protein; 3, particulate protein.

the protein bound to the column were eluted with buffer containing 0.15 M NaCl. The CaM-binding proteins were isolated from DE52 eluate by CaM-affinity chromatography, and the Cmp proteins were analyzed by Western blotting (data not shown). The Cmp1 and Cmp2 proteins of the wild-type sample were highly enriched in the CaM-binding protein fraction. These proteins were not present in the unadsorbed fraction of the affinity column, and neither of these proteins were detected in the CaM-binding proteins isolated from the null mutant (data not shown).

PP1, PP2A and PP2C with biochemical similarities to mammalian enzymes have been detected in the yeast cell extract. However, the presence of PP2B in the cell extract prepared from yeast was not clear [8]. We first measured PPase activity in the total cell extract using ^{32}P-labeled casein as substrate. About half of the PPase activity was inhibited by okadaic acid, an inhibitor of yeast PP1 and PP2A [8], suggesting that remaining activity contains those of PP2B and PP2C. However, the release of $^{32}P_i$ from casein in the presence of okadaic acid was stimulated about 2-fold by the presence of EGTA (data not shown). A similar extent of stimulation was observed in the absence of okadaic acid. These results may suggest that the accessibility of phosphoamino acids in casein to PPases changes with the presence or absence of Ca^{2+}. Thus, it was not possible to quantitatively estimate PP2B purification in the following experiments.

We next measured PP2B activity of the CaM-binding proteins isolated from the cell extract of the parental strain (HTT-8b). The affinity-purified sample contained both the catalytic and the regulatory subunits (Fig. 2B).

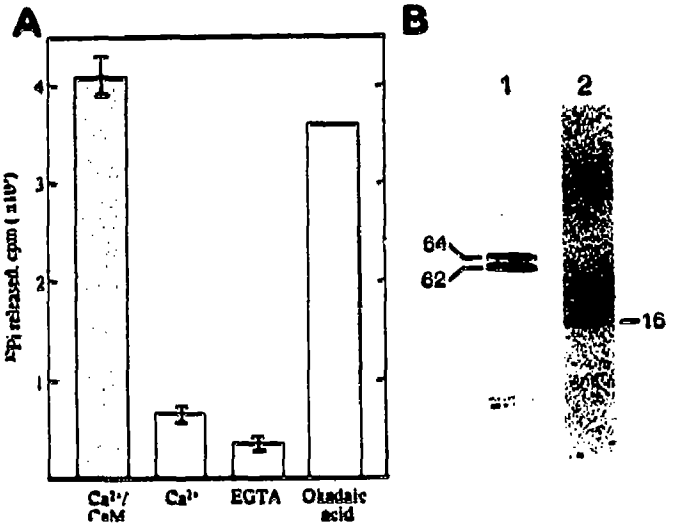

Fig. 2. PP2B of *S. cerevisiae*. (A) PPase activity of the CaM-binding proteins isolated from wild-type strain HHT-8b was measured with ^{32}P-labeled casein as substrate. (B) Two PP2B subunits of the affinity-purified sample were detected by Western blotting using antibody against the catalytic subunit (Cmp1 and Cmp2) of yeast PP2B in 10% gel (1), or the regulatory subunit of bovine brain calcineurin in 15% gel (2). Protein sizes in kDa are indicated.

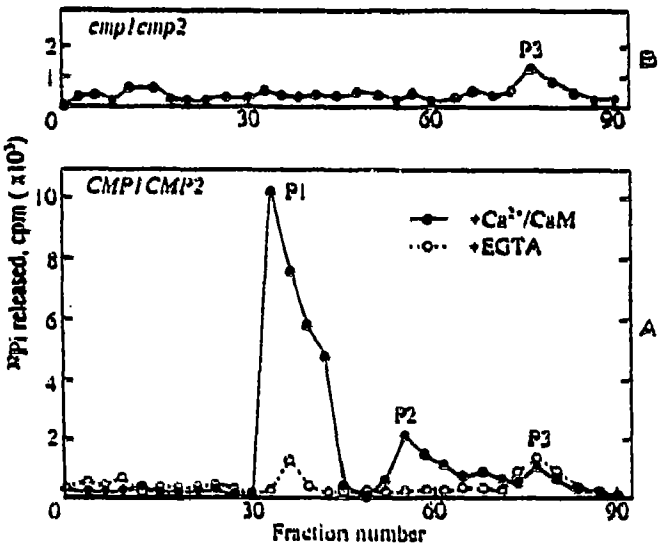

Fig. 3. Fractionation of the CaM-binding proteins by heparin agarose chromatography. (A) PPase of the sample prepared from the null mutant strain HTT-8c. Activity was measured in the presence (●) or absence (○) of Ca^{2+}/CaM. (B) PPase of the sample prepared from wild-type strain HTT-8b. Activity was measured in the presence of Ca^{2+}/CaM.

In a typical preparation, the PPase activity of this fraction was stimulated about 12–20-fold by the presence of Ca^{2+} and CaM (Fig. 2A). Both Ca^{2+} and CaM were required for maximal enzyme activity. The Ca^{2+}/CaM-stimulated activity was not inhibited by okadaic acid up to concentrations of 100 nM. PP1 and PP2A from mammals and yeasts, are inhibited by 30 nM and 0.1 nM okadaic acid, respectively [4,8]. More than 90% of mammalian PP2B remains active in the presence of 100 nM of okadaic acid [17]. Thus, the yeast PPases appear to be very similar to mammalian PPases in the pattern of sensitivity to okadaic acid.

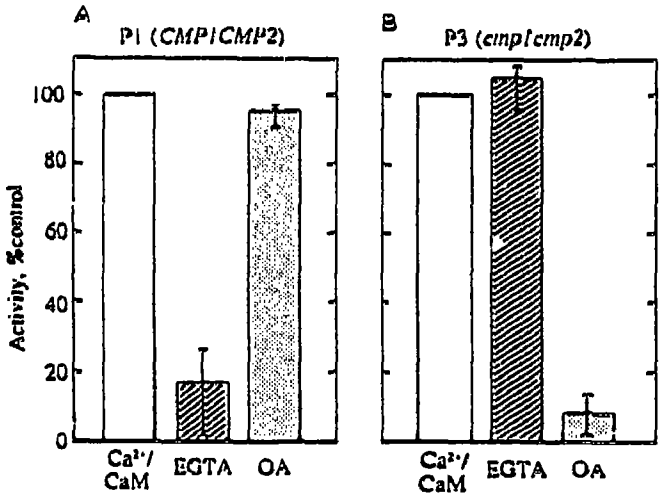

Fig. 4. Properties of PPases of P1 (from *CMP1 CMP2* strain) and P3 (from *cmp1 cmp2* strain) fractions of heparin-agarose chromatography. PPase activity of P3 from wild-type cells showed the same properties as those of the null mutant.

Initial attempts to measure PPase activity of the CaM-binding proteins isolated from a double null mutant HTT-8c indicated that the mutant contain much weaker PPase activity than the wild-type strain (less than 5%), and the activity of the mutant is independent of Ca^{2+}/CaM (data not shown). Thus, PPases of the CaM-binding proteins from wild type and the null mutant were further fractionated by heparin-agarose chromatography (Fig. 3B). The elution profile of wild type CaM-binding proteins showed three peaks (P1, P2 and P3) of PPase activity (Fig. 3B). The activity of P1 was stimulated about 20-fold by Ca^{2+}/CaM, and was insensitive to 100 nM okadaic acid (Fig. 4A). The properties of PPase of the P2 fraction was very similar to those of P1 activity. By Coomassie blue staining and Western blot analysis of P1 and P2 fractions, it was found that Cmp2 was the major protein of the P1 fraction with Cmp1 and an unknown protein of 66,000 Da as minor components. Several bands including Cmp1 and Cmp2 were present in P2. The weak activity of P3 was not stimulated by Ca^{2+}/CaM, and it was sensitive to okadaic acid (Fig. 4B). Cmp1 and Cmp2 proteins were not detectable by Western blot analysis of the P3 fraction (data not shown). These results suggest that the activity of the P3 fraction represents PPases of other types which are possibly associated with a CaM-binding protein.

The null mutant totally lacked the PPase activity corresponding to the P1 and P2 of wild-type enzymes, and only the P3 activity was present (Fig. 3A). The P3 PPase activity of the mutant, like that of the wild type, was not stimulated by Ca^{2+}/CaM, and the activity was sensitive to okadaic acid. CaM-binding proteins were also isolated from the particulate fraction of the mutant after solubilization of the membranes by *n*-octylglucoside. PP2B activity was not detectable in this fraction (data not shown). Based on these results, we concluded that PP2B activity is not detectable in the double null mutant using casein as substrate. These results indicate that *CMP1* and *CMP2* are the only genes that encode the catalytic subunit of PP2B, and the activity is not essential for growth. Consistent with this possibility, all our attempts to clone *CMP1/2* homologs by various procedures, including low-stringency Southern hybridization using DNA fragments corresponding to the regulatory domain of PP2B, PCR method based on conserved sequences of PP2B, and phenotypic complementation of a defect of a double null mutant (sensitivity to vanadate), were not successful (Liu et al., to be published).

In our previous paper [9], we have demonstrated that double disruption of *CMP1* and *CMP2* does not affect cell viability, and causes no distinct phenotypes, including changes in growth rate, sensitivity to heat shock, utilization of various carbon-sources, sensitivity to nitrogen starvation, mating and sporulation [9]. Considering the highly conserved structure of the yeast Cmp proteins through evolution, and the presence of a gene family, it is most likely that the yeast PP2B is involved in the regulation of basic cellular processes. However, the present results indicate that PP2B activity is not essential for the normal growth of *S. cerevisiae*. PPases of other types such as PP1, PP2A or PP2C may substitute for the loss of function of PP2B in the double null mutant. We recently found that growth of the double null mutant is sensitive to high concentrations of NaCl (1.5 M) or LiCl (0.1 M), and also sensitive to vanadate (5 mM) (unpublished results). Furthermore, the null mutant of PP2B is defective in the recovery from growth arrest induced by α-factor [11]. Thus, PP2B may be required only under certain stress conditions, and PPases of other types are not sufficient to replace the function of PP2B under these circumstances.

Acknowledgements: We thank Y. Liu, E. Tsuchiya and T. Yamada for helpful discussion.

REFERENCES

[1] Cohen, P. (1989) Ann. Rev. Biochem. 58, 453–508.
[2] Cyert, M.C. and Thorner, J. (1989) Cell 57, 891–893.
[3] Cohen, P. and Cohen, P.T.W. (1989) J. Biol. Chem. 264, 21435–21438.
[4] Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. and Yanagida, M. (1989) Cell 57, 97–107.
[5] Kinoshita, N., Ohkura, H. and Yanagida, M. (1990) Cell 63, 405–415.
[6] Sneddon, A.A., Cohen, P.T.W. and Stark, M.J.R. (1990) EMBO J. 9, 4339–4346.
[7] Ronne, H., Carlberg, M., Hu, G.-Z. and Nehlin, J.O. (1991) Mol. Cell. Biol. 11, 4876–4884.
[8] Cohen, P., Schelling, D.L. and Stark, M.J.R. (1989) FEBS Lett., 250, 601–606.
[9] Liu, Y., Ishii, S., Tsutsumi, H., Tokai, M., Ohki, O., Akada, R., Tanaka, K., Tsuchiya, E., Fukui, S. and Miyakawa, T. (1991) Mol. Gen. Genet. 227, 52–59.
[10] Klee, C.B., Draetta, G.F. and Hubbard, M.J. (1988) Adv. Enzymol. 61, 149–200.
[11] Cyert, M.S., Kunisawa, R., Kaim, D. and Thorner, J. (1991) Proc. Natl. Acad. Sci. USA 88, 7376–7380.
[12] Cohen, P., Klumpper, S. and Schelling, D.L. (1989) FEBS Lett. 250, 596–600.
[13] Jones, E.W., Zubenko, G.S. and Parker, R.R. (1982) Genetics 102, 665–677.
[14] Yokoyama, N. and Wang, J.W. (1991) J. Biol. Chem. 266, 14822–14829.
[15] Kuno, T., Mukai, H., Ito, A., Chang, C.-D., Kishima, K., Saito, N. and Tanaka, C. (1992) J. Neurochem. 58, 1643–1651.
[16] Kuno, T., Tanaka, H., Mukai, H., Chang, C.-D., Hiraga, K., Miyakawa, T. and Tanaka, C. (1991) Biochem. Biophys. Res. Commun. 180, 1159–1163.
[17] Bialojan, C. and Takai, A. (1988) Biochem. J. 256, 283–290.