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PHOSPHORYLATION OF Mg^{2+}-DEPENDENT PROTEIN PHOSPHATASE α (TYPE 2Cα) BY CASEIN KINASE II

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SUMMARY. In this study we show that rat Mg^{2+}-dependent protein phosphatase α (MPPα) expressed in *Saccharomyces cerevisiae* cells was phosphorylated on serine residues *in vivo*. The recombinant rat MPPα purified from *Escherichia coli* cells harboring an expression vector was phosphorylated *in vitro* by casein kinase II, but not by casein kinase I, to 1.5 mol phosphate per mol enzyme protein. Analysis by phosphopeptide mapping and amino acid analysis suggested that the sites of both *in vivo* and *in vitro* phosphorylation were the same and involved only serine residues. These results suggest that the rat MPPα expressed in yeast cells is phosphorylated by yeast casein kinase II in *vivo*. It is further proposed that the phosphorylation sites are located in the carboxyl terminal region of the enzyme molecule.

Mg^{2+}-dependent protein phosphatase (MPP[1]), also known as type 2C, is one of the four major protein serine / threonine protein phosphatases (types 1, 2A, 2B and 2C) and exists as a monomeric enzyme of 43-48 kDa (1, 2). cDNA clones encoding the two isotypes, MPPα and MPPβ, have been isolated from cDNA libraries of mammalian cells, and these two isotypes have been found to be encoded in two different genes (3-5). Recently it has been suggested that MPP is involved in the dephosphorylation of autophosphorylation sites of Ca^{2+}/ calmodulin-dependent protein kinase II in granule cells of rat cerebellum (6). However , little is known about the mechanism of regulation of MPP except that the enzyme activity is absolutely dependent on the

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[1]Based on preliminary recommendation of a nomenclature group on Protein Phosphatases (Copper Mountain, 1992), the name for type 2C phosphatase will become MPP as used here.

Abbreviations: MPP, Mg^{2+}-dependent protein phosphatase; TPCK, Nα-tosyl-L-phenylalanine chloromethyl ketone; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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presence of magnesium or manganese ion. Since there is an accumulation of evidence indicating that functions of certain number of protein kinases and protein phosphatases are regulated by phosphorylation (7-10), we intended to investigate whether MPPα is also phosphorylated on its specific sites by cellular protein kinases. In this present study we demonstrate that rat MPPα expressed in yeast cells is phosphorylated on serine residues *in vivo* and that casein kinase II is probably responsible for the phosphorylation.

MATERIALS AND METHODS

Plasmids and yeast strains - The construction of plasmid pJDB207 has been described previously (11). Yeast strains YPC101 and YPC102 were constructed by transforming A1002 [*MATα*, *ade* 8, *leu* 2, *met* 14, *trp* 1, *ura* 3, *aro* 7] with pJDBMPP (expression plasmid) and pJDB207 (vector), respectively.

^{32}P-labeling of yeast cells - Yeast cells were grown under the conditions as described previously (11). Then, the cells were harvested, washed three times in phosphate-free medium (5 mM sodium acetate (pH 5.5), 5% galactose, 0.2% sucrose, 26 mM $(NH_4)_2SO_4$, 10 mM L-asparagine, 2 mM $MgSO_4$, 8.6 mM NaCl and 2.3 mM $CaCl_2$) supplemented with amino acids and resuspended in the same medium containing [^{32}P]orthophosphate (0.5 mCi/ml). After incubation for 2 h at 30°C, the cells were harvested, washed three times in sterile distilled water, once with homogenization buffer (10 mM Tris-HCl (pH 7.4), 5 mM 2-mercaptoethanol, 2%(v/v) glycerol, 50 mM NaCl, 5 mM EDTA, 1 μM okadaic acid, 2 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 0.1 mM benzamidine, 0.1 mM TPCK, 0.5 mM TLCK, 1 mM PMSF and 1 mM EGTA) and resuspended in 0.1 ml of the same buffer. The cells were broken by glass beads and centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was used for immunoprepicitation as described below.

Antibodies - A polyclonal antipeptide antibody, AB102, directed against a synthetic peptide corresponding to amino acids 335-354 (NH_2-RTLASENIPSLPPGGELASK-COOH) of rat MPPα-conjugated hemocyanin, was raised in rabbits. Polyclonal antiserum against MPP purified from rat liver as described previously (13) was named AB101. Both AB101 and AB102 reacted selectively with rat MPPα, and not with any of the endogenous yeast or *E. coli* cellular proteins.

Immunoprecipitation - Crude extracts prepared as described above were incubated for 2 min at 95°C in the presence of 0.5% SDS. The incubated mixture was then diluted 10-fold with TBST solution (10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20) containing 5 mM EDTA and subjected to immunoprecipitation using antibody AB102 for 16 h at 4°C. After addition of pansorbin (insoluble proteinA), the mixture was incubated for 2 h at 4°C, washed three times in TBST solution and subjected to SDS-PAGE.

Phosphoamino acid analysis and phosphopeptide mapping - ^{32}P-labeled MPPα from both *in vivo* and *in vitro* experiments was subjected to phosphoamino acid and phosphopeptide analysis essentially as described (14) except that phosphoamino acids were separated by high- voltage paper electrophoresis instead of a thin-layer plate electrophoresis.

Purification of casein kinase I and casein kinase II - Casein kinase I was purified from rat brain as described by Dahmus (15). Casein kinase II was purified from bovine thymus using modifications of the procedures of Carmicheal *et al.* (16) and Kuenzel and Krebs (17).

In vitro *phosphorylation of rat MPPα* – The recombinant rat MPPα (1.5μg) protein purified from *E. coli* cells transformed by the expression vector as described previously (6) was incubated with 40 mM Tris-HCl (pH 7.7), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM [γ-^{32}P]ATP (1200 cpm/pmol) and purified casein kinase II (2μg) in a total volume of 100 μl. The reaction mixture was incubated for 2 min at 30°C. After addition of an equal volume of SDS-PAGE sample loading buffer, the mixture was incubated for 2 min at 100°C and subjected to SDS-PAGE.

Assay of protein phosphatase activity - Protein phosphatase activity was assayed by the release of [^{32}P]phosphate from [^{32}P]phosphohistone essentially as described previously (12).

RESULTS AND DISCUSSION

Phosphorylation of rat MPPα expressed in yeast cells - In order to determine whether MPPα is phosphorylated in vivo, we performed immunoprecipitation of MPPα after labeling the

yeast cells harboring the expression vector of MPPα with [^{32}P]orthophosphate. Strain YPC101 and its isogenic control YPC102 were labeled with [^{32}P]orthophosphate as described above, and immunoprecipitation was performed in the absence or presence of AB102 (Fig. 1). A phosphorylated band of 46 kDa was detected only when the antibody was added to the cell extract prepared from YPC101. Other phosphorylated bands observed in all lanes seemed to be cellular proteins which bound nonspecifically to protein A. On the basis of these results, we concluded that rat MPPα is a phosphoprotein. Phosphoamino acid analysis using high-voltage paper electrophoresis demonstrated that the phosphorylation was exclusively on serine residues (data not shown).

Phosphorylation of rat MPPα by casein kinase II in vitro - Rat MPPα contains 27 serine residues. Some of them are included in consensus phosphorylation site sequences (18) for cdc2 kinase (Ser135 and Ser216), Ca^{2+}/calmodulin-dependent protein kinase II (Ser116 and Ser127), protein kinase C (Ser88, Ser116, Ser127, Ser167, Ser280, Ser298 and Ser353), casein kinase I (Ser54, Ser285, Ser353, Ser375 and Ser377), and casein kinase II (Ser48, Ser227, Ser256 and Ser377). Among these consensus sites, the casein kinase I (Ser375 and Ser377) and casein kinase II (Ser377) sites, located in the carboxyl terminal region, attracted our attention because some of the phosphorylation sites in protein phosphatases regulated by phosphorylation are reported to be located in carboxyl terminal domains (9,10). When the recombinant MPPα purified

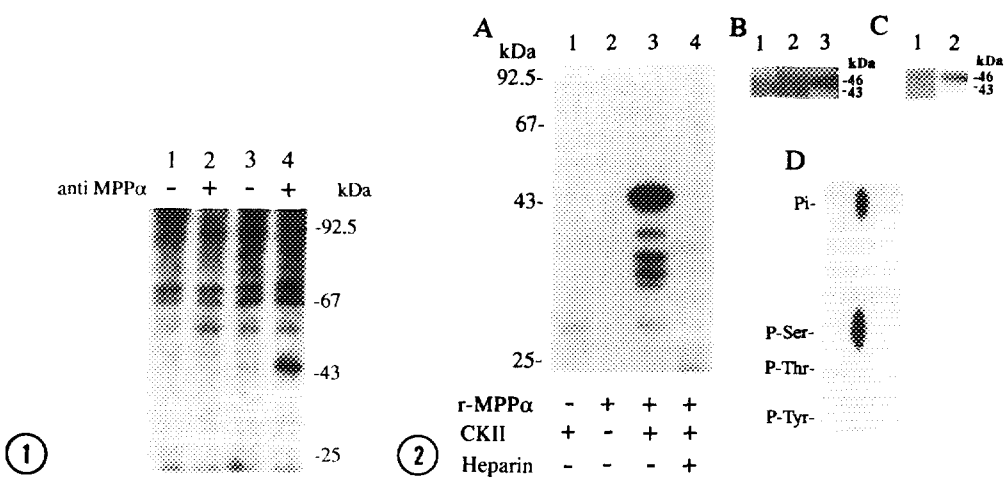

Fig. 1. Immunoprecipitation of rat MPPα from ^{32}P-labeled yeast cells using antipeptide antibody. Rat MPPα was immunoprecipitated with or without antibody AB102 from crude extracts of ^{32}P-labeled YPC102 (*lanes1* and *2*) and YPC101 (*lanes* 3 and *4*). At the top, the absence (-) or presence (+) of the antibody is indicated.

Fig. 2. Phosphorylation of rat MPPα by casein kinase II *in vitro*. In *panel A*, the recombinant rat MPPα purified from *E. coli* cells was phosphorylated as described in "MATERIALS AND METHODS". After the incubation, the samples were incubated with SDS-PAGE sample buffer and resolved by SDS-PAGE. The dried gel was exposed to X-ray film (Kodak XAR-5). At the *bottom*, the presence (+) or absence (-) of recombinant MPPα (r-MPPα), casein kinase II (CKII) or heparin is indicated. *Panel B* shows immunoblots of purified recombinant rat MPPα. The recombinant rat MPPα was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then cut into strips (1.0 x 8.0 cm). Each strip was either stained with amidoblack 10B (*lane 2*) or immunostained with antibody AB101 (*lane 1*) or AB102 (*lane 3*). *Panel C* shows the purified recombinant rat MPPα phosphorylated by casein kinase II and stained with Coomassie brilliant blue (*lane 1*), and the gel after exposure to X-ray film (*lane 2*). *Panel D* shows phosphoamino acid analysis of rat MPPα phosphorylated *in vitro*.

from an extract of transformed *E. coli* cells was incubated with casein kinase II under the conditions necessary for phosphorylation, incorporation of phosphate into recombinant MPPα was observed (Fig. 2A, lane 3). The phosphorylation was inhibited completely by 1.0 μM heparin (Fig. 2A, lane 4), confirming that casein kinase II was responsible. The phosphorylation stoichiometry value was 1.5 mol phosphate per mol recombinant rat MPPα (data not shown), and the phosphorylation was exclusively on serine residues (Fig. 2D). Several bands with molecular masses lower than 46 kDa were seen in lane 3 of Fig. 2A, but these were probably proteolytic products of rat MPPα, since the bands were immunostained by antibody AB102 (data not shown). Proteins of 27 kDa contained in the casein kinase II fraction were slightly phosphorylated by the kinase, as shown in lane 1 of Fig. 2A, and no phosphorylation was observed when the incubation was performed in the absence of casein kinase II (Fig. 2A, lane 2). These results demonstrated that the recombinant rat MPPα was phosphorylated stoichiometrically by casein kinase II. No significant change in the MPPα activity was observed after the phosphorylation as far as the artificial substrate (whole histone phosphorylated by cyclic AMP dependent protein kinase) was used as the substrate (data not shown). No phosphorylation of rat MPPα was observed when casein kinase I was employed instead of casein kinase II (data not shown).

By one-dimensional SDS-PAGE, the recombinant MPPα protein isolated from *E. coli* cells harboring the expression plasmid of rat MPPα was resolved into two bands with different mobilities upon staining with Coomassie brilliant blue (Fig. 2C, lane 1). We concluded that the lower band (43 kDa) was a proteolytic degradation product of the upper band (46 kDa), because both bands were stained by antibody AB101 (Fig. 2B, lane 1). However, phosphorylation by casein kinase II occurred only on the upper band (Fig. 2C, lane 2). Interestingly, only this upper band, and not the lower band, was stained by antibody AB102 (Fig. 2B, lane 3). Since antibody AB102 was raised against an oligopeptide (20 mer) located close to the carboxyl terminal (from Arg335 to Lys354) of rat MPPα, these results suggested that the carboxyl terminal region containing the full length or part of the peptide (Arg335 to Lys354) was missing in the 43 kDa protein . Judging from the apparent difference in the molecular mass (3 kDa) between the upper and lower bands, it is reasonable to assume that the carboxyl terminal peptide which was absent in the lower band (43 kDa) contained Ser377 but not Ser256 (Fig. 3A). Casein kinase II phosphorylated only the upper band, making it likely that the site of phosphorylation was Ser377 (Figs. 3A and 3B). This site displays characteristics typical of a casein kinase II phosphorylation site, where a phosphorylatable serine residue is followed by an acidic amino acid (+4 when the serine is +1) on the carboxyl terminal side (Fig. 3B). Considering that the stoichiometry of the phosphorylation of rat MPPα by casein kinase II was 1.5 mol phosphate per mol rat MPPα and that the phosphorylation was exclusively on serine, it seemed possible that Ser375 was also phosphorylated by the kinase. In such a case, the phosphorylation on Ser375 would probably have occurred after phosphorylation of Ser377 to provide casein kinase II with the necessary carboxyl terminal acidic environment (Fig. 3B), as described for the phosphorylation of DARPP-32 by casein kinase II (19).

Phosphopeptide mapping - Two-dimensional phosphopeptide mapping was performed to determine whether or not casein kinase II phosphorylated *in vitro* the same residues metabolically

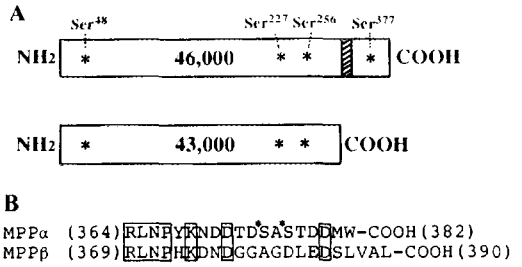

Fig. 3. Proposed position of phosphorylation sites of MPPα . *A.* Schematic representation of MPPα . Scheme of 46 kDa and 43 kDa proteins shown in the lane 1 of Fig. 2C is represented. The block with oblique lines indicates the region of MPPα against which antibody AB102 was raised. The positions of serine residue corresponding to the casein kinase II-consensus phosphorylation sequence are indicated by asterisks. *B.* Amino acid sequences of the carboxyl terminal regions deduced from the nucleotide sequences of rat MPPα (3) and rat MPPβ (5) cDNAs are shown. Identities are boxed and the putative phosphorylation sites are indicated by asterisks.

labeled in yeast cells. Tryptic phosphopeptides were prepared from both rat MPPα isolated from ^{32}P-labeled yeast cells and rat MPPα which was phosphorylated by casein kinase II *in vitro*, and the phosphopeptide maps for these two sets of tryptic peptides were compared (Figs. 4A and 4B). All of the spots observed on the map for *in vitro* phosphorylation (Fig. 4B) were also observed on the map for *in vivo* phosphorylation (Fig. 4A). These results demonstrated that casein kinase II hosphorylated the same peptide(s) as those phosphorylated *in vivo* and suggested that the phosphorylation sites were the same. The multiple spots on the phosphopeptide maps may have been due either to partial digestion or to the difference in phosphorylation state.

Casein kinase II is a cyclic nucleotide-independent Ca^{2+} -and calmodulin-insensitive serine/threonine-specific protein kinase (20, 21), which has been isolated from a variety of sources including mammals (15) and yeast (22). Many properties of yeast casein kinase II including approximate molecular mass (160 kDa), substrate specificity, inhibition by heparin and polyglutamic acid, stimulation by polyamines, and ability to use GTP in place of ATP as a phosphoryl donor are similar to those of mammalian casein kinase II (23, 24). These similarities

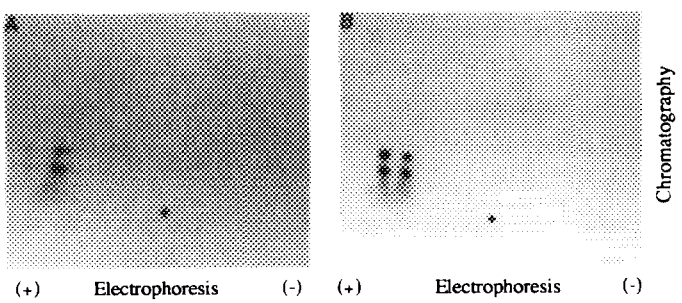

Fig. 4. Comparative phosphopeptide maps of rat MPPα. The rat MPPα phosphorylated *in vivo* (*panel A*) and *in vitro* (*panel B*), both of which had been isolated by immunoprecipitation, were digested by trypsin and then separated by electrophoresis at pH 8.9, followed by ascending chromatography in isobutyric acid buffer (isobutyric acid / n-butanol / pyridine / acetic acid / H_2O (65 : 2 : 5 : 3 : 29)). The positions of sample application are marked by +.

between the yeast and mammalian casein kinases II prompted us to conclude that yeast casein kinase II phosphorylates rat MPPα *in vivo*.

Recently, a cDNA clone encoding the full length of rat MPPβ has been isolated (5). According to the amino acid sequence deduced from the nucleotide sequence, there is no casein kinase II site in the carboxyl terminal domain of MPPβ (Fig. 3B). Although the physiological significance of the phosphorylation of MPPα has yet to be elucidated, this difference between MPPα and MPPβ may suggest that the two enzyme proteins may be under the control of separate systems and providing for the independent regulation of the isoforms.

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