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Isoform-Specific Phosphorylation of Fission Yeast Type 2C Protein Phosphatase

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Protein phosphatase 2C (PP2C) is one of the four major protein serine/threonine phosphatases of eukaryotes and is implicated in the regulation of various cellular functions. With the goal of elucidating the mechanism responsible for regulating PP2C functions, we investigated the significance of phosphorylation of fission yeast Ptc1, Ptc2, and Ptc3, the yeast orthologs of mammalian PP2C. Both Ptc2 and Ptc3 but not Ptc1 were phosphorylated stoichiometrically by casein kinase II on serine residues at their carboxy-terminal regions. Mutational analysis of Ptc2 and Ptc3 revealed that serine residues of the conserved sequence (Ser-X-Ser-X-X-Glu/Asp) of these proteins were the phosphorylation sites. Interestingly, the activities of Ptc2 and Ptc3 were decreased 25 ± 7.5% and increased 55 ± 3.7%, respectively, by phosphorylation. In addition, the same site(s) of Ptc2 was phosphorylated when the protein was expressed in fission yeast cells. These results suggest that phosphorylation of PP2C plays important physiological roles in fission yeast cells.

Protein phosphorylation plays a key role in the regulation of cellular functions, and protein phosphatases in combination with protein kinases control the phosphorylation levels of cellular proteins. Protein phosphatase 2C (PP2C) is one of four major protein serine/threonine phosphatases (PP1, PP2A, PP2B and PP2C) found in eukaryotic cells and implicated in various cellular functions (1). Genetic analysis of *Schizosaccharomyces pombe* revealed that it contained at least three genes encoding PP2C (Ptc1, Ptc2 and Ptc3) (2-4). *Saccharomyces cerevisiae* also has been found to contain five PP2C genes (PTC1/TPD1, PTC2, PTC3, YBR125c and YCR079w) (5, 6).

Molecular genetic studies of yeast cells have revealed that Ptc1 and Ptc3 of *S. pombe* as well as PTC1 and PTC3 of *S. cerevisiae* participate in the regulation of stress-activated protein kinase (SAPK) cascade (3, 5-8). The expression of Ptc1 but not that of Ptc2 or Ptc3 has been found to be enhanced by heat shock treatment of *S. pombe* cells (2). It was further clarified that the trancription factor Atf1, which is activated by SAPK (Spc1), participates in the stress-enhanced expression of Ptc1 (7). However, little is known about the regulation of the function of Ptc2 and Ptc3. Since we noticed that both Ptc2 and Ptc3 but not Ptc1 contained the sequence Ser-X-Ser-X-X-Glu/Asp which was similar to that of the phosphorylation sites of mammalian PP2Cα by casein kinase II (9, 10), we were interested in determining whether the activities of Ptc2 and Ptc3 were regulated by phosphorylation. Here, we demonstrate that both Ptc2 and Ptc3 are phosphorylated by casein kinase II and their activities are affected by the phosphorylation.

MATERIALS AND METHODS

Materials. Glutathione agarose beads, protein A agarose beads, partially hydrolyzed α-casein and whole histone were purchased from Sigma (St Louis, MO). [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Restriction enzymes and other modifying enzymes for DNA manipulation were from Takara (Kyoto, Japan). Anti-hemagglutinin (HA) antibody 12CA5 was purchased from Boehringer Mannheim (Mannheim, Germany). Anti-mouse IgG-alkaline phosphatase conjugate was from Promega (Madison, WI). Super Q toyopearl and Phenyl toyopearl were purchased from Toso (Tokyo, Japan). All other reagents were from Wako Pure Chemical (Osaka, Japan).

Strains and plasmids. *Escherichia coli* strain BL21 was used for the expression of GST-fusion proteins. *S. pombe* PR109 was used as the host strain for the overexpression of Ptc proteins. pGEX-4T was purchased from Pharmacia (Uppsala, Sweden). pREP1, pGEX-KG-ptc1$^+$, *S. pombe* ptc2$^+$ and ptc3$^+$ genomic DNAs (2, 4) have been kindly provided by Dr. P. Russell (The Scripps Research Institute, U.S.A.). Minimal medium MM was used for growing *S. pombe* PR109

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(11). Thiamine (2 mM) was added to the medium to derepress the nmt1 promoter of pREP1.

Construction of expression plasmids for glutathione S-transferase (GST)-Ptc isoforms. Plasmid pGEX-KG-ptc1$^+$ (2) was used for the expression of GST-Ptc1 protein.

The expression plasmid for GST-Ptc2 (pGEX-ptc2$^+$) was prepared as follows. Polymerase chain reaction (PCR) was performed to introduce *Bam*HI site into the N-terminal end of ptc2$^+$, and the PCR product was ligated between the *Bam*HI and *Sal*I sites of pGEX-4T. The 3′-portion of the ptc2$^+$ ORF (*Sal*I-*Not*I fragment) was then introduced between the *Sal*I and *Not*I sites of the plasmid. In order to prepare the expression plasmid for GST-Ptc3 (pGEX-ptc3$^+$), the two introns of the coding region of ptc3$^+$ were first removed by *in vitro* mutagenesis. A *Bam*HI site was introduced into the 3′-terminal of the 5′-portion of the ptc3$^+$ gene by PCR and the *Bam*HI-*Eco*RV fragment of the resultant product was ligated between the *Bam*HI and *Sma*I sites of pGEX-4T. This plasmid was termed pGEX-ptc3$^+$(5′). A *Not*I site was introduced into the 3′-end of the central region (*Sal*I-*Eco*RI fragment) of the ptc3$^+$ gene and ligated between the *Sal*I and *Not*I sites of pGEX-ptc3$^+$(5′). Finally, the 3′-portion of the ptc3$^+$ gene (*Eco*RI-*Not*I fragment) was ligated between the *Eco*RI and *Not*I sites of the plasmid.

GST-Ptc1, GST-Ptc2 and GST-Ptc3 were expressed in *E. coli* BL21 and purified using glutathione agarose beads and thrombin treatment (12). Since the Ptc proteins were rather susceptible to degradation by thrombin (which was used to release the Ptc portions from the GST-Ptc fusion proteins), thrombin and the degradation products were removed from the incubation mixture by column chromatography using Phenyl toyopearl (for Ptc1) or Super Q toyopearl (for Ptc2 and Ptc3) soon after digestion with thrombin.

Construction of expression plasmids for HA-Ptc1, HA-Ptc2, and HA-Ptc3 for use in yeast cells. In order to introduce a *Not*I site into the multicloning site of pREP1, two oligonucleotides, 5′-TAT GAC ACG GAT CCT GTG GTC GAC AGA GGC GGC CGC A-3′ and 5′-GAT CTG CGG CCG CCT CTG TCG ACC ACA GGA TCC GTG TCA-3′, were annealed and inserted between the *Nde*I and *Bam*HI sites of pREP1. This plasmid was termed pREP-*Not*I. PCR reaction was used to introduce HA-sequence and *Nde*I site into the N-terminal end of ORFs of ptc1$^+$, ptc2$^+$ and ptc3$^+$. After digestion with *Nde*I and *Bam*HI (ptc1$^+$) or *Nde*I and *Sal*I (ptc2$^+$ and ptc3$^+$), the PCR products were ligated between *Nde*I and *Bam*HI (ptc1$^+$) or *Nde*I and *Sal*I (ptc2$^+$ and ptc3$^+$) of pREP-*Not*I, resulting the plasmids pREP-ptc1$^+$(5′), pREP-ptc2$^+$(5′) and pREP-ptc3$^+$(5′), respectively. The 3′-portions of the ORFs of ptc1$^+$, ptc2$^+$ and ptc3$^+$ (*Bam*HI-*Not*I fragment of pGEX-KG-ptc1$^+$ or *Sal*I-*Not*I fragments of pGEX-ptc2$^+$ and ptc3$^+$) were ligated into pREP-ptc1$^+$(5′), pREP-ptc2$^+$(5′) and pREP-ptc3$^+$(5′), respectively. The final products were termed pREP-HA-ptc1$^+$, pREP-HA-ptc2$^+$ and pREP-HA-ptc3$^+$.

In vitro mutagenesis. In order to substitute serine residues with alanines in Ptc2 and Ptc3, *in vitro* mutagenesis was performed using the oligonucleotides, 5′-GGT GAT ACA TAC GAT GCC GAT GCA GAT GAT GAA ACC ATT GC-3′ (for ptc2$^+$-S375A/S377A) and 5′-GCC GAG GAG GAA AAT GCT GCA GCT GAA ACC GAT ATC GTA AAT TC-3′ (for ptc3$^+$-S389A/S390A/S391A). The expression plasmids of GST-ptc2$^+$-S375A/S377A and GST-ptc3$^+$-S389A/S390A/S391A for use in *E. coli* cells were prepared with the methods used in preparation of the expression plasmids of GST-ptc2$^+$ and GST-ptc3$^+$, respectively. The procedures for the preparation of the expression plasmids of HA-ptc2$^+$-S375A/S377A and HA-ptc3$^+$-S389A/S390A/S391A were the same as those for preparation of HA-ptc2$^+$ and HA-ptc3$^+$, respectively.

Phospholabeling of S. pombe cells. The yeast transformants were grown in 10 ml MM medium for 14 h at 30 °C and diluted with the same medium till the OD600 reched 0.1. After shaking for 4 h at 30 °C, the cells were harvested and washed with MM-P medium, which contained a lower concentration (2 mg/l) of K_2HPO_4 than MM. The cells were suspended in 10 ml MM-P medium containing 0.3 mCi [^{32}P]orthophosphate and incubated for 3 h at 30 °C. The cells were washed three times and broken with glass beads in 0.2 ml lysis buffer containing protein phosphatase inhibitors (50 mM NaF, 5 mM β-glycerophosphate and 1 μM OA). After centrifugation at 12,000 × g for 10 min, SDS was added to the supernatant to a final concentration of 0.5 % (w/v). The sample was diluted 10 times with lysis buffer and incubated with anti-HA antibody for 1 h at 25 °C. The immune complex was collected by adding protein A agarose beads. The precipitate was washed three times with lysis buffer and incubated at 100 °C for 2 min in SDS sample buffer.

Phosphorylation of Ptcs by casein kinase II. Casein kinase II was purified from rat liver nuclei using Sephadex G-200 and phosphocellulose P11 column chromatography as described by Takeda *et al.* (13). The recombinant mouse PP2Cα (14), *S. pombe* Ptc1, Ptc2 or Ptc3, was incubated with 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 0.5 mM ATP (10 μCi [γ-^{32}P]ATP), 0.1 mg/ml polylysine and purified casein kinase II (2 μg) in a total volume of 25 μl. The reaction mixture was incubated for 5 min at 30 °C and SDS-PAGE was performed following the termination of the reaction. The incorporated radioactivity was measured using a BAS2000 image-analyzer. When the effect of phosphorylation on catalytic activity was determined, [γ-^{32}P]ATP was removed from the reaction mixture. The reaction was stopped by the addition of an equal volume of 10 mM EDTA and 10 mg/ml BSA. Aliquots of the samples (2 μl) were removed and protein phosphatase activity was measured in the presence of 10 mM $MgCl_2$ and 1 μM OA.

Stress treatments. Heat shock, osmotic shock and oxidative stress treatments of *S. pombe* cells were performed as described previously (15).

Assay of protein phosphatase activity. [^{32}P]phosphocasein and [^{32}P]phosphohistone were prepared by incubating partially digested α-casein and whole histone, respectively, with the catalytic subunit of protein kinase A and [γ-^{32}P]ATP as described previously (16, 17). The catalytic subunit of protein kinase A was purified as described by Reimann and Beham (18). Protein phosphatase activity was assayed by measuring the release of [^{32}P]phosphate from the [^{32}P]phosphohistone or [^{32}P]phosphocasein, essentially as described previously (16).

RESULTS

Phosphorylation of recombinant Ptc2 and Ptc3 proteins by casein kinase II. Ptc1, Ptc2 and Ptc3 were expressed as GST-fusion proteins in *E. coli* cells. Their apparent molecular masses on SDS-PAGE after digestion with thrombin (Ptc1, 40 kDa; Ptc2, 42.5 kDa; Ptc3, 47.6 kDa) corresponded well to the calculated molecular masses of these proteins, although the migration of Ptc2 was somewhat slower than had been expected (Fig. 1A, lower panel) (2, 4). The purified recombinant Ptc1, Ptc2 and Ptc3 showed Mg^{2+}-dependent and OA-insensitive protein phosphatase activities (data not shown).

The recombinant Ptc1, Ptc2 and Ptc3 were incubated with purified rat casein kinase II and [γ-^{32}P]ATP. As shown in Fig. 1A, Ptc2 and Ptc3 were phosphorylated by casein kinase II, while no incorporation of phosphate into Ptc1 protein was observed, as had been expected. The stoichiometry of the phosphorylations for Ptc2 and Ptc3 were 1.48 and 1.27 mols phosphate per mol protein, respectively. Phosphoamino acid analysis revealed that the phosphorylation was almost exclusively on serine residues, although a trace of threonine phosphorylation was also observed (Fig. 1B).

In order to determine the phosphorylation sites for

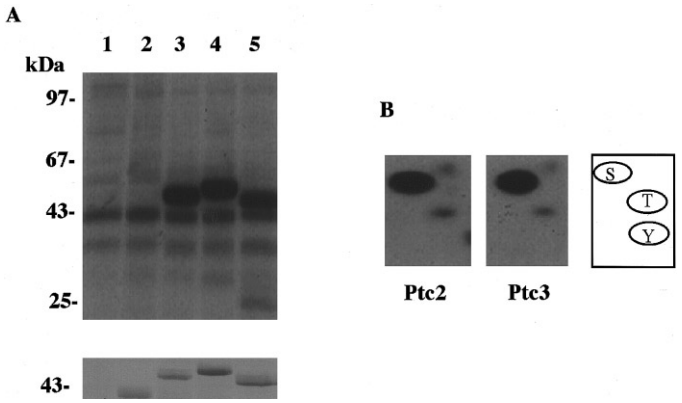

FIG. 1. Phosphorylation of recombinant Ptc proteins by casein kinase II. (A, upper panel) Recombinant Ptc1, Ptc2, Ptc3, and PP2Cα (21) (1.5 μg each) were incubated in the presence of [γ-^{32}P]ATP and casein kinase II as described under Materials and Methods. SDS-PAGE and autoradiography were performed after the phosphorylation reaction. (Lower panel) After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, no phosphatase; lane 2, Ptc1; lane 3, Ptc2; lane 4, Ptc3; lane 5, mouse PP2Cα. Size markers are shown on the left. (B) The results of phosphoamino acid analysis of the labeled Ptc2 and Ptc3 performed as described by Boyle *et al.* (22) are depicted.

casein kinase II, we then intended to prepare unphosphorylatable mutants of Ptc proteins by *in vitro* mutagenesis. Taking into account the fact that both Ptc2 and Ptc3 contained in their carboxy-terminal regions the sequence Ser-X-Ser-X-X-Asp/Glu which is similar to that of the sites of phosphorylation of PP2Cα, we aimed at the Ser-X-Ser sequences of Ptc2 and Ptc3. These serine residues were changed to alanines by *in vitro* mutagenesis as shown in Fig. 2A. Since another serine residue existed between the two serines in Ptc3, we changed all three serine residues to alanines. These mutations completely prevented the phosphorylation of Ptc2 and Ptc3 by casein kinase II as shown in Fig. 2B, demonstrating that these serine residues are the sites normally phosphorylated by casein kinase II.

Effect of in vitro phosphorylation on the catalytic activities of Ptc proteins. We wondered whether *in vitro* phosphorylation would affect the enzyme activities of Ptc2 and Ptc3, since no change in enzyme activity was observed when mammalian PP2Cα was phosphorylated by casein kinase II (9). In order to answer this question, we assayed the enzyme activities of Ptc1, Ptc2 and Ptc3 after incubation of these three proteins with casein kinase II and 0.5 mM ATP. As shown in Fig. 3, the phosphatase activity of Ptc2 against [^{32}P]phosphocasein was reduced by 25 $\pm$ 7.5 % after phosphorylation. In contrast, the phosphatase activity of Ptc3 increased by 55 $\pm$ 3.7 % after phosphorylation by casein kinase II. Incubation did not affect the phosphatase activity of Ptc1 protein. Similar results were obtained when [^{32}P]phosphohistone was used as the substrate (data not shown). The changes in the activities of Ptc2 and Ptc3 were not due to carryover of ATP from the phosphorylation reaction mixtures, because incubation with ATP in the absence of casein kinase II did not affect the activities of Ptc2 and Ptc3 (data not shown).

Phosphorylation of HA-Ptc2 expressed in S. pombe cells. In order to determine whether Ptc2 and Ptc3 are phosphorylated *in vivo*, we prepared expression constructs for HA-tagged Ptc1, Ptc2 and Ptc3 proteins. Since each ORF including the HA-tag sequence was introduced into the multicloning sites of vector pREP1 (19), expression could be derepressed by adding thia-

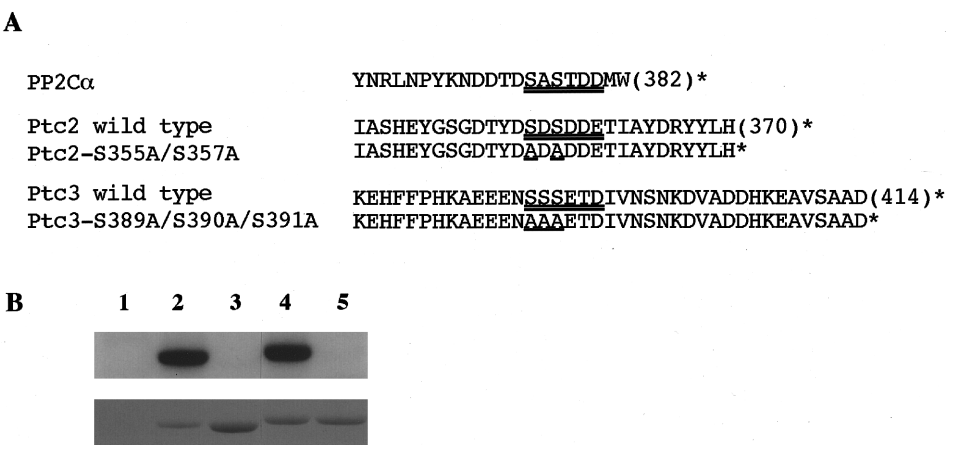

FIG. 2. Phosphorylation of mutant Ptc2 and Ptc3 proteins. (A) The Ser-X-Ser-X-X-Asp/Glu sequences found in the carboxy-terminal regions of PP2Cα, wild-type Ptc2, and Ptc3 are double underlined (4). The mutant amino acids are underlined. The numbers indicate the positions of the amino acid residues in PP2Cα, Ptc2, and Ptc3. (B, upper panel) Ptc2 (lane 2), Ptc2-S355A/S357A (lane 3), Ptc3 (lane 4), and Ptc3-S389A/S390A/S391A (lane 5) were incubated in the presence of [γ-^{32}P]ATP and casein kinase II. The incubation mixture for lane 1 contained no protein phosphatase. The results of autoradiography after SDS-PAGE are shown. (Lower panel) After electrophoresis, the gel was stained with Coomassie Brilliant Blue.

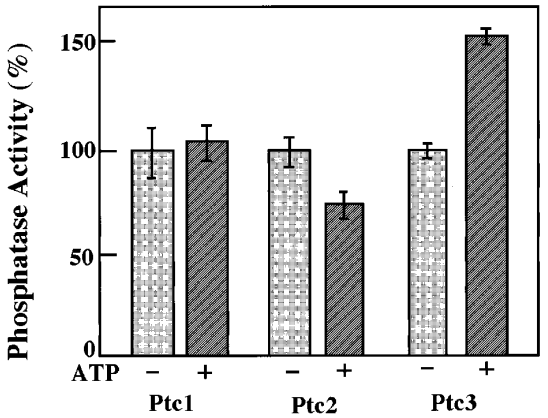

FIG. 3. Effect of phosphorylation by casein kinase II on the catalytic activities of Ptc proteins. Ptc1, Ptc2, and Ptc3 proteins (0.5 μg) were preincubated in the presence of ATP and casein kinase II, then their protein phosphatase activities were assayed. The relative phosphatase activities are shown. The activities of Ptc1, Ptc2, and Ptc3 without preincubation represent 100%. The values are means of three separate experiments ±S.D. of the means.

mine to the medium. In the cases of Ptc2 and Ptc3, the unphosphorylatable mutants were also expressed.

Using these transformants, we examined whether Ptc proteins were phosphorylated *in vivo*. After labeling the cellular proteins with [^{32}P]orthophosphate, the Ptc proteins were immunoprecipitated by the anti-HA antibody and analyzed by SDS-PAGE, followed by autoradiography. As shown in Fig. 4, Ptc2 was phosphorylated in yeast cells (lane 3). The phosphorylation site(s) was the same as for *in vitro* phosphorylation because mutant Ptc2 was not phosphorylated (lane 4). In contrast to Ptc2, Ptc3 was not phosphorylated in the yeast cells. Stress conditions such as heat shock, high osmolarity and oxidative stress did not cause enhanced phosphorylation of Ptc3 protein (data not shown).

DISCUSSION

The experiments involving phosphorylation of Ptc2 and Ptc3 and their mutants by casein kinase II demonstrated that both proteins were phosphorylated on the serine residues of the Ser-X-Ser-X-X-Asp/Glu sequences which are located in their carboxy-terminal regions, as was observed in PP2Cα (Figs. 1 and 2). The stoichiometry of the phosphorylation indicated that both serine residues in the sequence are phosphorylated. In the case of Ptc3, the extra serine residue existing between the usual two serines may also be phosphorylated, although it is not in the consensus sequence for phosphorylation by casein kinase II. The evidence that no phosphorylation was observed in the mutants of Ptc2 and Ptc3 may indicate that the phosphorylation of threonine, observed in the wild type Ptc2 and Ptc3 (Fig. 1B), was enhanced by the phosphorylation of the serine residues of the carboxy-terminal regions.

It appears that the consensus sequence (Ser/Thr-X-X-Asp/Glu) alone is not enough for phosphorylation because neither the Ser-Asn-Lys-Asp nor the Ser-Ala-Ala-Asp sequences downstream from the phosphorylation sites of Ptc3 was phosphorylated (Fig. 2B). Another feature of these phosphorylation sites, common to those of PP2Cα, Ptc2 and Ptc3, is that they are surrounded by acidic amino acids (Fig. 2A). This observation suggests that an acidic environment on both the carboxy-terminal and the amino-terminal sides is required for the phosphorylation of PP2Cs by casein kinase II. In this context, it is tempting to speculate that the sequence 439-Asp-Asp-Glu-Asp-Ser-Asp-Val-Thr-Asp-Glu-Glu from FEM-2 of *C. elegans* (20) may also be phosphorylated by casein kinase II on the threonine and possibly on the serine, too.

Unlike mouse PP2Cα, the phosphatase activities of Ptc2 and Ptc3 were affected by *in vitro* phosphorylation by casein kinase II (Fig. 3). Interestingly, phosphorylation of Ptc2 and Ptc3 produced opposing effects on their phosphatase activities. Similar results were obtained when [^{32}P]phosphohistone was used as the substrate. These observations suggest that in this case phosphorylation does not affect the substrate specificities of Ptc2 and Ptc3 but generally activates (Ptc3) or inactivates (Ptc2) these enzymes.

The *in vivo* phospholabeling of yeast transformants clearly demonstrated that Ptc2 proteins were phosphorylated in yeast cells. Phosphorylation did not occur when the mutant Ptc2 proteins were expressed, indicating that the phosphorylation site(s) is the same as that for *in vitro*

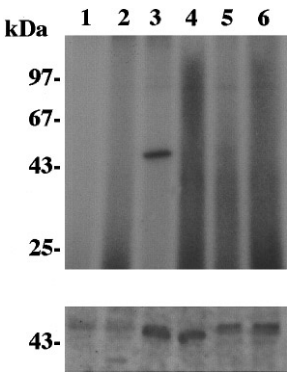

FIG. 4. Expression and phosphorylation of HA-tagged Ptc proteins in yeast cells. (Upper panel) Wild-type and mutant HA-Ptc proteins were expressed in *S. pombe* cells. The transformants were phospholabeled by adding [^{32}P]orthophosphate to the medium. The Ptc proteins were immunoprecipitated by the anti-HA antibody and subjected to SDS-PAGE. The autoradiography results are shown. Lane 1, pREP-1 (vector); lane 2, pREP-HA-ptc1$^+$; lane 3, pREP-HA-ptc2$^+$; lane 4, pREP-HA-ptc2$^+$-355A/357A; lane 5, pREP-HA-ptc3$^+$; lane 6, pREP-HA-ptc3$^+$-389A/390A/391A. (Lower panel) The results of the Western blot analysis of the cell extracts using the anti-HA antibody are shown.

phosphorylation by casein kinase II. Therefore, the regulation of Ptc2 activity by phosphorylation may well play an important physiological role *in vivo.*

In contrast to the case of Ptc2, no phosphorylation of Ptc3 proteins was demonstrated when they were expressed in yeast cells. Ptc3 protein may be phosphorylated only when greater activity is required. We speculated that such a situation might be caused by stress, since Shiozaki and Russell demonstrated that Ptc3 activity is required in the regulation of signal transduction processes which respond to stress (4). However, stress-treatment of the cells did not lead to the phosphorylation of this protein in our experiments. Therefore, condition which causes phosphorylation of Ptc 3 *in vivo* remains to be established.

In summary, we reported for the first time that the activity of fission yeast PP2C is regulated by phosphorylation *in vitro* and probably *in vivo,* too. It is likely that PP2C plays a key role as an in intracellular signal transducing factor in yeast cells, and elucidation of the upstream and downstream pathways of PP2C, especially those of Ptc2, is essential for verification of the physiological significance of the phosphorylation.

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