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Isoform specific phosphorylation of protein phosphatase 2C expressed in COS7 cells

Takayasu Kobayashi, Kazuyuki Kusuda, Motoko Ohnishi, Hong Wang, Shoko Ikeda, Masahito Hanada, Yuchio Yanagawa, Shinri Tamura*

Department of Biochemistry, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

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Abstract Of the six distinct isoforms of mouse protein phosphatase 2C (PP2C) (α, β-1, β-2, β-3, β-4 and β-5), PP2Cα was specifically phosphorylated on the serine residue(s) when expressed in COS7 cells. Analysis of phosphorylation sites using site-directed mutagenesis demonstrated that Ser-375 and/or Ser-377 were phosphorylated in vivo. These serine residues were the sites of phosphorylation by casein kinase II in vitro. Phosphorylation of PP2Cα was enhanced two-fold by the addition of okadaic acid to the culture medium, but addition of cyclosporin A had no such effect. These results suggest that the expressed PP2Cα is phosphorylated by a casein kinase II-like protein kinase and dephosphorylated by PP1 and/or PP2A in COS7 cells.

Key words: Protein phosphatase 2C; Phosphorylation; Site-directed mutagenesis; Okadaic acid

1. Introduction

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. The catalytic subunits of the major protein serine/threonine phosphatases found in eukaryotic cells have been divided into four classes, types 1, 2A, 2B and 2C (PP1, PP2A, PP2B and PP2C, respectively) [1]. To date the presence of five distinct PP2C genes (PP2Cα, PP2Cβ, PP2Cγ, Wip1 and FIN13) has been reported in mammalian cells [2–6]. In addition, mouse PP2Cβ has been found to have five distinct isoforms (β-1, β-2, β-3, β-4 and β-5) which are splicing variants originating from a single pre-mRNA [7–9]. The presence of three isoforms of human PP2Cα has also been reported [10].

Recently, information on the function of mammalian PP2C has accumulated. PP2Cα has been implicated in regulation of the activities of AMP-activated protein kinase and Ca^{2+}/calmodulin-dependent protein kinase II, and both Wip1 and FIN13 have been found to participate in regulation of cell growth [5,6,11,12]. In this context, we have demonstrated that expression of rat PP2Cα in *Saccharomyces cerevisiae* cells caused suppression of the DNA excision repair system [13]. In the same experimental system, we also found that the expressed PP2Cα was phosphorylated on serine residue(s) in the yeast cells [14]. The phosphorylation site(s) was predicted to be in the carboxy-terminal region of PP2Cα. However, demonstration of phosphorylation of PP2Cα in mammalian cells, determination of isoform specificity of the phosphorylation and identification of the phosphorylated serine residue(s) have yet to be accomplished.

In this study, we demonstrate that of the six distinct mouse PP2C isoforms (α, β-1, β-2, β-3, β-4 and β-5) PP2Cα was specifically phosphorylated on Ser-375 and/or Ser-377 in COS7 cells. We also provide evidence indicating that PP2Cα is dephosphorylated by PP1 and/or PP2A in the cells.

2. Materials and methods

2.1. Materials

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

2.2. Strains and plasmids

Escherichia coli strain BL21 was used for the expression of GST fusion proteins. Plasmids pEUK-C-1, pGEX-2T and pKF18k were purchased from Clontech Laboratories (Palo Alto, CA, USA), Pharmacia (Uppsala, Sweden) and Takara, respectively.

2.3. Construction of epitope tagged PP2C expression plasmids for COS7 cells

Expression constructs of HA epitope (YPYDVPDYA) tagged PP2Cα (pEUK-C-HA-PP2Cα) and of HA-PP2Cβ isoforms (pEUK-C-HA-PP2Cβ-1, -2, -3, -4 and -5) were prepared essentially as described previously [15,16].

2.4. In vitro mutagenesis

The *Eco*RI fragment (1.1 kbp) of mouse PP2Cα cDNA [17] was subcloned into pKF18k. The mutagenesis was performed by the oligonucleotide-directed dual amber method [18] using the following primers: 5′-TGA TTC TGC GGC AAC CGA TGA-3′ (for PP2Cα-S377A), 5′-CGA CAC TGA TGC TGC GTC AAC-3′ (for PP2Cα-S375A) and 5′-CGA CAC TGA TGC TGC GGC AAC CGA TGA-3′ (for PP2Cα-S375A/S377A). The *Eco*RI fragments of pEUK-C-HA-PP2Cα and pGEX-PP2Cα were replaced by the mutated *Eco*RI fragments to make expression plasmids for COS7 and *E. coli* cells, respectively. The final products were termed pEUK-C-HA-PP2Cα-375A (for PP2Cα-S375A), pEUK-C-HA-PP2Cα-377A (for PP2Cα-S377A), pEUK-C-HA-PP2Cα-375A/377A (for PP2Cα-S375A/S377A), pGEX-375A (for PP2Cα-S375A), pGEX-377A (for PP2Cα-S377A) and pGEX-375A/377A (for PP2Cα-S375A/S377A).

2.5. Expression of recombinant PP2Cα and its mutants in E. coli cells

The GST-PP2C isoforms were expressed in *E. coli* BL21 and purified using glutathione agarose beads and thrombin treatment [17].

2.6. DNA sequencing

The DNA fragments generated by PCR or in vitro mutagenesis were examined using an ABI DNA sequencer, model 373A.

*Corresponding author. Fax: (81) (22) 717-8476
E-mail: tamura@idac.tohoku.ac.jp

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2.7. Cell culture and transfection

The COS7 cells were grown in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin. The HA tagged PP2C cDNAs were transiently expressed in COS7 cells using the DEAE-dextran transfection procedure [17]. The cells were washed in ice-cold PBS 48 h after transfection and fixed with 10% (w/v) trichloroacetic acid for 10 min at 4°C. The fixed cells were collected using a cell scraper and centrifuged at 12 000×*g* for 1 min. Sodium dodecyl sulfate (SDS) sample buffer was added to the precipitate and homogenized by sonication.

2.8. Phospholabeling of COS7 cells

The COS7 cells were washed with 150 mM NaCl and incubated with phosphate-free DMEM containing [^{32}P]orthophosphate (60 μCi/ml) 48 h after transfection. The dish was incubated for 5 h at 37°C and the phospholabeled cells were harvested after washing with ice-cold PBS. The cells were lysed using TNE buffer (10 mM Tris-HCl, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA and 1 mM PMSF) containing 50 mM NaF and 0.1 μM okadaic acid (OA). After centrifugation, the anti-HA antibody 12CA5 was added to the supernatant and the PP2C-antibody complex was recovered using protein A agarose beads and subjected to SDS-PAGE analysis.

2.9. Phosphorylation of PP2C by casein kinase II

Casein kinase II was purified from rat liver nuclei as described by Takeda et al. [19]. The recombinant mouse PP2Cα and its point mutants were 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 incorporated radioactivity was measured after SDS-PAGE using a BAS2000 image analyzer.

2.10. Western blot analysis

Western blotting was performed by the standard procedure, using the anti-HA antibody 12CA5 and alkaline phosphatase conjugated anti-mouse IgG. The blots were developed by chemiluminescence using CSPD as substrate. In order to demonstrate the amount of HA-PP2C protein precipitated by the anti-HA antibody, biotin conjugated anti-HA antibody and streptavidin-peroxidase conjugate was used instead of alkaline phosphatase conjugated anti-mouse IgG, because HA-PP2Cα protein migrated almost simultaneously with the heavy chain of the immunoglobulin on SDS-PAGE, so that the HA-PP2Cα band was hidden by the band of the heavy chain of the IgG when anti-mouse IgG antibody was used as the secondary antibody.

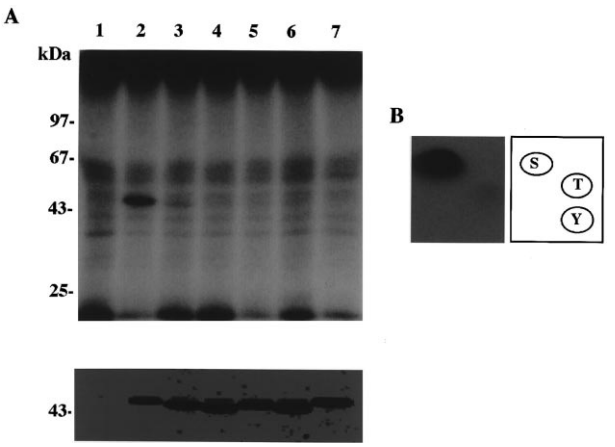

Fig. 1. Phosporylation of HA tagged PP2C isoforms in COS7 cells. A: Upper panel: COS7 cells transfected with pEUK-C-1 (lane 1), pEUK-C-HA-PP2Cα (lane 2), pEUK-C-HA-PP2Cβ-1 (lane 3), pEUK-C-HA-PP2Cβ-2 (lane 4), pEUK-C-HA-PP2Cβ-3 (lane 5), pEUK-C-PP2Cβ-4 (lane 6) and pEUK-C-PP2Cβ-5 (lane 7) were phospholabeled by adding [^{32}P]orthophosphate to the culture medium. The cell extracts were immunoprecipitated by the anti-HA antibody and the immunoprecipitates were subjected to SDS-PAGE. The results of autoradiography are shown. Lower panel: Western blot analysis was performed using the anti-HA antibody. B: The protein band for phospho-HA-PP2Cα (as shown in A) was excised from the gel and subjected to phosphoamino acid analysis.

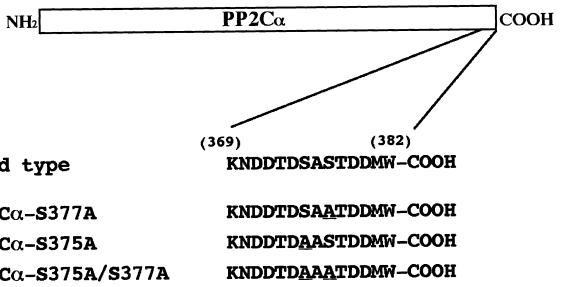

Fig. 2. Diagram of in vitro mutagenesis of PP2Cα. The mutant amino acids are underlined. The numbers indicate the positions of the amino acid residues in mouse PP2Cα.

2.11. Phosphoamino acid analysis

^{32}P-labeled PP2Cα was subjected to phosphoamino acid analysis as described by Boyle et al. [20].

2.12. Assay of protein phosphatase activity

[^{32}P]Phosphohistone and [^{32}P]phosphocasein were prepared by incubating whole histone and partially digested α-casein, respectively, with the catalytic subunit of protein kinase A and [γ-^{32}P]ATP as described previously [21,22]. The catalytic subunit of protein kinase A was purified as described by Reimann and Beham [23]. 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 [21]. One unit of activity was defined as the amount of enzyme which catalyzes the release of 1 nmol phosphate per min.

3. Results

3.1. Phosphorylation of HA-PP2C isoforms expressed in COS7 cells

Cell extracts from COS7 cells transfected with expression plasmids for six distinct mouse PP2C isoforms (α, β-1, β-2, β-3, β-4 and β-5) were analyzed by Western blotting using the anti-HA monoclonal antibody (12CA5). Protein bands of 46–48 kDa were observed only when the cells were transfected with the expression plasmids (Fig. 1A, lower panel). In order to determine whether the HA tagged PP2C isoforms exhibited the protein phosphatase activities characteristic of PP2C, the activities of the immunoprecipitates were measured using [^{32}P]phosphohistone phosphorylated by protein kinase A as the substrate. All the immunoprecipitates from the cell extracts that had been transfected with the expression vectors showed substantial levels of Mg^{2+} dependent and OA insensitive histone phosphatase activity (data not shown). However, no protein phosphatase activity was detected in immunoprecipitates from cells transfected only with the control vector plasmid.

In order to test whether PP2C isoforms were phosphorylated when expressed in mammalian cells, we performed phospholabeling of COS7 cells expressing HA tagged PP2C proteins. COS7 cells transfected with the expression plasmids were metabolically labeled with [^{32}P]orthophosphate and lysed using a buffer containing Triton X-100. The PP2C isoforms were immunoprecipitated with the anti-HA antibody and analyzed by SDS-PAGE, and the radioactivities in the gel were visualized by autoradiography. As shown in Fig.

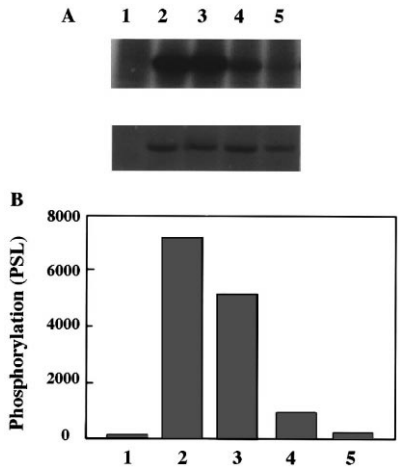

Fig. 3. Phosphorylation of mutant PP2Cα proteins by casein kinase II. A: Upper panel: Wild-type and mutant PP2Cα were expressed in *E. coli* cells, purified by glutathione agarose beads and thrombin treatment, and phosphorylated by casein kinase II. The results of autoradiography after SDS-PAGE are shown. Lower panel: The gel was stained with Coomassie brilliant blue after SDS-PAGE. B: The radioactivities incorporated into the PP2Cα proteins (A) were determined using a BAS 2000 image analyzer. Lane 1, no PP2C protein; lane 2, PP2Cα; lane 3, PP2Cα-S377A; lane 4, PP2Cα-S375A; lane 5, PP2Cα-S375A/S377A.

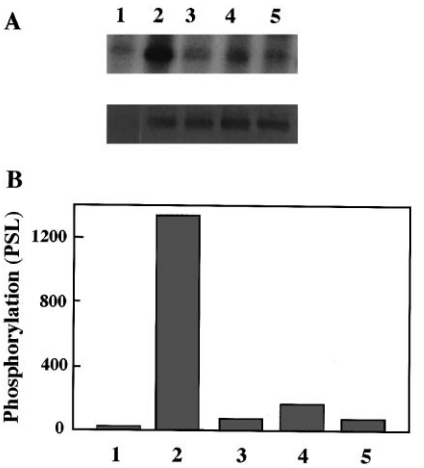

Fig. 4. Phosphorylation of mutant HA tagged PP2Cα proteins in COS7 cells. A: Upper panel: Wild-type and mutant HA-PP2Cα proteins were transiently expressed in COS7 cells and metabolically labeled by [^{32}P]orthophosphate. The labeled proteins were immunoprecipitated by the anti-HA antibody and subjected to SDS-PAGE. The radioactivities incorporated into the PP2Cα proteins were visualized by autoradiography. Lower panel: Western blot analysis was performed using the anti-HA antibody. B: The radioactivities incorporated into the PP2Cα proteins were determined using a BAS 2000 image analyzer. Lane 1, pEUK-C-1; lane 2, pEUK-C-HA-PP2Cα; lane 3, pEUK-C-HA-PP2Cα-377A; lane 4, pEUK-C-HA-PP2Cα-375A; lane 5, pEUK-C-HA-PP2Cα-375A/377A.

1A, PP2Cα, but no other isoform, was phosphorylated in the cells. Phosphoamino acid analysis using high voltage thin layer chromatography demonstrated that phosphorylation of PP2Cα occurred almost exclusively on serine residues (Fig. 1B). A trace amount of threonine phosphorylation was observed, but no tyrosine phosphorylation could be detected.

3.2. *Phosphorylation of mutant PP2Cα in vitro and in vivo*

The putative phosphorylation sites of rat PP2Cα suggested by our previous study were in the carboxy-terminal region, which contained two serine residues (Ser-375 and Ser-377) [14]. The sequence of the carboxy-terminus including Ser-375 and Ser-377 was conserved in mouse PP2Cα (Fig. 2) [17]. In order to determine the actual phosphorylation sites, we introduced a mutation from serine to alanine at these two serine sites by site-directed mutagenesis (Fig. 2). Fragments of mutant PP2Cα were introduced into either the pGEX-2T vector (for GST fusion) or pEUK-C vector (for COS7 expression). All three mutants were expressed as effectively as wild-type PP2Cα in both *E. coli* (Fig. 3A, lower panel) and COS7 cells (Fig. 4A, lower panel).

The recombinant PP2Cα mutant proteins were incubated with casein kinase II and [γ-^{32}P]ATP (Fig. 3A,B). The wild-type mouse PP2Cα was phosphorylated by casein kinase II, confirming our previous observation with rat PP2Cα [14]. The stoichiometry of the phosphorylation was 1.2 mol phosphate per mol protein. The Ser-375→Ala-375 mutation (PP2Cα-S375A) caused an 87% decrease in the phosphorylation of PP2Cα (lane 4). However, the decrease in phosphorylation produced by the Ser-377→Ala-377 mutation (PP2Cα-S377A) was only 28% compared with the control (lane 3). The mutant in which both serine residues were changed to alanine (PP2Cα-S375A/S377A) was not phosphorylated (lane 5). These results indicate that the major phosphorylation site for casein kinase II is Ser-375. Ser-377 was also phosphorylated by casein kinase II but to a much smaller extent.

Fig. 4 shows the results of the in vivo experiments. As in the in vitro experiments, the double mutant HA-PP2Cα-S375A/S377A was not phosphorylated when expressed in COS7 cells (lane 5), indicating that at least one of these two serines was phosphorylated in COS7 cells. However, in contrast to the in vitro experiments, essentially no phosphoryla-

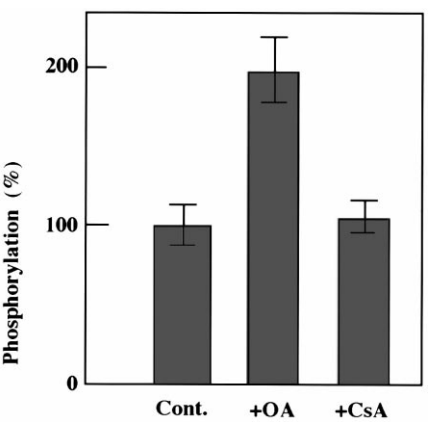

Fig. 5. Effects of phosphatase inhibitors on the phosphorylation of HA tagged PP2Cα in COS7 cells. COS7 cells harboring the expression vector for HA-PP2Cα were labeled by [^{32}P]inorganic phosphate. OA (1 μM) or CsA (1 μg/ml) was added to the phospholabeling medium 1 h before harvesting. The cells were then collected and lysed. The HA-PP2Cα proteins were immunoprecipitated from the cell extracts by the anti-HA antibody. After SDS-PAGE of the immunoprecipitates, the radioactivities incorporated into the PP2Cα proteins were determined using a BAS 2000 image analyzer. The results represent the means of three separate experiments ± S.D.

tion was observed in HA-PP2Cα-S377A (lane 3) while HA-PP2Cα-S375A (lane 4) was slightly phosphorylated in COS7 cells.

3.3. Effect of phosphatase inhibitors on the phosphorylation of PP2Cα

We next determined whether PP2Cα phosphorylated in COS7 cells was dephosphorylated by endogenous PPs. To this end we tested the effects of protein phosphatase inhibitors on the phosphorylation of PP2Cα expressed in COS7 cells. Following incubation of the COS7 cells with [^{32}P]orthophosphate, 1 μM OA or 1 μg/ml cyclosporin A (CsA) was added to the labeling mixture and incubated for another 1 h. The labeled PP2Cα was immunoprecipitated with the anti-HA antibody and the level of phosphorylation was determined using a BAS 2000 image analyzer after SDS-PAGE (Fig. 5). The phosphorylation of PP2Cα was increased two-fold by the addition of OA. However, the addition of CsA did not affect phosphorylation. These results indicated that endogenous PPs responsible for the dephosphorylation of PP2Cα in COS7 cells were PP1 and/or PP2A.

4. Discussion

In our previous study, we have demonstrated that rat PP2Cα expressed in *S. cerevisiae* cells was phosphorylated on serine residues of its carboxy-terminal region [14]. However, whether the phosphorylation was a physiological phenomenon was not known since the experiments were performed using a heterologous system, namely expression of mammalian PP2C in yeast cells. In addition, the putative phosphorylation sites of rat PP2Cα were not conserved in the *S. cerevisiae* PP2Cs. In this study, we demonstrated for the first time the isoform specific phosphorylation of mammalian PP2C in mammalian cells, indicating that the phosphorylation of PP2Cα is a physiologically occurring event.

Since the double mutant of PP2Cα (S375A/S377A) was not phosphorylated both in vivo and in vitro (Figs. 3 and 4), it was suggested that PP2Cα was phosphorylated by casein kinase II or casein kinase II-like protein kinase in COS7 cells. However, a marked difference was observed in the phosphorylation level of Ser-375 between in vivo and in vitro experiments when phosphorylation of a single mutant (S377A) was examined. The reason for this discrepancy is not known. One unique feature of the in vivo condition is that endogenous PP(s) may affect the phosphorylation of recombinant PP2Cα. It was indicated that PP1 and/or PP2A are responsible for the dephosphorylation of PP2Cα in COS7 cell. Therefore, it seems reasonable to speculate that the difference in the phosphorylation level between the in vivo and in vitro experiments was caused by a higher susceptibility of phosphoserine-375 in the single mutant S377A to endogenous PP1 and/or PP2A than that in wild-type PP2Cα. This may have been caused by the absence of phosphoserine-377 in the mutant molecule. Alternatively, phosphorylation of Ser-377 may be a prerequisite for the phosphorylation of Ser-375 in vivo.

Peruski et al. isolated the human cDNAs encoding three isoforms of PP2Cα (α1, α2 and α3) by RT-PCR from HL60 monocytes [10]. They reported that PP2Cα1 was the dominant isoform, comprising 75% of the PP2Cα cDNAs generated, and that the amounts of PP2Cα2 and PP2Cα3 were relatively low. The deduced amino acid sequences indicated that the three isoforms were similar but a significant difference was observed at their carboxy-terminal regions. Of these three human PP2Cα isoforms PP2Cα1 was most homologous to rat and mouse PP2Cα. Interestingly, the two serines corresponding to Ser-375 and Ser-377 of mouse PP2Cα are conserved in human PP2Cα1 and PP2Cα3 but not in PP2Cα2, in which the amino-terminal side Ser is changed to Tyr. Therefore, human PP2Cα1 and PP2Cα3 but not PP2Cα2 may also be phosphorylated in vivo.

In our previous study, we demonstrated that the phosphorylation by casein kinase II did not affect the enzyme activity of PP2Cα as far as artificial substrates (phosphohistone or phosphocasein) were used in the enzyme assay. However, it still seems possible that the activity against physiological substrate(s) is regulated by the phosphorylation. Alternatively, the phosphorylation may affect the binding of PP2Cα to other proteins in the cells or it may regulate the subcellular localization of PP2Cα. Studies to verify these hypotheses are under way now.

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References

[1] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[2] Tamura, S., Lynch, K.R., Larner, J., Fox, J., Yasui, A., Kikuchi, K., Suzuki, Y. and Tsuiki, S. (1989) Proc. Natl. Acad. Sci. USA 86, 1796–1800.
[3] Wenk, J., Trompeter, H.I., Pettrich, K.G., Cohen, P.T.W., Campbell, D.G. and Mieskes, G. (1992) FEBS Lett. 297, 135–138.
[4] Travis, S.M. and Welsh, M.J. (1997) FEBS Lett. 412, 415–419.
[5] Guthridge, M.A., Bellosta, P., Tavoloni, N. and Basilico, C. (1997) Mol. Cell. Biol. 17, 5485–5498.
[6] Fiscella, M., Zhang, H., Fan, S., Sakaguchi, K., Shen, S., Mercer, W.E., Vande Woude, G.F., O'Connor, P.M. and Appella, E. (1997) Proc. Natl. Acad. Sci. USA 94, 6048–6053.
[7] Terasawa, T., Kobayashi, T., Murakami, T., Ohnishi, M., Kato, S., Tanaka, O., Kondo, H., Yamamoto, H., Takeuchi, T. and Tamura, S. (1993) Arch. Biochem. Biophys. 307, 342–349.
[8] Hou, E.W., Kawai, Y., Miyasaka, H. and Li, S.S.-L. (1994) Biochem. Mol. Biol. Int. 32, 773–780.
[9] Kato, S., Terasawa, T., Kobayashi, T., Ohnishi, M., Sasahara, Y., Kusuda, K., Yanagawa, Y., Hiraga, A., Matsui, Y. and Tamura, S. (1995) Arch. Biochem. Biophys. 318, 387–393.
[10] Peruski, L.F., Wadzinski, B.E. and Johnson, G.L. (1993) Adv. Protein Phosphatases 7, 9–30.
[11] Moore, F., Weekes, J. and Hardie, D.G. (1991) Eur. J. Biochem. 199, 691–697.
[12] Fukunaga, K., Kobayashi, T., Tamura, S. and Miyamoto, E. (1993) J. Biol. Chem. 268, 133–137.
[13] Kobayashi, T., Yasui, A., Ohnishi, M., Kato, S., Sasahara, Y., Kusuda, K., Chida, N., Yanagawa, Y., Hiraga, A. and Tamura, S. (1995) Mutat. Res. 362, 213–217.
[14] Kobayashi, T., Kanno, S., Terasawa, T., Murakami, T., Ohnishi, M., Ohtsuki, K., Hiraga, A. and Tamua, S. (1993) Biochem. Biophys. Res. Commun. 195, 484–489.
[15] Kusuda, K., Kobayashi, T., Ikeda, S., Ohnishi, M., Chida, N., Yanagawa, Y., Shineha, R., Nishihira, T., Satomi, S., Hiraga, A. and Tamura, S. (1998) Biochem. J. (in press).
[16] Kobayashi, T., Kusuda, K., Ohnishi, M., Chida, N. and Tamura, S. (1998) Methods Mol. Biol. (in press).
[17] Kato, S., Kobayashi, T., Terasawa, T., Ohnishi, M., Sasahara, Y., Kanamaru, R. and Tamura, S. (1994) Gene 145, 311–312.

[18] Hashimoto-Gotoh, T., Mizuno, T., Ogasahara, Y. and Nakagawa, M. (1995) Gene 152, 271–275.
[19] Takeda, M., Matsumura, S. and Nakaya, Y. (1974) J. Biochem. 75, 743–751.
[20] Boyle, W., van der Geer, P. and Hunter, T. (1991) Method Enzymol. 201, 110–149.
[21] Tamura, S., Kikuchi, K., Hiraga, A., Kikuchi, H., Hosokawa, M. and Tsuiki, S. (1978) Biochim. Biophys. Acta 524, 349–356.
[22] McGowan, C.H. and Cohen, P. (1988) Methods Enzymol. 159, 416–425.
[23] Reimann, E.M. and Beham, R.A. (1983) Methods Enzymol. 99, 51–55.