Phosphorylation of Protein Phosphatase 2Cζ by c-Jun NH_2-Terminal Kinase at Ser^{92} Attenuates Its Phosphatase Activity†

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ABSTRACT: The protein phosphatase 2C (PP2C) family represents one of the four major protein Ser/Thr phosphatase activities in mammalian cells and contains at least 13 distinct gene products. Although PP2C family members regulate a variety of cellular functions, mechanisms of regulation of their activities are largely unknown. Here, we show that PP2Cζ, a PP2C family member that is enriched in testicular germ cells, is phosphorylated by c-Jun NH_2-terminal kinase (JNK) but not by p38 in vitro. Mass spectrometry and mutational analyses demonstrated that phosphorylation occurs at Ser^{92}, Thr^{202}, and Thr^{205} of PP2Cζ. Phosphorylation of these Ser and Thr residues of PP2Cζ ectopically expressed in 293 cells was enhanced by osmotic stress and was attenuated by a JNK inhibitor but not by p38 or MEK inhibitors. Phosphorylation of PP2Cζ by TAK1-activated JNK repressed its phosphatase activity in cells, and alanine mutation at Ser^{92} but not at Thr^{202} or Thr^{205} suppressed this inhibition. Taken together, these results suggest that specific phosphorylation of PP2Cζ at Ser^{92} by stress-activated JNK attenuates its phosphatase activity in cells.

Stress-activated protein kinases (SAPKs), a subfamily of the mitogen-activated protein kinase (MAPK) superfamily, relay signals from various extracellular stimuli, including environmental stresses and inflammatory cytokines (*1*). In mammalian cells, MAPK kinase kinases (MKKKs), including transforming growth factor β-activated kinase 1 (TAK1) and apoptosis signal-regulating kinase 1 (ASK1), phosphorylate and activate MAPK kinases (MKKs) (*2, 3*). MKKs then phosphorylate two distinct classes of SAPKs, the c-Jun N-terminal kinases (JNK) and the p38 MAPK, at conserved tyrosine and threonine residues in their catalytic domains (*1, 4*). Finally, the activated SAPKs phosphorylate their substrates at serine and/or threonine residues within a Ser/Pro (SP) or Thr/Pro (TP) motif.

PP2C[1] (also termed PPM) is one of four major protein Ser/Thr phosphatase families (PP1, PP2A, PP2B, and PP2C) found in mammalian cells. At least 13 distinct PP2C gene products (PP2Cα, PP2Cβ, PP2Cγ/FIN13, PP2Cδ/ILKAP, PP2Cε, PP2Cζ, PP2Cη, PP2Cκ, Wip1, CaMKPase/hFEM2/POPX2, CaMKP-N/POPX1, NERPP-2C, and SCOP/PHLPP) have been identified (*5–10*), and they exhibit Mg^{2+}- and/or Mn^{2+}-dependent protein phosphatase activity and are insensitive to inhibition by okadaic acid. Structurally, all PP2C family members contain six conserved motifs that form a β-sandwich structure to hold two divalent cations in the catalytic site (*11*). Mammalian PP2C family members are classified into two subgroups according to differences in the primary structure of conserved motif 1 (*12*). Subgroup 2 consists of PP2Cζ, PP2Cη, and NERPP-2C (*13*), while subgroup 1 includes all other PP2C members.

Four members of the PP2C family, PP2Cα, PP2Cβ, PP2Cε, and Wip1, have been implicated in negative regulation of SAPK signaling pathways (*14–19*). We have shown that PP2Cβ-1 selectively suppresses the stress-induced activation of p38 and JNK through direct association and dephosphorylation of TAK1 (*14, 15*). Takekawa et al. demonstrated that PP2Cα-2 dephosphorylates and inactivates MKK4, MKK6, and p38 both in vivo and in vitro (*16*). They also found that Wip1 selectively inhibits p38 activation and suppresses the subsequent activation of p53 (*17*). Recently, we have reported that ectopic expression of PP2Cε in mammalian cells represses the activity of TAK1 and ASK1 (*18, 19*). In quiescent cells, PP2Cε associates with and dephosphorylates TAK1 and ASK1 to maintain these kinases in an inactive state. However, this association was transiently suppressed in response to treatment of the cells with IL-1 and H_2O_2, thereby inducing the activation of these MKKKs.

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[1] Abbreviations: PP2C, protein phosphatase 2C; TAK1, transforming growth factor β-activated kinase 1; ASK1, apoptosis regulating kinase 1; HBS, HEPES-buffered saline; MBP, maltose binding protein; IR, ischemia reperfusion.

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Expression of three PP2C family members, Wip1, PP2Cδ/ILKAP, and PP2Cα, is induced by extracellular stimuli. Wip1 is induced by ionizing radiation in a p53-dependent manner, and PP2Cδ/ILKAP mRNA is induced in response to heat shock, ethanol treatment, and UV irradiation (*20, 21*). Recently, the level of expression of PP2Cα was shown to be increased by TNF in skeletal muscle, resulting in dephosphorylation of AMPK (*22*). However, little is known regarding the regulation of PP2C activity in cells.

PP2Cζ, a member of PP2C subgroup 2, is expressed specifically in testicular germ cells but not in Leidig or Sertoli cells (*23*). PP2Cζ interacts with the SUMO-conjugating enzyme, UBC9, and this association is enhanced by coexpression of the small ubiquitin-related modifier, SUMO-1 (*23*). However, the physiological function and regulation of PP2Cζ in cells remain to be elucidated.

By sequence analysis, we found that PP2Cζ has a unique region containing multiple SP/TP sites, potential phosphoacceptor sites for SAPKs, between conserved motifs 2 and 3. Therefore, in this study, we have tested the possibility that the multiple SP/TP sites in PP2Cζ might be targets of JNK and/or p38 in the stress-activated signaling pathway. We provide evidence that specific phosphorylation of PP2Cζ at an SP site by stress-activated JNK attenuates its phosphatase activity in cells.

EXPERIMENTAL PROCEDURES

Materials. Modifying enzymes used for DNA manipulation, amylase resin, and λ-phosphatase were obtained from New England Biolabs (Beverly, MA). Lipofectamine 2000 and precast 4 to 12% SDS−polyacrylamide electrophoresis gels were purchased from Invitrogen (Carlsbad, CA). Glutathione Sepharose-4B, polyvinylidene difluoride (PVDF) membrane, ECL kits, and [γ-^{32}P]ATP were obtained from GE Healthcare (Buckinghamshire, U.K.). Anti-phospho Thr-Pro, anti-phospho-JNK, anti-phospho-p38, and anti-phospho-ERK antibodies and HRP-labeled secondary antibody were obtained from Cell Signaling (Davers, MA). S-Protein agarose beads, U0126, SB203580, zwittergent 3-16, and cellulose plates for thin layer chromatography were obtained from Merck (Darmstadt, Germany). SP600125 was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Microcystin-LR was obtained from Alexis Biochemicals (Lausen, Switzerland). A Ser/Thr phosphatase assay kit was purchased from Promega (Madison, WI). All other reagents were purchased from Wako Pure Chemical (Osaka, Japan).

Generation of Antibodies Recognizing PP2Cζ. A phospho-specific antibody recognizing PP2Cζ phosphorylated at Ser^{92} (termed antibody pS92, α-pS92) was raised in rabbits against peptide CRAVQpSPPDTG. The antibody was affinity purified on NSH-Sepharose covalently coupled to the phosphorylated peptide and passed through a column of NSH-Sepharose coupled to the nonphosphorylated peptide. An antibody recognizing PP2Cζ was raised in rabbits against a mixture of two peptides, CGTSSQRSKSPDLPNA and CAQETPKSSREKPGNQV (corresponding to residues 17−31 and 38−53 of mouse PP2Cζ, respectively).

Expression of Proteins. Constructs encoding MBP (maltose binding protein)−PP2Cζ, His−JNK, His−p38, GST−MKK4, GST−MKK6, and GST−MKK7 proteins were transformed into the *Escherichia coli* BL21(DE3) strain harboring the pLysS plasmid. Transformants were grown at 25 °C (MBP−PP2Cζ) or 30 °C (all other proteins) for 3 h in the presence of 1 mM isopropyl β-D-galactoside (IPTG), and the expressed proteins were purified on either maltose resin (MBP−PP2Cζ), Ni-NTA resin (His−JNK and His−p38), or glutathione Sepharose (GST−MKK4, GST−MKK6, and GST−MKK7). GST−TAK1 protein was coexpressed with HA-TAB1 in HEK293 cells, and the active TAK1 was purified with glutathione Sepharose. To obtain active JNK and p38, His−JNK or His−p38 protein bound to Ni-NTA resin was incubated with GST−MKK4, GST−MKK7, and active GST−TAK1 (for activation of JNK) or GST−MKK6 and active GST−TAK1 (for activation of p38) at 30 °C for 1 h in the presence of 100 μM ATP. After the upstream kinases had been washed away, activated His−JNK or His−p38 protein was eluted with 250 mM imidazole. The specific activities of the activated JNK and p38 toward GST−c-jun (1−79) and GST−ATF2 (1−107) protein were 70 and 290 units/mg of protein, respectively.

Phosphopeptide Mapping of MBP−PP2Cζ Protein Phosphorylated by JNK. MBP−PP2Cζ protein (2 μg) was incubated with active JNK in a buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (1000 cpm/pmol) in a total volume of 20 μL for 45 min at 30 °C. The reactions were then terminated by adding 1% (w/v) SDS and 10 mM dithiothreitol followed by heating at 95 °C for 3 min. After the mixture had cooled, 4-vinylpyridine was added to a concentration of 1% (v/v) to alkylate cysteine residues. The samples were subjected to electrophoresis on precast 4 to 12% SDS−polyacrylamide gels and analyzed using a BAS2000 imaging analyzer (Fuji Film). The band corresponding to the phosphorylated MBP−PP2Cζ protein was excised, and the gel piece was washed with 1 mL each of a 1:1 mixture of H_2O and acetonitrile, 0.1 M ammonium bicarbonate, a 1:1 mixture of 0.2 M ammonium bicarbonate and acetonitrile, and acetonitrile. The gel piece was dried and incubated with cleavage buffer [50 mM ammonium bicarbonate (pH 8.3) and 0.05% (w/v) zwittergent 3-16] containing 10 ng/mL modified trypsin for 16 h at 30 °C. The supernatant, containing ~80% of the radioactivity, was chromatographed on a Vydac 218TP54 C_{18} column equilibrated with 0.1% (v/v) trifluoroacetic acid in water. The column was developed with a liner acetonitrile gradient at a flow rate of 0.8 mL/min, and 0.4 mL fractions were collected. The phosphopeptides were analyzed by MALDI-TOF mass spectrometry (Voyager Biospectrometry, ABI) using 10 mg/mL α-cyanocinnamic acid as a matrix. Phosphoamino acid analysis of ^{32}P-labeled peptides was performed as described previously (*24*).

Cell Culture and Immunoblotting. HEK293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum. Cells transfected with the indicated expression plasmids were washed twice with HEPES-buffered saline (HBS), containing 20 mM HEPES (pH 7.5) and 150 mM NaCl, and lysed with ice-cold lysis buffer, containing 20 mM Tris-HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 0.1% (v/v) 2-mercaptoethanol, and a cocktail of protease inhibitors. The samples were subjected to 10% (w/v) SDS−PAGE and transferred onto PVDF membranes. The membranes were

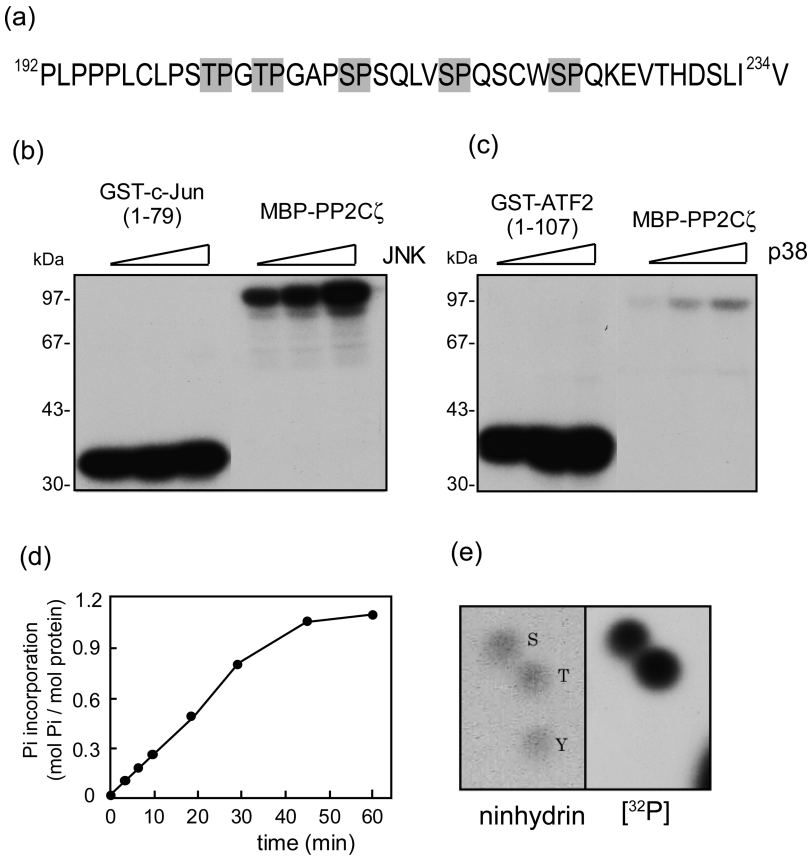

FIGURE 1: Phosphorylation of PP2Cζ by JNK. (a) Amino acid sequences of PP2Cζ surrounding several SP/TP motifs. (b) Two micrograms of GST−c-Jun (1−79) or MBP−PP2Cζ protein was incubated with increasing amounts of JNK (0.06, 0.18, and 0.54 unit/mL) in a buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (1000 cpm/pmol) in a total volume of 20 μL for 10 min at 30 °C. Incorporation of phosphate into the substrate protein was assessed following the electrophoresis of samples via 10% SDS−PAGE and autoradiography of the gel. GST−c-Jun (1−79), the physiological substrate of JNK, was used as a positive control. (c) Two micrograms of GST−ATF2 (1−107) or MBP−PP2Cζ protein was incubated with p38 (0.06, 0.18, and 0.54 unit/mL) and analyzed as described for panel b. GST−ATF (1−107) protein, the physiological substrate of p38, was used as a positive control. (d) MBP−PP2Cζ protein (2 μg) was phosphorylated with 0.18 unit/mL JNK at 30 °C in the presence of 100 μM [γ-^{32}P]ATP. After incubation for the indicated times, the reactions were terminated and the incorporation of phosphate into PP2Cζ was quantitated. (e) Two-dimensional autoradiography of MBP−PP2Cζ protein that had been phosphorylated by JNK. The left panel depicts ninhydrin staining, which shows the position of phosphoamino acids (S, phospho-Ser; T, phospho-Thr; and Y, phospho-Tyr).

incubated with a primary antibody for 1 h at 25 °C and with a HRP-conjugated secondary antibody for 1 h at 25 °C and were developed by chemiluminescence using an ECL kit.

Protein Phosphatase Assays. Cells transfected with an expression plasmid for S-epitope-tagged PP2Cζ were washed with HBS and lysed with ice-cold lysis buffer. Cell lysate containing 200 μg of protein was incubated with 5 μL of S-protein agarose beads for 0.5 h at 4 °C on a shaking platform, and the beads were then washed twice with lysis buffer and twice with HBS containing 0.1 mg/mL BSA. S-PP2Cζ was purified to near homogeneity as judged by silver staining of the purified protein (see the Supporting Information). Reaction mixtures for protein phosphatase assays (50 μL), containing the washed S-pull down sample, 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 0.2 mM EGTA, 0.02% (v/v) 2-mercaptoethanol, 0.1 mg/mL BSA, and 0.1 mM substrate (RRApTVA) or 2 mg/mL α-casein, were incubated for 10 min (phosphopeptide) or 1 h (α-casein) at 30 °C, with continuous agitation to keep the beads in suspension. The reaction was stopped by adding 50 μL of a molybdate/malachite green dye mixture (Promega), and the absorbance at 600 nm was determined. One unit of enzyme activity is defined as phosphatase activity that releases 1 nmol of phosphate in 1 min at 30 °C. The kinetic parameters, V_{max} (maximum velocity) and $S_{0.5}$ (substrate concentration at half-saturation), were obtained using KaleidaGraph (Synergy Software, Reading, PA).

RESULTS

Phosphorylation of PP2Cζ by JNK in Vitro. PP2Cζ has a unique region containing multiple SP/TP sites, potential phosphoacceptor sites for SAPKs, between conserved motifs 2 and 3 (Figure 1a). We therefore examined whether PP2Cζ could be phosphorylated by JNK and/or p38 in vitro. Mouse PP2Cζ was expressed in *E. coli* cells as a MBP fusion protein, purified on amylose resin, and used as a substrate for in vitro kinase assays. MBP−PP2Cζ protein was readily phosphorylated by JNK, reaching a stoichiometry of 1 mol of phosphate/mol of protein after incubation for 45 min (Figure 1b,d). Phosphoamino acid analysis of the phosphorylated protein demonstrated that JNK phosphorylated PP2Cζ at both Ser and Thr residues (Figure 1e). In contrast, MBP−PP2Cζ protein was poorly phosphorylated by p38 (Figure 1c). These results suggest that PP2Cζ is rather selectively phosphorylated by JNK in vitro.

Mapping of the JNK Phosphorylation Sites within PP2Cζ. MBP−PP2Cζ protein that had been phosphorylated by JNK

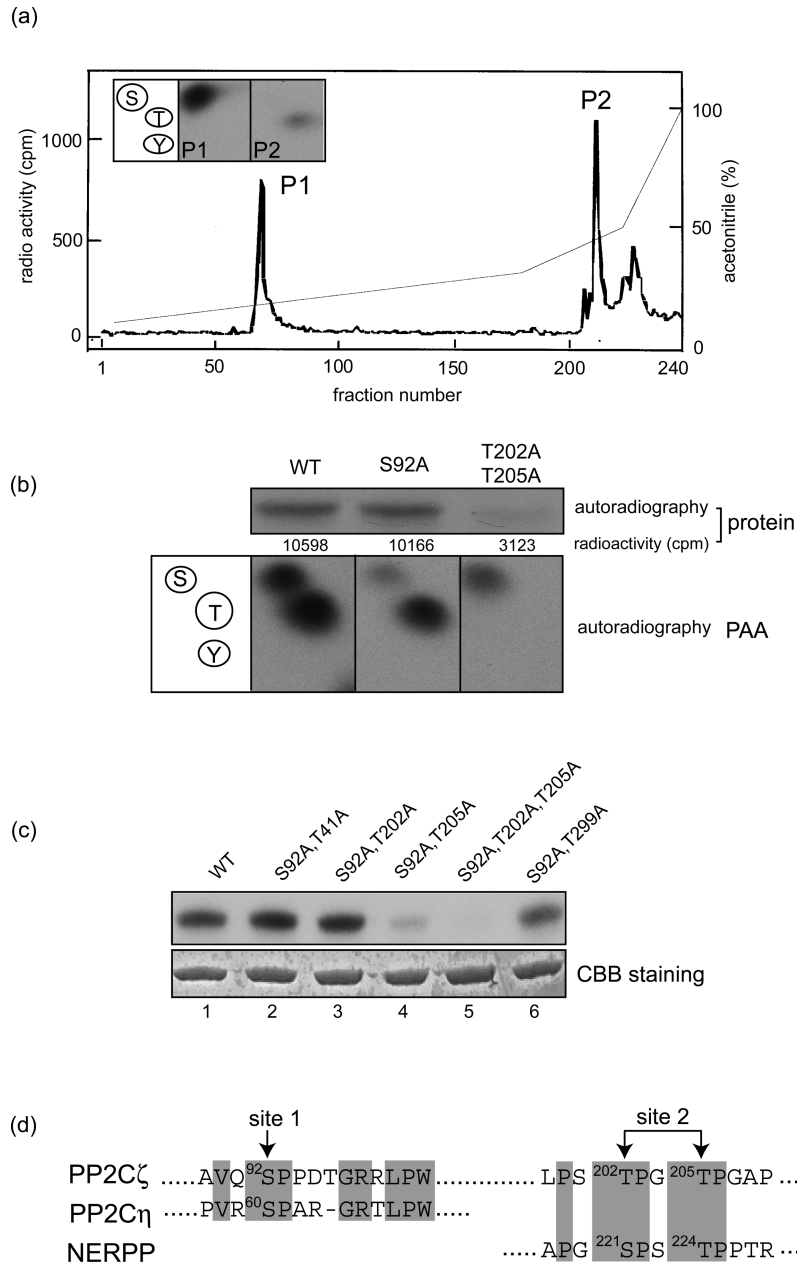

FIGURE 2: Mapping of the JNK phosphorylation sites within PP2Cζ. (a) Chromatography of MBP−PP2Cζ protein that had been phosphorylated by JNK in vitro. The inset shows phosphoamino acid analysis of the major ^{32}P-labeled peptides (P1 and P2). (b) Two micrograms of MBP−PP2Cζ, MBP−PP2Cζ[S92A], or MBP−PP2Cζ[T202A/T205A] protein was phosphorylated by 0.18 unit/mL JNK as described in the legend of Figure 1a and autoradiographed following electrophoresis via 10% SDS−PAGE (top panel). Incorporation of phosphate into PP2Cζ was assessed by Cherenkov counting. Phosphoamino acid analysis (PAA) was performed following the extraction of PP2Cζ and its mutants from the gel (bottom panel). (c) MBP fusion proteins with the wild type and indicated PP2Cζ mutants were incubated with JNK (0.18 unit/mL) for 10 min in the presence of 100 μM [γ-^{32}P]ATP, and the incorporation of phosphate into PP2Cζ was assessed with a BAS-2000 imaging analyzer (top panel). Coomassie brilliant blue staining of proteins is shown in the bottom panel. (d) Alignment of the phosphorylation sites of PP2Cζ and putative phosphorylation sites of PP2Cη and NERPP by MAPK.

in vitro was electrophoresed, excised from the gel, and digested with trypsin. C_{18} column chromatography showed two major tryptic phosphopeptides, termed P1 and P2, that eluted at 17 and 47% acetonitrile, respectively (Figure 2a). Phosphoamino acid analysis of these peptides showed that P1 contained phosphoserine, whereas P2 contained phosphothreonine (Figure 2a, insets). The molecular mass of P1 (1107.62 Da), determined by MALDI-TOF mass spectrometry, was identical to that expected for a tryptic phosphopeptide comprising residues 89−98 that is phosphorylated at Ser^{92} (theoretical mass of 1027.6537 Da for AVQSPTDTGR plus 79.9663 Da for phosphate). Consistent with these results, replacement of Ser^{92} with Ala resulted in a substantial reduction in the amount of phosphoserine phosphorylation by JNK (Figure 2b, bottom panel). Interestingly, however, the overall phosphorylation level was not affected (Figure 2b, top panel). Despite multiple trials, the molecular mass of peptide P2 could not be determined by mass spectrometry. We therefore generated PP2Cζ mutants in which Ser^{92} and one of four threonine residues followed by proline (Thr^{41}, Thr^{202}, Thr^{205}, and Thr^{299}) were replaced with alanine. PP2Cζ[S92A/T41A], PP2Cζ[S92A/T202A], and PP2Cζ[S92A/T299A] were phosphorylated by JNK to an extent similar to that of wild-type PP2Cζ (Figure 2c, lanes 2, 3, and 6); in

contrast, PP2Cζ[S92A/T205A] was poorly phosphorylated (Figure 2c, lane 4), suggesting that PP2Cζ was phosphorylated by JNK at Thr^{205} in vitro. Phosphorylation of PP2Cζ[S92A/T202A/T205A] was completely abolished, suggesting that Thr^{202} was weakly phosphorylated by JNK (Figure 2c, lane 5; see also Figure 2b). We refer to Ser^{92} as site 1 and Thr^{202} and Thr^{205} as site 2 (Figure 2d). All of these sites are located within typical JNK phosphorylation motifs (Hyd-X-Thr/Ser-Pro or Pro-X-Thr/Ser-Pro, where Hyd represents any hydrophobic amino acid).

PP2Cζ Is Phosphorylated by JNK in Cells. To detect phosphorylation of site 1, we generated a phosphospecific antibody against a phosphopeptide containing the sequence surrounding phosphorylated Ser^{92} (α-pS92). The specificity of this antibody was established by confirming that it strongly recognized wild-type PP2Cζ but not the PP2Cζ[S92A] mutant, following their phosphorylation in vitro by JNK (Figure 3a). To detect phosphorylation of site 2 (Thr$^{202/205}$), we used a commercially available antibody specific for phospho-TP (α-pTP). The specificity of this antibody was established by confirming that it recognized wild-type PP2Cζ but not the PP2Cζ[T202A/T205A] mutant after phosphorylation by JNK in vitro (Figure 3b).

S-Epitope-tagged PP2Cζ was ectopically expressed in HEK293 cells, which were then treated with sorbitol, an inducer of osmotic shock and activator of JNK. S-PP2Cζ was purified and immunoblotted with the α-pS92 (for site 1) and α-pTP (for site 2) antibodies described above, as well as with an antibody recognizing PP2Cζ.

The level of phosphorylation of endogenous JNK, p38, and ERK increased in a time-dependent manner following sorbitol treatment (Figure 3c, fourth, fifth, and bottom panels). In unstimulated cells, a very low level of phosphorylation at either site of PP2Cζ was observed, but the level of phosphorylation of both sites 1 and 2 increased in response to sorbitol, reaching a plateau at 30 min and remaining elevated for 3 h (Figure 3d). Similar results were obtained when the cells were treated with anisomycin (5 μg/mL) or irradiated with UV (20 J/m^2) (results not shown).

SP60125, a specific inhibitor of JNK, substantially reduced the level of sorbitol-induced phosphorylation of PP2Cζ at both sites 1 and 2 (Figure 3d, lanes 5 and 6, and Figure 3e). In contrast, the phosphorylation of these sites was not prevented by U0126, a MEK inhibitor (Figure 3d, lanes 7 and 8, and Figure 3e), and was enhanced by the p38 inhibitor SB203580 (Figure 3d, lanes 9 and 10, and Figure 3e). These results suggest that the phosphorylation of these sites was mediated by JNK, but not by ERK or p38 in cells.

Phosphorylation of Ser^{92} Attenuates PP2Cζ Phosphatase Activity. We next examined whether the phosphorylation of PP2Cζ affected its catalytic activity. To this end, phosphorylation of PP2Cζ was induced by coexpression of S-PP2Cζ, TAK1, and TAB1, a TAK1 activating protein, in HeLa cells; this resulted in more extensive phosphorylation of both PP2Cζ sites in comparison to the amount of phosphorylation with osmotic shock (Figure 4a, right panel, and results not shown). S-PP2Cζ was affinity-purified, and its phosphatase activity was determined using a specific phosphopeptide (RRApTVA) or α-casein as the substrate. The phosphatase activity of PP2Cζ coexpressed with TAK1 and TAB1 toward both phosphopeptide and α-casein was much lower than that of PP2Cζ expressed alone (Figure 4a, left panel, lanes 1 and 2, and Figure 4b, left panel).

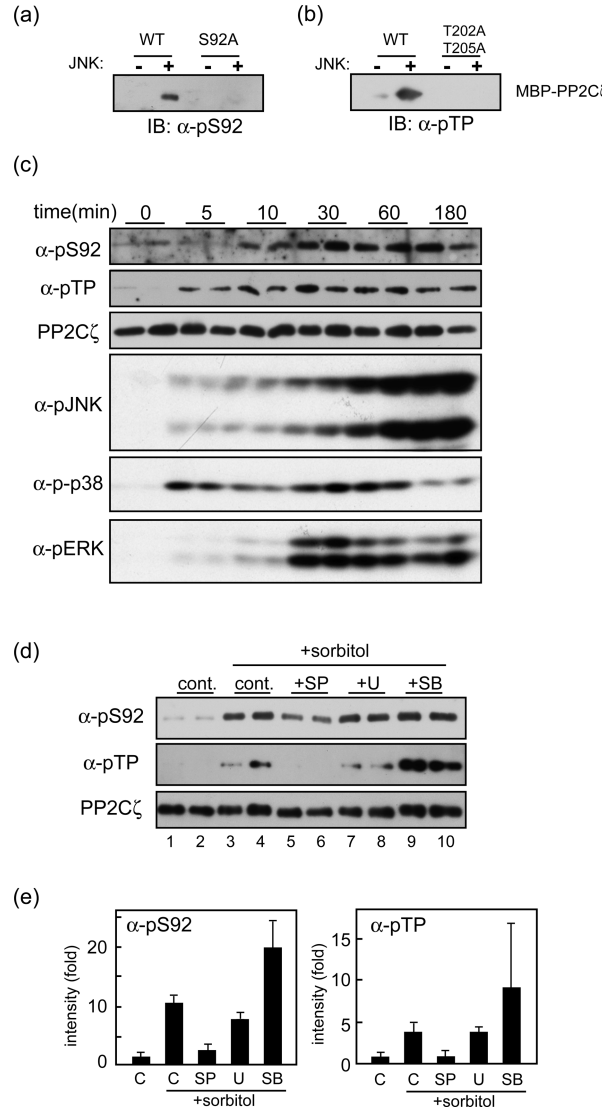

FIGURE 3: JNK phosphorylates PP2Cζ in cells. (a) MBP−PP2Cζ and MBP−PP2Cζ[S92A] proteins (25 ng) were incubated with 100 μM ATP in the absence or presence of JNK. The proteins were then immunoblotted with α-pS92 antibody. (b) Like panel a, except that the T202A/T205A mutant was used instead of the S92A mutant. The proteins were then immunoblotted with α-pTP antibody. (c) HEK293 cells were transfected with a plasmid expressing S-PP2Cζ and treated with 0.4 M sorbitol for the indicated times. Isolated S-PP2Cζ was immunoblotted with α-pS92, α-pTP, and anti-PP2Cζ antibodies. The lysates were also immunoblotted with phospho-specific JNK, phospho-specific p38, and phospho-specific ERK antibodies. (d) HEK293 cells transfected with the expression plasmid for S-PP2Cζ were pretreated for 1 h with either DMSO (control), 20 μM SP600125, 10 μM U0126, or 10 μM SB203580 prior to stimulation with 0.4 M sorbitol for 30 min. S-PP2Cζ was isolated and immunoblotted with α-pS92, α-pTP, and anti-PP2Cζ antibodies. (e) Graphic presentation of the effect of inhibitors on phosphorylation of PP2Cζ shown in panel d. The band intensities for each sample from three independent experiments performed in duplicate were quantified and normalized to PP2Cζ protein level, and the means ± the standard error are depicted.

To examine whether phosphorylation at site 1 (Ser^{92}) and/or site 2 (Thr$^{202/205}$) was involved in the attenuation of phosphatase activity, we carried out phosphatase assays using the S92A and T202A/T205A mutants. While expression of TAK1 and TAB1 inactivated S-PP2Cζ[T202A/T205A], it

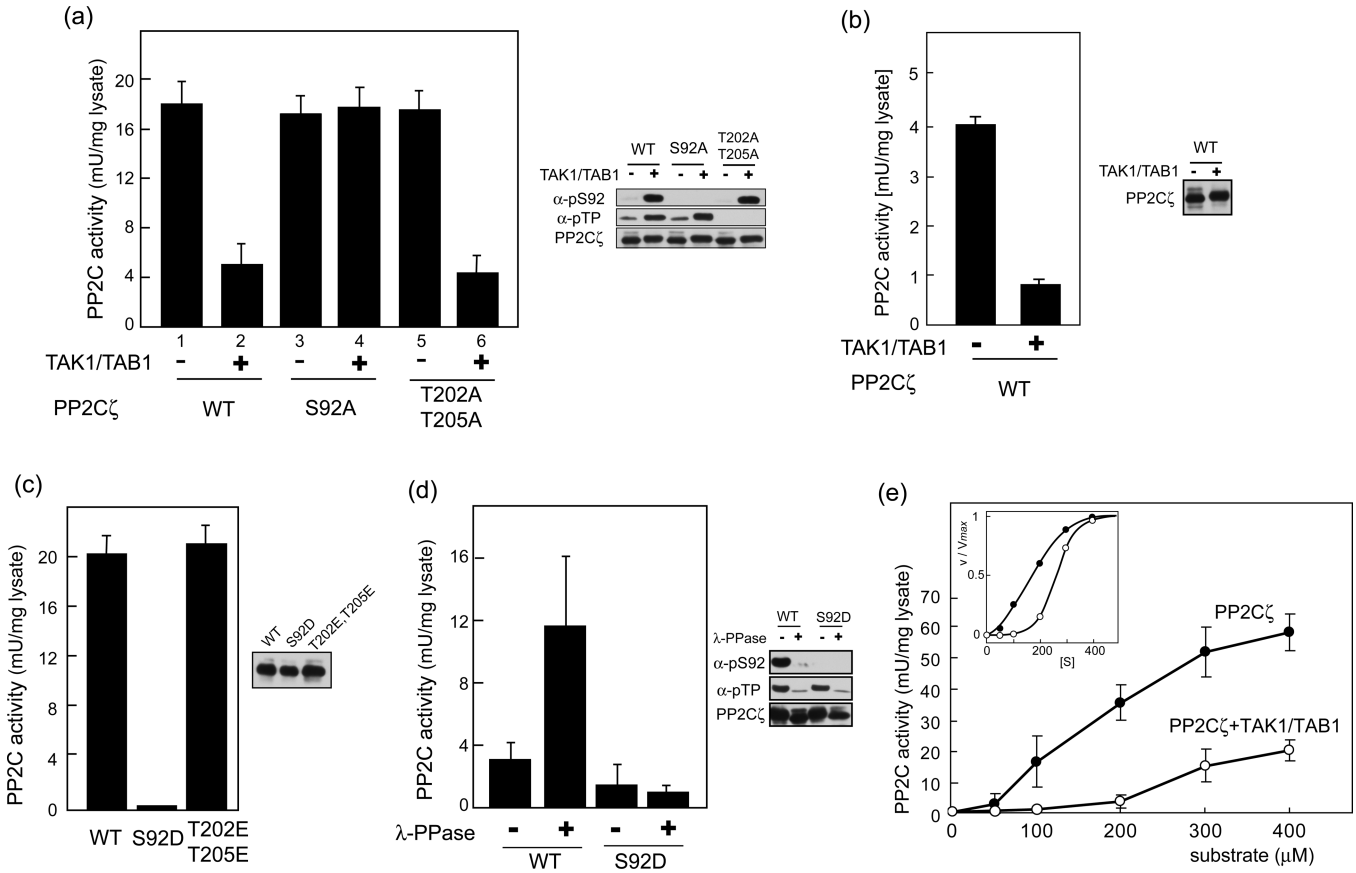

FIGURE 4: Phosphorylation of Ser[92] attenuates the phosphatase activity of PP2Cζ. (a) In the left panel, HeLa cells were transfected with a plasmid expressing S-PP2Cζ together with or without expression plasmids for GST−TAK1 and HA−TAB1 proteins. S-PP2Cζ was isolated by S-pull down, and its phosphatase activity was measured using the RRApTVA peptide as the substrate. In the right panel, the isolated proteins were also immunoblotted with α-pS92, α-pTP, or anti-PP2Cζ antibodies. (b) Like panel a, except that the phosphatase activity was measured using α-casein as a substrate. (c) In the left panel, HeLa cells were transfected with expression plasmids for wild-type S-PP2Cζ, PP2Cζ[S92D], or PP2Cζ[T202E/T205E], and phosphatase activity was measured as described for panel a. In the right panel, the expression levels of PP2Cζ proteins are shown. (d) In the left panel, HeLa cells were cotransfected with expression plasmids for GST−TAK1, HA−TAB1, and S-PP2Cζ or S-PP2Cζ[S92D]. S-PP2Cζ and S-PP2Cζ[S92D] were isolated from the cell extracts by S-pull down and incubated with λ-phosphatase, and their phosphatase activity was measured as described for panel a. In the right panel, the levels of phosphorylation of S-PP2Cζ and S-PP2Cζ[S92D] were assessed by immunoblotting with α-pS92 and α-pTP antibodies. (e) The indicated concentrations of the RRApTVA peptide were dephosphorylated by S-PP2Cζ (●) or S-PP2Cζ phosphorylated at Ser[92] (○), prepared as described for panel a, at 30 °C for 5 min. Each point shows the mean ± the standard error of three independent experiments performed in triplicate. The inset shows the experimental data were calculated using KaleidaGraph, and the curves that best fit the experimental data are presented. The reaction rate (v) as a fraction of V_{max} is graphed as a function of substrate concentration, [S].

had no effect on the phosphatase activity of S-PP2Cζ[S92A] (Figure 4a, lanes 3−6). Furthermore, PP2Cζ harboring an aspartic acid substitution at Ser[92] (S92D), which mimics phosphorylation, completely lost its phosphatase activity, while PP2Cζ[T202E/T205E] exhibited normal activity (Figure 4c, left panel). These results strongly suggest that phosphorylation of site 1 (Ser[92]) is involved in suppression of PP2Cζ catalytic activity. Inhibition of S-PP2Cζ phosphatase activity resulting from coexpression of TAK1 and TAB1 was reversed by incubation with λ-phosphatase, whereas that of S-PP2Cζ[S92D] was not affected after incubation with λ-phosphatase, suggesting that phosphorylation of site 1 (Ser[92]) is indeed involved in the inhibition of phosphatase activity (Figure 4d, left panel).

Finally, we carried out a kinetic analysis to probe the mechanism of inhibition by phosphorylation at Ser[92]. Interestingly, PP2Cζ expressed in HeLa cells had kinetics that did not follow the rectangular hyperbola of the Michaelis−Menten equation (Figure 4e). Instead, the kinetic curve for PP2Cζ was sigmoidal, irrespective of the presence or absence of coexpressed TAK1 and TAB1. The V_{max} and $S_{0.5}$ values calculated for PP2Cζ expressed alone were 60.4 milliunits/mg of lysate and 175 μM, respectively. In contrast, coexpression of TAK1 and TAB1 caused a 67% decrease in V_{max} (20.2 milliunits/mg of lysate) and a 47% increase in $S_{0.5}$ (258 μM) for PP2Cζ, suggesting that the conformational change induced by phosphorylation of PP2Cζ affects both of these kinetic parameters and inactivates PP2Cζ.

DISCUSSION

In this study, we have provided evidence that PP2Cζ is phosphorylated rapidly by JNK in vitro and have established that Ser[92] and Thr[205] are the major phosphorylation sites, although Thr[202] is also weakly phosphorylated. Incorporation of phosphate into PP2Cζ (1 mol of phosphate/mol of protein) could be underestimated because a specific inhibitor for PP2C could not be utilized to prevent autodephosphorylation. While mutation at site 2 (Thr[202/205]) resulted in a reduction in the level of phosphorylation by JNK, the S92A mutation did not affect the overall phosphorylation of PP2Cζ, despite a substantial reduction in the level of Ser phosphorylation

(Figure 2b,c). These results suggest that mutation of Ser^{92} to Ala may lead to conformational change in which JNK is easily phosphorylated at site 2 ($Thr^{202/205}$) in vitro. However, the significance of this observation is unclear because the S92A mutation did not affect the phosphorylation of site 2 ($Thr^{202/205}$) in vivo (Figure 4a, right panel).

We have also shown that PP2Cζ is phosphorylated at these sites in vivo in response to stimuli that activate MAPKs. The findings that sorbitol-induced phosphorylation of these sites was prevented by the JNK inhibitor, SP600125, but not the MEK inhibitor, U0126, and was enhanced by the p38 inhibitor, SB203580, indicate that phosphorylation is catalyzed by JNK but not by p38 or ERK in these cells. Enhancement of the phosphorylation by SB203580 may be due to the activation of JNK by suppression of the feedback inhibition of the upstream kinase TAK1 by p38 as previously reported by Cheung et al. (*25*). The level of phosphorylation of PP2Cζ in response to sorbitol reached a plateau at 30 min, and the high level of phosphorylation was maintained for up to 3 h; on the other hand, the level of phosphorylation of JNK increased continuously during this period, suggesting that the relatively small increase in JNK activity is sufficient for full phosphorylation of endogenous PP2Cζ in the cells.

Although expression of some PP2C family members is regulated by extracellular stimuli (*20–22*), little is known about the short-term regulation of the activity of PP2C family members. In this study, we show that specific phosphorylation of PP2Cζ at Ser^{92} by JNK attenuates its phosphatase activity in cells. To the best of our knowledge, this provides the first evidence of posttranslational regulation of a PP2C family member.

Enzyme kinetic analysis of PP2Cζ expressed in HeLa cells showed a sigmoidal kinetic curve irrespective of the presence or absence of coexpressed TAK1 and TAB1. Although the molecular mechanism underlying the sigmoidal kinetics has yet to be elucidated, one possibility is that the activity of PP2Cζ is allosterically regulated by its substrate. In this case, PP2Cζ may be composed of multiple subunits that function cooperatively. Coexpression of PP2Cζ with TAK1 and TAB1 gave a decreased V_{max} and an increased $S_{0.5}$. Considering that Ser^{92} is located proximal to PP2C-conserved motif 1, which participates in holding the divalent metal ions essential for catalytic function (*11*), phosphorylation at Ser^{92} may alter the conformation around the active site, inactivating PP2Cζ and altering the kinetic parameters.

Lysiak et al. (*26*) reported that ischemia reperfusion (IR) of the murine testis results in germ cell-specific apoptosis and that IR upregulates interleukin 1β expression which results in activation of JNK and phosphorylation of its downstream transcription factor, c-Jun. Activation of the JNK pathway then leads to an increase in the level of E-selectin expression and neutrophil recruitment to the testis (*26*). Although the signaling pathway which mediates E-selectin expression has yet to be studied, PP2Cζ may be involved in regulation of the germ cell-specific apoptosis induced by IR.

The sequence surrounding Ser^{92} is conserved in PP2Cη, which also belongs to PP2C subgroup 2 (Figure 2d). It would be interesting to test whether the activity of PP2Cη is also regulated by stress-activated JNK. Although the sequence surrounding site 2 ($Thr^{202/205}$) is conserved in NERPP, the significance of site 2 phosphorylation in PP2Cζ is still obscure. Further studies are required to elucidate the possible generality of the regulation of PP2C subgroup 2 by phosphorylation.

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SUPPORTING INFORMATION AVAILABLE

Silver staining of the enzyme preparation used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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