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Selective suppression of stress-activated protein kinase pathway by protein phosphatase 2C in mammalian cells

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Abstract Protein phosphatase 2Cα (PP2Cα) or PP2Cβ-1 expressed in COS7 cells suppressed anisomycin- and NaCl-enhanced phosphorylations of p38 co-expressed in the cells. PP2Cα or PP2Cβ-1 expression also suppressed both basal and stress-enhanced phosphorylations of MKK3b and MKK6b, which are upstream protein kinases of p38, and of MKK4, which is one of the major upstream protein kinases of JNK. Basal activity of MKK7, another upstream protein kinase of JNK, was also suppressed by PP2Cα or PP2Cβ-1 expression. However, basal as well as serum-activated phosphorylation of MKK1a, an upstream protein kinase of ERKs, was not affected by PP2Cβ or PP2Cβ-1. A catalytically inactive mutant of PP2Cβ-1 further enhanced the NaCl-stimulated phosphorylations of MMK3b, MKK4 and MKK6b, suggesting that this mutant PP2Cβ-1 works as a dominant negative form. These results suggest that PP2C selectively inhibits the SAPK pathways through suppression of MKK3b, MKK4, MKK6b and MKK7 activities in mammalian cells.

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Key words: Protein phosphatase 2C;
Stress-activated protein kinase signal pathway

1. Introduction

Stress-activated kinases (SAPKs) constitute a subfamily of the mitogen-activated protein (MAP) kinase superfamily [1], and c-Jun N-terminal kinases (JNK1, 2 and 3) and p38 SAPKs (p38 α, β, γ and δ) have been reported to be present in mammalian cells [1–7]. Cell biological studies have revealed that these SAPKs are activated by extracellular stress, including osmotic shock, heat shock, oxidative stress, protein synthesis inhibitors, ultraviolet irradiation and anti-cancer drugs [1]. SAPKs have also been reported to be activated by some cytokines [1]. During the course of activation, these SAPKs are phosphorylated at the conserved tyrosine and threonine residues in the catalytic domain by dual-specific protein kinases called MAP kinase kinases (MKKs; MKK3, MKK4, MKK6 and MKK7) [1,8–11]. Substrate specificity studies revealed that MKK3 and MKK6 are responsible for phosphorylation and activation of p38 SAPKs and that MKK4 and MKK7 are the major protein kinases phosphorylating JNKs [1–11]. These MKKs are also phosphorylated at their conserved serine and threonine residues in response to extracellular stress. Recently, several MKK kinases (MKKKs) that activate MKKs have been identified, including MEKK1, MEKK2, MEKK3, MEKK4, the mixed lineage kinase (MLK) family, TGF β-activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1) and MAP three kinase 1 (MTK1) [1,9,12–24].

The SAPKs are highly conserved among eukaryotes. In the budding yeast *Saccharomyces cerevisiae*, the Hog1p (SAPK) pathway is activated by hyperosmotic shock [25,26]. In the fission yeast *Schizosaccharomyces pombe*, the Spc1 (SAPK) pathway is activated by heat shock, oxidative stress, nutrient stress and osmotic shock [25,27], and the resulting activated Spc1 induces the expression of various genes through the activation of Atf1 transcription factor [25].

Molecular genetic studies have indicated that yeast SAPK pathways are negatively regulated by protein phosphatase 2C [PP2C, one of the four major protein serine/threonine phosphatases (PP1, 2A, 2B and 2C)] [25]. The target of PP2C (PTC1 and PTC3 of *S. cerevisiae* and Ptc1 and Ptc3 of *S. pombe*) in the SAPK system was first predicted to be SAPK (Hog1) itself or a component upstream of SAPK in *S. cerevisiae* cells. However, a recent study showed that the target of Ptc1 in *S. pombe* cells lies downstream of SAPK (Spc1) [28].

Studies on the functions of mammalian PP2C have suggested that PP2C is involved in the regulation of AMP-activated protein kinase [29] and Ca^{2+}/calmodulin-dependent protein kinase II [30], but whether it also participates in the regulation of SAPK systems in mammalian cells is unknown. In this study we have addressed this question using transient expression of recombinant proteins in COS7 cells and provide evidence indicating that PP2C selectively suppresses the SAPK signal pathways in COS7 cells.

2. Materials and methods

2.1. Materials

The restriction enzymes and other modifying enzymes used for DNA manipulation were from Takara (Kyoto, Japan). Anti-6×His and anti-HA antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). Anti-phospho-p38, anti-phospho-JNK, anti-phospho-ERK1/2, anti-phospho-MKK4 and anti-phospho-MKK3/6 antibodies were from New England Biolabs (Beverly, MA, USA). Anti-Flag (M2) antibody was purchased from IBI Kodak (New Haven, CT, USA). Anisomycin was purchased from Sigma (St. Louis, MO, USA), anti-mouse IgG-alkaline phosphatase conjugate was from Promega (Madison, WI, USA) and all other reagents used were from Wako Pure Chemical (Osaka, Japan).

2.2. Expression plasmids

The HA-PP2Cα and HA-PP2Cβ-1 cDNAs [31] were subcloned into pcDNA3 (Invitrogen, Netherlands) and the resultant plasmids were named pcDNA3-HA-PP2Cα and pcDNA3-HA-PP2Cβ-1, respectively. Point mutants of PP2Cβ-1, which have no protein phosphatase activity, were prepared as described elsewhere [32]. HA tag was in-

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serted into the amino-terminal end of each point mutant of PP2Cβ-1 by overlap polymerase chain reaction (PCR). The cDNAs encoding HA-tagged PP2Cβ-1 point mutants were subcloned into pcDNA3. Dr. Jiahuai Han (The Scripps Research Institute) provided pcDNA3-Flag-p38, pcDNA3-MKK3b and pcDNA3-MKK6b [8]. MKK1a cDNA was purchased from American Type Culture Collection. ERK1 cDNA was from Dr. Jacques Pouyssegur (Université de Nice). JNK1 cDNA was from Dr. Michael Karin (University of California at San Diego, CA, USA). MKK4 and MKK7 cDNAs were prepared using PCR. All the cDNAs were subcloned into expression vector pcDNA3 (Invitrogen, Netherlands), and epitope tags were added by synthesized oligonucleotides. The plasmid pGEX-2T-glutathione *S*-transferase (GST)-ATF2 (1–96) was a gift from Dr. Roger J. Davis (University of Massachusetts) [33] and pGEX-2T-GST-c-Jun was provided by Dr. Masahiko Hibi (Osaka University) [2].

2.3. Cell culture and transfection

COS7 cells were grown in Dulbecco's minimum Eagle's medium (Gibco BRL, Rockville, USA) supplemented with 10% (v/v) fetal calf serum (FCS) and transfected by the DEAE-dextran method [34] or using lipofectamine (Gibco BRL).

2.4. Immune complex kinase assay

The COS7 cells were lysed in buffer A (20 mM HEPES, pH 7.4, 1% (v/v) Triton X-100, 137 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM NaF, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and aliquots of the lysates (100 μg protein) were subjected to the immune complex kinase assays, as described previously [8], using GST-activating transcription factor 2 (ATF2) (for p38 activity assay), GST-c-Jun (for JNK activity assay) and MBP (for ERK activity assay) as the substrates. The radioactivities incorporated into the substrates were determined using BAS 2000 image analyzer (Fuji, Japan).

2.5. Western blot analysis

Western blot analysis was performed by the standard procedure, using the anti-HA, anti-phospho-p38, anti-Flag, anti-6×His, anti-phospho-MKK3/6, anti-phospho-MKK4 and anti-phospho MKK1 antibodies. The chemiluminescence of each blot was detected with enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

2.6. Protein phosphatase activity assay

The COS7 cells were lysed in NaF-free buffer A and the PP2C activities (Mg^{2+}-dependent and okadaic acid-insensitive protein phosphatase activities) of the cell extracts were assayed using [^{32}P]phosphohistone as the substrate, as described previously [35].

3. Results and discussion

3.1. PP2C suppresses stress-enhanced tyrosine phosphorylation of p38

As p38 SAPK has been established to be the mammalian ortholog of *S. cerevisiae* HOG1 [1,25,26] and *S. pombe* Spc1 [25,27], first, we examined whether PP2C overexpression in COS7 cells affected basal and/or stress-enhanced tyrosine phosphorylation of p38 which was co-expressed in the cells. When HA-tagged PP2Cα was expressed transiently in COS7 cells, the PP2C activity of the cell extracts increased about three-fold (data not shown). Then, we co-expressed Flag-tagged p38 and HA-PP2Cα in COS7 cells and determined the tyrosine phosphorylation of Flag-p38 before and after treating the cells with anisomycin or NaCl. The results demonstrated that PP2Cα suppressed basal and anisomycin- and NaCl-enhanced tyrosine phosphorylation of p38 (Fig. 1A). Similar results were obtained when PP2Cβ-1, another PP2C isoform, was expressed in the cells instead of PP2Cα. No differences in expression levels of HA-PP2Cα and HA-PP2Cβ-1 were observed between the untreated control and stress inducer-treated cells. The suppression of both activity and tyrosine phosphorylation of endogenous p38 was also observed when HA-PP2Cα or HA-PP2Cβ-1 alone was expressed in the COS7 cells (data not shown).

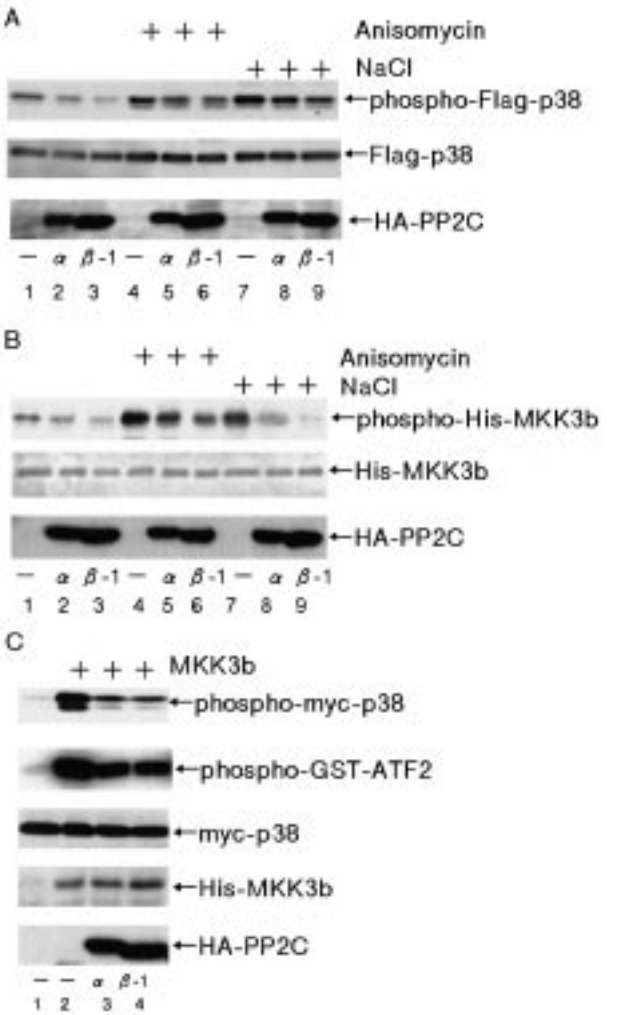

Fig. 1. Effect of expression of PP2C on phosphorylation and activity of p38 and MKK3b co-expressed in COS7 cells. A: The expression plasmids of Flag-p38 and HA-PP2C were co-transfected into COS7 cells in the ratio 1:4. The total amounts of DNA were adjusted with empty vector (pCDNA3). Forty-eight hours after the transfection, the cells were treated with anisomycin (10 mg/ml, 30 min at 37°C, lanes 4–6) or NaCl (0.7 M, 30 min at 37°C, lanes 7–9). The cells without treatment were used as the control (lanes 1–3). The aliquots of the cell extracts (3 μg protein) were immunoblotted with anti-phospho-p38 antibody recognizing tyrosine phosphorylated p38 (top panel), anti-Flag antibody (middle panel) and anti-HA antibody (bottom panel). B: The expression plasmids of His-MKK3b and HA-PP2C were co-transfected into COS7 cells in the ratio 1:4. The cells were treated with anisomycin (lanes 4–6) or NaCl (lanes 7–9). The cells without treatment were used as the control (lanes 1–3). Aliquots of the lysates (3–10 μg protein) were immunoblotted with anti-phospho-MKK3/6 antibody (top panel), anti-His antibody (middle panel) and anti-HA antibody (bottom panel). (C) The expression plasmids of myc-p38, His-MKK3b and HA-PP2C were co-transfected into COS7 cells in the ratio 1:1:2. The cells were lysed and aliquots of the lysates (100 μg protein) were immunoprecipitated with anti-myc antibody, and immune complex kinase assays were performed with GST-ATF2 as the substrate. Aliquots of the lysates (5–10 μg) were also immunoblotted with anti-phospho-p38, anti-myc, anti-His and anti-HA antibodies. The results represent one of at least three reproducible experiments.

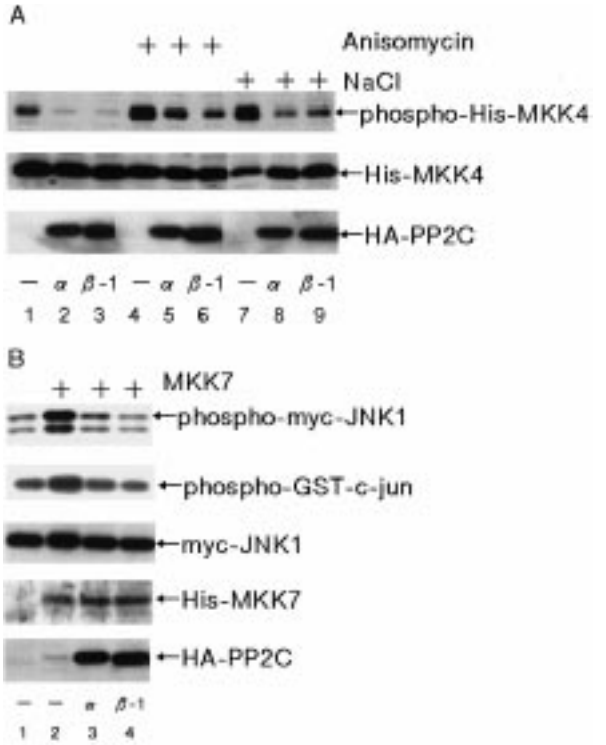

Fig. 2. Effects of PP2C on phosphorylation of MKK4 and activity of MKK7 co-expressed in COS7 cells. A: The expression plasmids of His-MKK4 and HA-PP2C were co-transfected into COS7 cells in the ratio 1:4. The cells were treated with anisomycin (lanes 4–6) or NaCl (lanes 7–9). The cells without treatment were used as the control (lanes 1–3). Aliquots of the lysates (3–10 μg protein) were immunoblotted with anti-phospho-MKK4 antibody (top panel), anti-His antibody (middle panel) and anti-HA antibody (bottom panel). B: The expression plasmids of myc-JNK1, His-MKK7 and HA-PP2C were co-transfected into COS7 cells in the ratio 1:1:2 (lanes 2–4). Cells transfected with the expression plasmid of myc-JNK1 alone (lane 1) or those of myc-JNK1 and His-MKK7 (lane 2) were also used. The cells were lysed and aliquots of the lysates (100 μg protein) were immunoprecipitated with anti-myc antibody, and immune complex kinase assays were performed with GST-c-jun as the substrate. Aliquots of the lysates (5–10 μg) were immunoblotted with anti-phospho-JNK, anti-myc, anti-His and anti-HA antibodies. The results represent one of at least three reproducible experiments.

3.2. PP2C suppresses phosphorylation and activity of MKK3 and MKK6

As PP2C is known to have high specificity for phosphoserine and phosphothreonine residues, we speculated that the target molecule of PP2Cs in the p38 MAPK pathway might lie upstream of p38. Since MKK3 and MKK6 have been found to be the major MKKs responsible for p38 activation [1,14–16], we anticipated that phosphorylation of these MKKs would be affected by PP2C expression. In order to verify speculation, we co-expressed HA-PP2Cα or HA-PP2Cβ-1 and His-MKK3b in COS7 cells and investigated whether PP2C suppressed the phosphorylation of MKK3b in the presence and absence of stress inducers. The phosphorylations of MKK3b (Fig. 1B) in the absence of stress inducers were suppressed by the co-expressed PP2Cα or PP2Cβ-1. In parallel with the decrease in the phosphorylation levels of MKK3b, the activity of MKK3b was reduced by the expression of PP2Cα or PP2Cβ-1 as determined by phosphorylation and activation of Myc-p38 co-expressed in the cells (Fig. 1C). The anisomycin- or NaCl-enhanced phosphorylation of MKK3b was also suppressed by the exogenous PP2Cα and PP2Cβ-1 (Fig. 1B). Essentially the same results were obtained when His-MKK6b was co-expressed with PP2Cα or PP2Cβ-1 in COS7 cells (Fig. 4, data not shown).

3.3. PP2C suppresses phosphorylation and/or activity of MKK4 and MKK7 but not those of MKK1

In order to determine whether PP2C has an effect only on the p38 pathway or also affects the other MAP kinase pathways, such as ERK and JNK pathways, we tested the effect of PP2C expression on phosphorylations of MKK1 (an upstream protein kinase of ERK) and MKK4 (a major upstream protein kinase of JNK).

When His-tagged MKK4 was co-expressed with PP2Cα or PP2Cβ-1 in COS7 cells, the basal threonine phosphorylation of MKK4 was suppressed (Fig. 2A). In parallel with the decreased phosphorylation, the activity of MKK4 was suppressed by PP2Cα and PP2Cβ-1 as determined by the phosphorylation and activation of the co-expressed myc-JNK1

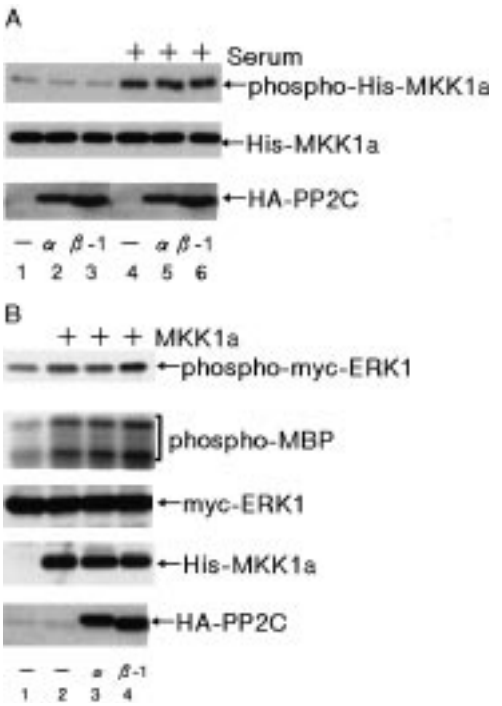

Fig. 3. Effects of PP2C expression on phosphorylation and activity of MKK1a co-expressed in COS7 cells. A: The expression plasmids of His-MKK1a and HA-PP2C were co-transfected into COS7 cells in the ratio 1:4. Twelve hours after the transfection, the cells were serum starved for 36 h, then treated with 20% (v/v) FCS for 10 min (lanes 4–6). The cells without serum treatment were used as the control (lanes 1–3). Aliquots of the lysate (5 μg protein) were immunoblotted with anti-phospho-MKK1/2 antibody (top panel), anti-His antibody (middle panel) and anti-HA antibody (bottom panel). B: The expression plasmids of Myc-ERK1, His-MKK1a and HA-PP2C were co-transfected into COS7 cells in the ratio 1:1:2 (lanes 3 and 4). Cells harboring the expression plasmid of myc-ERK1 alone (lane 1) or those of myc-ERK1 and His-MKK1a (lane 2) were also used. The cells were lysed and aliquots of the lysates (100 μg protein) were immunoprecipitated with anti-myc antibody, and immune complex kinase assays were performed with MBP as the substrate. Aliquots of the lysates (5–10 μg) were immunoblotted with anti-phospho-ERK1/2, anti-myc, anti-His and anti-HA antibody. The results represent one of at least three reproducible experiments.

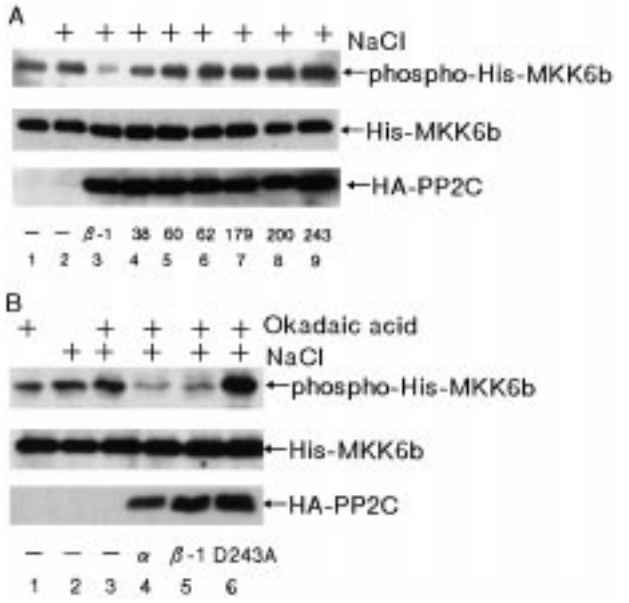

Fig. 4. Effects of expression of point mutants of PP2Cβ-1 on phosphorylation of MKK6B co-expressed in COS7 cells. A: The expression plasmids of His-MKK6b and wild type HA-PP2Cβ-1 or the points mutants of PP2Cβ-1 (D38A, D60A, H62L, R179G, R200G and D243A) were co-transfected into COS7 cells in the ratio 1:4. The cells were treated with NaCl (lanes 2–9). The cells without treatment were used as the control (lane 1). Aliquots of the lysates (3–10 μg protein) were immunoblotted with anti-phospho-MKK3/6 antibody (top panel), anti-His antibody (middle panel) and anti-HA antibody (bottom panel). B: The expression plasmids of His-MKK6b and wild type HA-PP2C of PP2Cβ-1(D243A) were co-transfected into COS7 cells in the ratio 1:4. The cells were treated with NaCl (lanes 2–5) in the presence (lanes 1, 3–6) or absence (lane 2) of 15 μM okadaic acid. Aliquots of the lysates (3–10 μg protein) were immunoblotted with anti-phospho-MKK3/6 antibody (top panel), anti-His antibody (middle panel) and anti-HA antibody (bottom panel). The results represent one of at least three reproducible experiments.

(data not shown). Both anisomycin and NaCl treatments of the cells stimulated the phosphorylation of His-MKK4 and the stress-enhanced phosphorylation of MKK4 was suppressed by the expression of PP2Cα or PP2Cβ-1 (Fig. 2A).

The activity of His-tagged MKK7 (a major upstream protein kinase of JNK) expressed in the COS7 cells was also suppressed by the co-expression of PP2Cα or PP2Cβ-1 as determined by the phosphorylation and activation of myc-JNK1 co-expressed in the cells (Fig. 2B).

Next, we co-expressed His-tagged MKK1a and HA-PP2Cα or HA-PP2Cβ-1 in COS7 cells and tested the effect of PP2C on the serine phosphorylation of MKK1a before and after serum treatment of the cells. The basal phosphorylation of His-MKK1a was affected very little by co-expression of PP2Cα or PP2Cβ-1 (Fig. 3A). The activity of His-MKK1a was not affected by the expression of PP2Cα or PP2Cβ-1 as determined by phosphorylation and activation of myc-ERK1 co-expressed in the cells (Fig. 3B). Serum treatment of the cells stimulated the phosphorylation of MKK1a, and the expression of PP2Cα or PP2Cβ-1 did not have any influence on the serum-enhanced phosphorylation of MKK1a (Fig. 3A).

Taken together, these results indicate that PP2Cα and PP2Cβ-1 selectively suppress the SAPK pathways (p38 and JNK pathways) but do not affect the ERK pathway.

3.4. PP2Cβ-1 point mutant (D243A) enhances the phosphorylation of MKK3, MKK4 and MKK6

We have previously reported that the activities of six distinct point mutants of PP2Cβ-1 (D38A, D60A, H62L, R179G, R200G and D243A) were less than 3% of that of wild type PP2Cβ-1 [32]. Each of these six point mutants was co-expressed with MKK6 in COS7 cells and the effects of these mutants on the NaCl-enhanced phosphorylation of MKK6 were determined. Five of the six point mutants (D38A, D60A, H62L, R179G and R200G) had little effect on the NaCl-enhanced phosphorylation of MKK6, demonstrating that PP2Cβ-1 activity was required for the suppression of the phosphorylation of MKK6 (Fig. 4A). In contrast, the expression of D243A stimulated further ($60 \pm 9\%$, mean ± S.E.M., $n = 4$) the NaCl-enhanced phosphorylation of MKK6 (Fig. 4A). The stress-enhanced phosphorylations of MKK3 and MKK4 were also stimulated further by the expression of D243A (data not shown). The stimulatory effect of D243A on MKK6 phosphorylation was also observed when 1 μM okadaic acid was present in the culture medium (Fig. 4B). Therefore, it was strongly suggested that D243A worked as a dominant negative form of PP2Cβ-1 and stimulated further the stress-enhanced phosphorylation of MKK6 by inhibiting the activity of endogenous PP2C, but not those of okadaic acid-sensitive phosphatases such as PP1 and PP2A, of COS7 cells. The serum-stimulated phosphorylation of MKK1a was not affected by the co-expression of D243A (data not shown). These results suggest that the endogenous PP2C in fact participates in the selective regulation of SAPK signalling pathways.

We have previously reported the existence of at least five distinct isoforms of 2Cβ (β-1 to β-5) in mammalian cells whose structural differences are only in the 10–20 amino acids of their carboxy-terminal regions [35]. We have recently tested the effects of expression of each of these five PP2Cβ isoforms on the NaCl-enhanced phosphorylation of MKK6a co-expressed in COS7 cells and found all five isoforms suppressed similarly the phosphorylation of MKK6a (unpublished observation). Therefore, the inhibitory effect on SAPK signalling pathways is likely to be a common feature of PP2Cα and PP2Cβ isoforms.

It has been reported that the expression level of Ptc1 of fission yeast cells increased following hyperosmotic shock treatment of the cells and that the expressed Ptc1 then suppressed the function of Atf1 to prevent overproduction of glycerol [28]. We have determined using immunoblot analysis whether the expression levels of endogenous PP2Cα and PP2Cβ-1 of COS7 cells were also affected by hyperosmotic stress. However, no alteration of their expression levels was observed up to 24 h after NaCl treatment of the cells (unpublished observation). Therefore, whether the function of PP2C is regulated by upstream signal(s) remains to be elucidated.

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