Regulation of apoptosis signal-regulating kinase 1 by protein phosphatase 2C ϵ

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ASK1 (apoptosis signal-regulating kinase 1), a MKKK (mitogen-activated protein kinase kinase kinase), is activated in response to cytotoxic stresses, such as H₂O₂ and TNF α (tumour necrosis factor α). ASK1 induction initiates a signalling cascade leading to apoptosis. After exposure of cells to H₂O₂, ASK1 is transiently activated by autophosphorylation at Thr⁸⁴⁵. The protein then associates with PP5 (protein serine/threonine phosphatase 5), which inactivates ASK1 by dephosphorylation of Thr⁸⁴⁵. Although this feedback regulation mechanism has been elucidated, it remains unclear how ASK1 is maintained in the dephosphorylated state under non-stressed conditions. In the present study, we have examined the possible role of PP2C ϵ (protein phosphatase 2C ϵ), a member of PP2C family, in the regulation of ASK1 signalling. Following expression in HEK-293 cells (human embryonic kidney cells), wild-type PP2C ϵ inhibited ASK1-induced activation of an AP-1 (activator protein 1) reporter gene. Conversely, a dominant-negative PP2C ϵ mutant enhanced AP-1 activity.

Exogenous PP2C ϵ associated with exogenous ASK1 in HEK-293 cells under non-stressed conditions, inactivating ASK1 by decreasing Thr⁸⁴⁵ phosphorylation. The association of endogenous PP2C ϵ and ASK1 was also observed in mouse brain extracts. PP2C ϵ directly dephosphorylated ASK1 at Thr⁸⁴⁵ *in vitro*. In contrast with PP5, PP2C ϵ transiently dissociated from ASK1 within cells upon H₂O₂ treatment. These results suggest that PP2C ϵ maintains ASK1 in an inactive state by dephosphorylation in quiescent cells, supporting the possibility that PP2C ϵ and PP5 play different roles in H₂O₂-induced regulation of ASK1 activity.

Key words: apoptosis, apoptosis signal-regulating kinase 1 (ASK1), hydrogen peroxide (H₂O₂), mitogen-activated protein kinase (MAPK), protein phosphatase 2C (PP2C), protein serine/threonine phosphatase 5 (PP5), stress-activated protein kinase (SAPK).

INTRODUCTION

SAPKs (stress-activated protein kinases), a subfamily of the MAPK (mitogen-activated protein kinase) superfamily, are highly conserved from yeast to mammals. SAPKs relay signals from various extracellular stimuli, including environmental stresses and inflammatory cytokines [1]. In mammalian cells, two distinct classes of SAPKs have been described: the c-Jun N-terminal kinases (JNK1, JNK2 and JNK3) and the p38 MAPKs (p38 α , p38 β , p38 γ and p38 δ) (Figure 1A) [1,2]. SAPKs are activated by phosphorylation of conserved tyrosine and threonine residues in the catalytic domain by dual-specificity protein kinases of the MKK (MAPK kinase) family. MKK3 and MKK6 phosphorylate p38, MKK7 phosphorylates JNK, and MKK4 can phosphorylate both targets. These MKKs in turn are activated by phosphorylation of conserved serine and threonine residues [1]. Several MKK-activating MKKKs (MKK kinases) have been identified [1], some of which, including ASK1 (apoptosis signal-regulating kinase 1), TAK1 (transforming-growth-factor- β -activated kinase 1), MEKK1 (MAPK/extracellular-signal-regulated kinase kinase 1) and MLK3 (mixed-lineage kinase 3) are also activated by phosphorylation [3–7].

In the absence of an activating signal, the constituents of the SAPK cascade return to an inactive dephosphorylated state, suggesting a role for phosphatases in the regulation of the SAPK pathway. Protein phosphatases are classified into three subgroups based on phospho-amino-acid specificity: PPs (protein serine/threonine phosphatases), protein serine/threonine/tyrosine phosphatases [DSPs (dual-specificity phosphatases)] and PTPs (protein tyrosine phosphatases). Dephosphorylation of SAPK signalling pathway components on serine/threonine and threonine/tyrosine residues requires the participation of multiple phosphatase subtypes. JNK and p38 can both be dephosphorylated by PTPs and DSPs [8]. Both p38 and MKK4 can be dephosphorylated by PP2C α , a member of the PP2C family [9]. The PP2C family contains at least 12 different gene products: PP2C α , PP2C β , PP2C γ /FIN13, PP2C δ /ILKAP, PP2C ϵ , PP2C ζ , PP2C η , Wip1, CaMKPase/hFEM2/POPX2, CaMKP-N, NERPP-2C and SCOP/PHLPP [10–34]. In the regulation of MKKKs, both PP2C β and PP2C ϵ associate with and dephosphorylate TAK1 directly in quiescent cells [30,35]. Interestingly, PP2C ϵ transiently dissociates from TAK1 upon IL-1 (interleukin-1) treatment, suggesting that PP2C ϵ associates with and dephosphorylates TAK1 to maintain the TAK1 signalling pathway in an inactive

Abbreviations used: AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; DSP, dual-specificity phosphatase; GST, glutathione S-transferase; HA, haemagglutinin; HEK-293 cell, human embryonic kidney cell; HRP, horseradish peroxidase; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEKK1, MAPK/extracellular-signal-regulated kinase kinase 1; MKK, MAPK kinase; MKKK, MKK kinase; NHS, N-hydroxysuccinimido; PP, protein serine/threonine phosphatase; PP2C, protein phosphatase 2C; PTP, protein tyrosine phosphatase; SAPK, stress-activated protein kinase; TAK1, transforming-growth-factor- β -activated kinase 1; TNF, tumour necrosis factor; Trx, thioredoxin.

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Figure 1 PP2Cε associates with ASK1

(A) Schematic diagram showing the SAPK signalling pathways. ATF2, activating transcription factor 2; MLK, mixed lineage kinase. (B) HEK-293 cells were transiently transfected with an expression plasmid encoding Myc-TAK1 or ASK1-Myc in the presence or absence of an expression plasmid encoding HA-PP2Cε. Proteins were immunoprecipitated (IP) from cell lysates with an anti-HA antibody (αHA); precipitates were immunoblotted (WB) with an anti-Myc antibody (αMyc). The cell lysates were also immunoblotted with anti-Myc or anti-HA antibodies. The results are from one of two reproducible experiments. (C) Proteins immunoprecipitated (IP) from mouse brain cell extracts with normal rabbit IgG (Cont) or an anti-PP2Cε antibody (αPP2Cε) were immunoblotted (WB) with an anti-ASK1 antibody (αASK1). The results are from one of two reproducible experiments.

state [30]. In an examination of the role of PP2Cε in the regulation of MKKKs, we observed that ASK1 expressed in HEK-293 cells (human embryonic kidney cells) had an affinity for PP2Cε that was similar to that of TAK1 (J.-i. Saito, T. Kobayashi and S. Tamura, unpublished work).

ASK1 is activated in response to various cytotoxic stresses, including H₂O₂, Fas ligation, TNF (tumour necrosis factor), serum withdrawal and antitumour reagents [36–40]. ASK1 kinase activ-

ity is tightly regulated within cells. Under non-stressed conditions, ASK1 is inhibited by association with its physiological inhibitor Trx (thioredoxin) [41]. Upon exposure of cells to H₂O₂ or TNFα, ROS (reactive oxygen species)-dependent oxidation of Trx induces dissociation of ASK1 from Trx, allowing the activation of the kinase by autophosphorylation at Thr⁸⁴⁵ [7]. Morita et al. [42] have demonstrated that PP5, a member of the PP family, could dephosphorylate ASK1 at Thr⁸⁴⁵. PP5 also associates with ASK1 following H₂O₂ or TNFα treatment, suggesting that PP5 participates in the feedback inhibition of ASK1 activity.

In the present study, we have determined the role of PP2Cε in the regulation of ASK1 activity. We have also investigated the difference in the roles of PP2Cε and PP5 in the regulation of ASK1 activity. We provide evidence that PP2Cε negatively regulates ASK1 in a manner different from that of PP5.

EXPERIMENTAL

Materials

Restriction enzymes and DNA-modifying enzymes used for DNA manipulation were obtained from TaKaRa. Human TNFα and Lipofectamine™ were purchased from Techne and Gibco BRL respectively. The luciferase and β-galactosidase enzyme assay systems were supplied by Promega. Glutathione-Sepharose-4B, Protein G-agarose beads, NHS (N-hydroxysuccinimido)-activated Sepharose 4B, PVDF membranes, ECL® (enhanced chemiluminescence) plus kits and [γ-³²P]ATP were obtained from GE Healthcare. Anti-HA (haemagglutinin) and anti-FLAG antibodies were purchased from Roche Molecular Biochemicals and Sigma-Aldrich respectively. Anti-(phospho-JNK), anti-(phospho-p38), anti-(phospho-MKK4), anti-(phospho-MKK3/6) and anti-[phospho-ASK(Thr⁸⁴⁵)] antibodies and HRP (horseradish peroxidase)-conjugated secondary antibodies were obtained from Cell Signaling Technology. Anti-Myc and anti-ASK1 antibodies were from Santa Cruz Biotechnology. All other reagents were purchased from Wako Pure Chemicals.

Construction of expression plasmids

Full-length cDNA clones encoding mouse PP2Cε and a dominant-negative point mutant [PP2Cε(DA)] were prepared as described previously [30]. The preparation of expression plasmids encoding ASK1 and TAK1 has also been described previously [30,41,43]. To express MKK6, p38 and PP2Cε in bacteria, cDNAs encoding these proteins were subcloned into the pGEX vector (GE Healthcare) to generate GST (glutathione S-transferase) fusion proteins.

Cell culture and transfection

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal bovine serum. At approx. 80% confluency, cells in 12-well plates were transfected with 1 μg of total DNA per well using Lipofectamine™. After transfection, cells were cultured for 24–48 h before harvesting.

Production of an anti-PP2Cε antibody

The pGEX-4T construct encoding GST-PP2Cε was transfected into *Escherichia coli* BL21 cells, and the transformant was grown in the presence of 0.1 mM IPTG (isopropyl β-D-thiogalactoside) for 3 h at 30°C. The cells were harvested and lysed by sonication, and GST-PP2Cε was collected using glutathione-Sepharose beads. The PP2Cε portion was then cleaved from the GST

carrier by incubation with the site-specific protease thrombin (25 units/ml) for 1 h at 25°C. Purified protein (1 mg) was used to immunize rabbits. The PP2C ϵ antiserum generated was affinity-purified by NHS-activated Sepharose 4B covalently coupled with PP2C ϵ .

Immunoprecipitation and immunoblot analysis

After two washes in PBS, cells transfected with the indicated expression plasmids were lysed in ice-cold lysis buffer [20 mM Tris/HCl (pH 7.5), 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM NaF, 1 mM dithiothreitol and 1 mM PMSF]. Proteins were immunoprecipitated using the indicated antibodies and Protein G-agarose beads. Immunoprecipitates were subjected to SDS/PAGE [10% (w/v) gels] and transferred on to PVDF membranes. Membranes were incubated with the indicated primary antibodies for 1 h at 25°C, treated with HRP-conjugated secondary antibodies for 1 h at 25°C and visualized using an ECL[®] plus kit.

In vitro association assay

HEK-293 cells were transfected with empty vector or the expression plasmid encoding ASK1-FLAG and cultured for 48 h at 37°C before harvesting. ASK1-FLAG in cell lysates was immunopurified using Protein G-agarose beads with an anti-FLAG antibody. ASK1-bound Protein G-agarose beads were incubated with recombinant PP2C ϵ for 2 h at 4°C. The association of PP2C ϵ with ASK1 *in vitro* was identified by Western blot analysis of proteins bound to the Protein G-agarose beads.

Reporter gene assay

Reporter gene activity assays were performed as described previously [30]. AP-1 (activator protein 1)-dependent transcriptional activity was measured using the pGL3-AP-1/luciferase reporter gene. Luciferase activity was determined using the Luciferase Assay System (Promega). A β -galactosidase reporter plasmid, in which expression of the enzyme was controlled by the β -actin promoter, was co-transfected to facilitate normalization of transfection efficiencies.

In vitro coupled kinase assay

Following expression in HEK-293 cells, ASK1 was immunoprecipitated from cell lysates. The precipitated proteins were incubated with 40 μ l of pre-incubation mixture [50 mM Tris/HCl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, 1 μ M microcystin-LR, 0.1 mM sodium orthovanadate, 0.1 mM EGTA, 10 mM MgCl₂, 0.1 mg/ml BSA, 7.5 μ g/ml recombinant GST-MKK6, 100 μ g/ml recombinant GST-p38 and 0.1 mM unlabelled ATP] for 15 min at 30°C. A portion of the reaction mixture (3 μ l) was then incubated with 47 μ l of incubation mixture {50 mM Tris/HCl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, 1 μ M microcystin-LR, 0.1 mM sodium orthovanadate, 0.1 mM EGTA, 10 mM MgCl₂, 0.1 mg/ml BSA, 0.4 mg/ml MBP (myelin basic protein) and 0.1 mM [γ -³²P]ATP} for 10 min at 30°C. The reaction was terminated by applying the reaction mixture on to phosphocellulose P81 paper, followed by washing with 75 mM phosphoric acid. The radioactivity incorporated into MBP was quantified by Cerenkov counting.

RESULTS

PP2C ϵ associates with ASK1 *in vivo*

ASK1 expressed in HEK-293 cells was able to bind to ectopically expressed PP2C ϵ with a similar affinity as TAK1 (Figure 1B).

Figure 2 PP2C ϵ inhibits ASK1-induced AP-1 activation

HEK-293 cells were transfected with an AP-1 luciferase reporter plasmid (0.1 μ g/well) in combination with empty vector (0.3 μ g/well; lane 1) or an expression plasmid encoding ASK1-Myc (0.3 μ g/well; lanes 2–6) in the presence or absence of various amounts of expression plasmid encoding HA-PP2C ϵ (lanes 1 and 2, none; lane 3, 0.1 μ g/well; lane 4, 0.2 μ g/well; and lane 5, 0.3 μ g/well) or HA-PP2C ϵ (DA) (lane 6, 0.3 μ g/well). The luciferase activities of cell lysates were determined and normalized on the basis of β -galactosidase expression 48 h after transfection. Results are the means $\pm$ S.E.M. ($n = 3$). Cell lysates were also immunoblotted with anti-HA (α HA) or anti-Myc (α Myc) antibodies. The results are from one of three reproducible experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the control experiment in which only ASK1 was expressed.

We therefore investigated whether endogenous PP2C ϵ associated with endogenous ASK1 in cells. PP2C ϵ was immunoprecipitated from mouse brain extracts with an anti-PP2C ϵ antibody, and the immunoprecipitates were then examined by immunoblotting with an anti-ASK1 antibody. The results demonstrated that endogenous PP2C ϵ associated with ASK1 in the brain (Figure 1C).

PP2C ϵ suppresses the ASK1-induced activation of both MKK3 and MKK4

To study the effect of PP2C ϵ expression on ASK1 function, we investigated the effect of PP2C ϵ expression on ASK1-induced activation of the AP-1 reporter gene. We observed a 9-fold increase in AP-1 activity when ASK1 was expressed in HEK-293 cells (Figure 2, lane 1 compared with lane 2). PP2C ϵ expression suppressed ASK1-mediated AP-1 activation in a concentration-dependent manner (Figure 2, lanes 2–5). In contrast, expression of a dominant-negative mutant of PP2C ϵ , PP2C ϵ (DA), stimulated AP-1 activity (Figure 2, lane 2 compared with lane 6), indicating that PP2C ϵ phosphatase activity was required for the inactivation of the ASK1-AP-1 signalling pathway. These results also suggest that endogenous PP2C ϵ suppresses the ASK1 signalling pathway.

ASK1 activates AP-1 by stimulating MKK4/7-JNK-mediated pathways [36]. ASK1 has also been reported to be responsible for activation of the MKK3/6-p38 pathway [36]. In our previous study [30], we determined that PP2C ϵ was not able to dephosphorylate either MKK3 or MKK4. On the basis of these observations, suppression by PP2C ϵ of ASK1-enhanced activation of both MKK3 and MKK4 would suggest that PP2C ϵ directly or indirectly inactivates ASK1. We expressed ASK1 alone or in combination with PP2C ϵ in HEK-293 cells and determined the phosphorylation status of endogenous MKK3 and MKK4 by immunoblot analysis using anti-(phospho-MKK3) and anti-(phospho-MKK4) antibodies respectively. The phosphorylation levels of these two signalling components were substantially decreased by the co-expression of PP2C ϵ (Figure 3), which is consistent with the hypothesis that ASK1 is the target of PP2C ϵ .

Figure 3 PP2Cε inhibits ASK1-enhanced phosphorylation of MKK3 and MKK4

HEK-293 cells were transfected with empty vector or an expression plasmid encoding ASK1-Myc in the presence or absence of an expression plasmid encoding HA-PP2Cε. Immunoblotting of cell lysates was performed using anti-(phospho-MKK3) (αpMKK3), anti-MKK3 (αMKK3), anti-(phospho-MKK4) (αpMKK4), anti-MKK4 (αMKK4), anti-Myc (αMyc) or anti-HA (αHA) antibodies. The results are from one of two reproducible experiments.

PP2Cε inactivates ASK1 by dephosphorylating Thr⁸⁴⁵

H₂O₂ treatment of cells activates ASK1 by inducing ASK1 auto-phosphorylation at Thr⁸⁴⁵ [7]. To clarify the mechanism by which PP2Cε suppresses ASK1 signalling pathway in more detail, we examined the effect of PP2Cε expression on the H₂O₂-enhanced phosphorylation of ASK1 at Thr⁸⁴⁵. H₂O₂ treatment of HEK-293 cells enhanced exogenous ASK1 phosphorylation at Thr⁸⁴⁵ (Figure 4A, lanes 1 and 2 compared with lanes 3 and 4). HA-PP2Cε expression decreased Thr⁸⁴⁵ phosphorylation in a concentration-dependent manner (Figure 4A, lanes 5–10).

To determine whether ASK1 activity decreased in parallel with the PP2Cε-induced decrease in Thr⁸⁴⁵ phosphorylation, we expressed ASK1-FLAG in HEK-293 cells in the presence or absence of HA-PP2Cε. Exogenous ASK1 was then immunoprecipitated with an anti-FLAG antibody. An *in vitro* kinase assay of the immunoprecipitates using MKK6 as substrate indicated that PP2Cε expression resulted in a 65% decrease in ASK1 activity (Figure 4B). In addition to the decrease in ASK1 activity, Thr⁸⁴⁵ phosphorylation was decreased by approx. 65% following PP2Cε expression (Figure 4B).

We next determined whether PP2Cε was able to dephosphorylate ASK1 Thr⁸⁴⁵ directly *in vitro*. Following expression in HEK-293 cells, ASK1-FLAG was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitated ASK1-FLAG was then incubated *in vitro* with bacterially expressed PP2Cε. Immunoblotting with the anti-[phospho-ASK(Thr⁸⁴⁵)] antibody demonstrated that PP2Cε was able to dephosphorylate Thr⁸⁴⁵ directly (Figure 5).

H₂O₂ induces transient dissociation of PP2Cε from ASK1

Morita et al. [42] have demonstrated that ASK1 is negatively regulated by PP5. In a manner similar to that demonstrated for PP2Cε, PP5 dephosphorylated ASK1 at Thr⁸⁴⁵. Expression of PP5, however, had a minimal effect on the basal or H₂O₂-induced activity of ASK1. ASK1 activity, however, decreased gradually with time in

Figure 4 PP2Cε induces decreased phosphorylation of ASK1 at Thr⁸⁴⁵

(A) HEK-293 cells (5×10^5 cells/well) were transfected with an expression plasmid encoding ASK1-HA (0.3 μg/well) in the presence or absence of various amounts of an expression plasmid encoding HA-PP2Cε (lanes 1–4, none; lane 5, 0.05 μg/well; lane 6, 0.1 μg/well; lane 7, 0.2 μg/well; lane 8, 0.3 μg/well; lane 9, 0.4 μg/well; and lane 10, 0.5 μg/well). After treatment of cells with 1 mM H₂O₂ for 15 min, cell lysates were analysed by immunoblot analyses with anti-[phospho-ASK(Thr⁸⁴⁵)] (αpASK1), anti-HA (αHA) or anti-PP2Cε (αPP2Cε) antibodies. The results are from one of three reproducible experiments. (B) HEK-293 cells were transfected with an expression plasmid encoding ASK1-Myc in the presence or absence of an expression plasmid encoding HA-PP2Cε. ASK1-Myc from cell lysates was immunoprecipitated with an anti-Myc antibody, and isolated proteins were then analysed by an *in vitro* kinase assay (top panel). Results are the means $\pm$ S.E.M. ($n = 3$). Cell lysates were also immunoblotted with anti-[phospho-ASK(Thr⁸⁴⁵)], anti-Myc or anti-PP2Cε antibodies. The results are from one of two reproducible experiments.

cells expressing exogenous PP5. The time course of ASK1 inactivation correlated well with that of ASK1-PP5 association, suggesting that PP5 participates in negative-feedback regulation of the ASK1 signalling pathway [42]. The H₂O₂-induced association of PP5 with ASK1 was observed in both HeLa and HEK-293 cells [42].

The association of PP2Cε with ASK1 under non-stressed conditions prompted us to determine whether PP2Cε behaves differently from PP5 following H₂O₂ treatment of cells. We expressed ASK1-FLAG alone or ASK1-FLAG and Myc-PP2Cε together in HEK-293 cells prior to treatment with 1 mM H₂O₂. We observed a stable association of PP2Cε with ASK1 until 5 min after H₂O₂ treatment (Figure 6A, lanes 2, 4 and 6), followed by transient dissociation of PP2Cε from ASK1 (Figure 6A, lanes 8, 10 and 12). The dissociation reached a maximum at 30 min after treatment (Figure 6A, lane 10); re-association was observed by 60 min after

Figure 5 PP2C ϵ directly dephosphorylates ASK1 *in vitro*

HEK-293 cells were transiently transfected with an expression plasmid encoding ASK1-FLAG. Exogenously expressed ASK1-FLAG was then immunoprecipitated (IP) with an anti-FLAG antibody (α FLAG) and incubated with various amounts of recombinant PP2C ϵ (rec. PP2C ϵ ; lanes 1 and 2, none; lane 3, 0.1 μ g; and lane 4, 0.3 μ g) *in vitro*. Incubation mixtures were immunoblotted (WB) with anti-[phospho-ASK(Thr⁸⁴⁵)] (α pASK1) or anti-FLAG (α FLAG) antibodies. Coomassie Brilliant Blue (C.B.B.) staining of recombinant PP2C ϵ following blotting on to PVDF membranes is also shown (bottom panel). The results are from one of two reproducible experiments.

Figure 6 H₂O₂ induces the transient dissociation of PP2C ϵ from ASK1

(A) HEK-293 cells were transiently transfected with an expression plasmid encoding ASK1-FLAG alone (lanes 1, 3, 5, 7, 9 and 11) or together with an expression plasmid for Myc-PP2C ϵ (lanes 2, 4, 6, 8, 10 and 12). Transfectants were cultured for 48 h, and then treated with 1 mM H₂O₂ for the indicated times. Exogenously expressed ASK1-FLAG was immunoprecipitated (IP) with an anti-FLAG antibody (α FLAG); immunoprecipitates were immunoblotted (WB) with anti-Myc (α Myc) or anti-FLAG (α FLAG) antibodies. Cell lysates were also immunoblotted with anti-PP2C ϵ (α PP2C ϵ) or anti-FLAG (α FLAG) antibodies. The results are from one of three reproducible experiments. (B) HEK-293 cells were transiently transfected with expression plasmids encoding ASK1-FLAG and Myc-PP2C ϵ , cultured for 48 h and treated with 50 ng/ml TNF α for the indicated times. Exogenously expressed ASK1-FLAG was immunoprecipitated (IP) with an anti-FLAG antibody (α FLAG); the resulting immunoprecipitates were immunoblotted (WB) with anti-PP2C ϵ (α PP2C ϵ) or anti-FLAG (α FLAG) antibodies. Cell lysates were also immunoblotted with anti-PP2C ϵ or anti-FLAG antibodies. The results are from one of two reproducible experiments.

treatment (Figure 6A, lane 12). These results indicate that PP2C ϵ and PP5 behave differently upon H₂O₂ stimulation of cells.

ASK1 is also activated by TNF α stimulation of cells [41]. Therefore we determined whether the transient dissociation of PP2C ϵ from ASK1 in HEK-293 cells was also observed following treatment with TNF α . The results indicated that TNF α also induced the transient dissociation of PP2C ϵ from ASK1

(Figure 6B), although the rate of re-association was lower than that seen in cells treated with H₂O₂.

DISCUSSION

In the present study, we have demonstrated that exogenous PP2C ϵ associated with exogenous ASK1 in HEK-293 cells. This interaction resulted in the inactivation of ASK1 by decreasing Thr⁸⁴⁵ phosphorylation under non-stressed conditions. PP2C ϵ inactivated ASK1 directly by dephosphorylating Thr⁸⁴⁵ of ASK1, as confirmed by *in vitro* assays. In addition, PP2C ϵ transiently dissociated from ASK1 upon H₂O₂ treatment of cells. Collectively, these results indicate that PP2C ϵ maintains ASK1 in an inactive state by dephosphorylating ASK1 in quiescent cells. These observations are consistent with the hypothesis that PP2C ϵ and PP5 play different physiological roles in the negative regulation of ASK1.

We have demonstrated previously [30] that PP2C ϵ directly associates with and dephosphorylates TAK1 in quiescent cells, transiently dissociating from TAK1 upon IL-1 treatment. In the present study, we have established that PP2C ϵ dissociated transiently from ASK1 upon both H₂O₂ and TNF α treatment of cells (Figure 6). These observations raise the possibility that the broad substrate specificity of PP2C ϵ for MKKKs contributes to the generalized maintenance of MKKKs in an inactive state in quiescent cells. Supporting this conclusion is the observation that exogenous MEKK1 expressed in HEK-293 cells could be co-immunoprecipitated with exogenous PP2C ϵ (J.-i. Saito, T. Kobayashi and S. Tamura, unpublished work). Dissociation of PP2C ϵ from MKKKs may be a cellular mechanism conserved throughout the stress- or pro-inflammatory-cytokine-induced activation of multiple MKKKs.

The conclusion that PP2C ϵ participates in maintaining ASK1 in an inactive state in quiescent cells is reminiscent of the role of Ptc1, a yeast orthologue of mammalian PP2C, in the osmotic stress response of the yeast *Saccharomyces cerevisiae* [44]. Three Ptc family members, Ptc1, Ptc2 and Ptc3, have been implicated in the regulation of the MAPK Hog1 signalling pathway after the exposure of cells to high-osmolarity environments [44–47]. Deletion of *PTC1* led to a high basal activity of Hog1, indicating that Ptc1 is responsible for maintaining the Hog1 pathway in an inactive state under non-stressed conditions [44]. In contrast, deletion of *PTC2* and *PTC3* led to increased Hog1 activation upon osmotic stress than was observed in wild-type strains, although no obvious change in basal Hog1 activity was observed [47]. These observations indicate that, in contrast with Ptc1, Ptc2 and Ptc3 limit maximal Hog1 activity. Similarly to Ptc1 in the yeast Hog1 system, PP2C ϵ participates in maintaining ASK1 in an inactive state in quiescent cells. The molecular mechanisms governing the regulation of maximal ASK1 activation following H₂O₂ treatment are not well understood. It would be interesting to test whether other PP2C family members, similarly to Ptc2 and Ptc3 in the yeast Hog1 pathway, are involved in the regulation of maximal ASK1 activation upon H₂O₂ treatment of cells.

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