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

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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 (tumour necrosis factor). 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 PP2Cε (protein phosphatase 2Cε), a member of the PP2C family, in the regulation of ASK1 signalling. Following expression in HEK-293 cells (human embryonic kidney cells), wild-type PP2Cε inhibited ASK1-induced activation of an AP-1 (activator protein 1) reporter gene. Conversely, a dominant-negative PP2Cε mutant enhanced AP-1 activity.

Exogenous PP2Cε associated with exogenous ASK1 in HEK-293 cells under non-stressed conditions, inactivating ASK1 by decreasing Thrα95 phosphorylation. The association of endogenous PP2Cε and ASK1 was also observed in mouse brain extracts. PP2Cε directly dephosphorylated ASK1 at Thrα95 in vitro. In contrast with PP5, PP2Cε transiently dissociated from ASK1 within cells upon H2O2 treatment. These results suggest that PP2Cε maintains ASK1 in an inactive state by dephosphorylation in quiescent cells, supporting the possibility that PP2Cε 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 (p38α, p38β, p38γ and p38δ) (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 phosphorlates 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 MKKs (MKKs) 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 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 tyrosine/threonine 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 PP2Ca, a member of the PP2C family [9]. The PP2C family contains at least 12 different gene products: PP2Ca, PP2Cβ, PP2Cγ/FIN13, PP2Cζ/ILKAP, PP2Cɛ, PP2Cζ, Wip1, CaM kinase II regulatory subunit FEM2/POPX2, CaMKP-N, NERPP-2C and SCOP/PHLPP [10–34]. In the regulation of MKKs, both PP2Ca and PP2Cγ associate with and dephosphorylate TAK1 directly in quiescent cells [30,35]. Interestingly, PP2Cε transiently dissociates from TAK1 upon IL-1 (interleukin-1) treatment, suggesting that PP2Cε associates with and dephosphorylates TAK1 to maintain the TAK1 signalling pathway in an inactive state.

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, MAPK kinase; NHE, N-heptadecanoylphosphatidylethanolamine; P2, 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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state [30]. In an examination of the role of PP2Ce in the regulation of MKKks, we observed that ASK1 expressed in HEK-293 cells (human embryonic kidney cells) had an affinity for PP2Ce 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 activity 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 PP2Ce in the regulation of ASK1 activity. We have also investigated the difference in the roles of PP2Ce and PP5 in the regulation of ASK1 activity. We provide evidence that PP2Ce 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 PP2Ce and a dominant-negative point mutant [PP2Ce(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 PP2Ce 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-PP2Ce antibody**

The pGEX-4T construct encoding GST–PP2Ce 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–PP2Ce was collected using glutathione–Sepharose beads. The PP2Ce 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 PP2Ce antiserum generated was affinity-purified by NHS-activated Sepharose 4B covalently coupled with PP2Ce.

**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 37°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 PP2Ce for 2 h at 4°C. The association of PP2Ce with ASK1 in vitro was identified by Western blot analysis of proteins bound to the Protein G–agarose beads.

**Reporters 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 µl of pre-incubation mixture [50 mM Tris/HCl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, 1 µM microcystin-LR, 0.1 mM sodium orthovanadate, 0.1 mM EDTA, 10 mM MgCl₂, 0.1 mg/ml BSA, 7.5 µg/ml recombinant GST–M KK6, 100 µg/ml recombinant GST–p38 and 0.1 mM unlabelled ATP] for 15 min at 30°C. A portion of the reaction mixture (3 µl) was then incubated with 47 µl of incubation mixture [50 mM Tris/HCl (pH 7.5), 0.1% (v/v) 2-mercaptoethanol, 1 µM microcystin-LR, 0.1 mM sodium orthovanadate, 0.1 mM EDTA, 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 adding 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**

**PP2Ce associates with ASK1 in vivo**

ASK1 expressed in HEK-293 cells was able to bind to ectopically expressed PP2Ce with a similar affinity as TAK1 (Figure 1B).

**PP2Ce suppresses the ASK1-induced activation of both MKK3 and MKK4**

To study the effect of PP2Ce expression on ASK1 function, we investigated the effect of PP2Ce 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). PP2Ce 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 PP2Ce, PP2Ce(DA), stimulated AP-1 activity (Figure 2, lane 2 compared with lane 6), indicating that PP2Ce phosphatase activity was required for the inactivation of the ASK1–AP-1 signalling pathway. These results also suggest that endogenous PP2Ce 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 PP2Ce was not able to dephosphorylate either MKK3 or MKK4. On the basis of these observations, suppression by PP2Ce of ASK1-enhanced activation of both MKK3 and MKK4 would suggest that PP2Ce directly or indirectly inactivates ASK1. We expressed ASK1 alone or in combination with PP2Ce 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 PP2Ce (Figure 3), which is consistent with the hypothesis that ASK1 is the target of PP2Ce.

We therefore investigated whether endogenous PP2Ce associated with endogenous ASK1 in cells. PP2Ce was immunoprecipitated from mouse brain extracts with an anti-PP2Ce antibody, and the immunoprecipitates were then examined by immunoblotting with an anti-ASK1 antibody. The results demonstrated that endogenous PP2Ce associated with ASK1 in the brain (Figure 1C).
PP2Ce 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 PP2Ce suppresses ASK1 signalling pathway in more detail, we examined the effect of PP2Ce 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–PP2Ce expression decreased Thr845 phosphorylation in a concentration-dependent manner (Figure 4A, lanes 3–10).

To determine whether ASK1 activity decreased in parallel with the PP2Ce-induced decrease in Thr845 phosphorylation, we expressed ASK1–FLAG in HEK-293 cells in the presence or absence of HA–PP2Ce. Exogenous ASK1 was then immunoprecipitated with an anti-FLAG antibody. An in vitro kinase assay of the immunoprecipitates using MKK6 as substrate indicated that PP2Ce 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 PP2Ce expression (Figure 4B).

We next determined whether PP2Ce was able to dephosphorylate ASK1 Thr845 directly in vitro. Following expression in HEK-293 cells, ASK1–FLAG was immunoprecipitated with an anti-FLAG antibody. Immunopurified ASK1–FLAG was then incubated in vitro with bacterially expressed PP2Ce. Immunoblotting with the anti-[phospho-ASK(Thr845)] antibody demonstrated that PP2Ce was able to dephosphorylate Thr845 directly (Figure 5).

H2O2 induces transient dissociation of PP2Ce from ASK1

Morita et al. [42] have demonstrated that ASK1 is negatively regulated by PP5. In a manner similar to that demonstrated for PP2Ce, 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 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 PP2Ce with ASK1 under non-stressed conditions prompted us to determine whether PP2Ce behaves differently from PP5 following H2O2 treatment of cells. We expressed ASK1–FLAG alone or ASK1–FLAG and Myc–PP2Ce together in HEK-293 cells prior to treatment with 1 mM H2O2. We observed a stable association of PP2Ce with ASK1 until 5 min after H2O2 treatment (Figure 6A, lanes 2, 4 and 6), followed by transient dissociation of PP2Ce 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 3 PP2Ce 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 an expression plasmid encoding HA–PP2Ce. Immunoblotting of cell lysates was performed using anti-(phospho-MKK3) (αMKK3), 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.

Figure 4 PP2Ce decreases phosphorylation of ASK1 at Thr845

(A) HEK-293 cells (5 × 10⁵ 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–PP2Ce (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-PP2Ce (αPP2Ce) 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–PP2Ce. 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 ± S.E.M. (n = 3). Cell lysates were also immunoblotted with anti-[phospho-ASK(Thr845)], anti-Myc or anti-PP2Ce antibodies. The results are from one of two reproducible experiments.
treatment (Figure 6A, lane 12). These results indicate that PP2Ce and P5P behave differently upon H2O2 stimulation of cells.

ASK1 is also activated by TNFα stimulation of cells [41]. Therefore we determined whether the transient dissociation of PP2Ce from ASK1 in HEK-293 cells was also observed following treatment with TNFα. The results indicate that TNFα also induced the transient dissociation of PP2Ce 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 PP2Ce associated with exogenous ASK1 in HEK-293 cells. This interaction resulted in the inactivation of ASK1 by decreasing Thr454 phosphorylation under non-stressed conditions. PP2Ce inactivated ASK1 directly by dephosphorylating Thr454 of ASK1, as confirmed by in vitro assays. In addition, PP2Ce transiently dissociated from ASK1 upon H2O2 treatment of cells. Collectively, these results indicate that PP2Ce maintains ASK1 in an inactive state by dephosphorylating ASK1 in quiescent cells. These observations are consistent with the hypothesis that PP2Ce and PP5 play different physiological roles in the negative regulation of ASK1.

We have demonstrated previously [30] that PP2Ce 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 PP2Ce dissociated transiently from ASK1 upon both H2O2 and TNFα treatment of cells (Figure 6). These observations raise the possibility that the broad substrate specificity of PP2Ce 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 PP2Ce (J.-i. Saito, T. Kobayashi and S. Tamura, unpublished work). Dissociation of PP2Ce from MKKKs may be a cellular mechanism conserved throughout the stress- or pro-inflammatory-cytokine-induced activation of multiple MKKKs.

The conclusion that PP2Ce participates in maintaining ASK1 in an inactive state in quiescent cells is reminiscent of the role of Ptc1, a yeast orthologue of mammalian PP2Ce, 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, PP2Ce 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.

**REFERENCES**


