Catalytic activation of mitogen-activated protein (MAP) kinase phosphatase-1 by binding to p38 MAP kinase: critical role of the p38 C-terminal domain in its negative regulation

Dorothy HUTTER, Peili CHEN, Janice BARNES and Yusen LIU[1]

Stress Signaling Unit, Laboratory of Cellular and Molecular Biology, National Institute on Aging, NIH, 5600 Nathan Shock Drive, Baltimore, MD 21224, U.S.A

Mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1) is the archetypal member of the dual-specificity protein phosphatase family, the expression of which can be rapidly induced by a variety of growth factors and cellular stress. Since MKP-1 protein localizes in the nucleus, it has been suggested to play an important role in the feedback control of MAP kinase-regulated gene transcription. Recently it has been demonstrated that the interaction of several cytosolic MAP kinase phosphatases with MAP kinases can trigger the catalytic activation of the phosphatases. It is unclear whether such a regulatory mechanism can apply to nuclear MAP kinase phosphatases and serve as an additional apparatus for the feedback control of MAP kinase-mediated gene expression. Here we have shown that MKP-1 associates directly with p38 MAP kinase both *in vivo* and *in vitro*, and that this interaction enhances the catalytic activity of MKP-1. The point mutation Asp-316 → Asn in the C-terminus of p38, analogous to the ERK2 (extracellular-signal-regulated kinase 2) *sevenmaker* mutation, dramatically decreases its binding to MKP-1 and substantially compromises its stimulatory effect on the catalytic activity of this phosphatase. Consistent with its defective interaction with MKP-1, this p38 mutant also displays greater resistance to dephosphorylation by the phosphatase. Our studies provide the first example of catalytic activation of a nuclear MAP kinase phosphatase through direct binding to a MAP kinase, suggesting that such a regulatory mechanism may play an important role in the feedback control of MAP kinase signalling in the nuclear compartment.

Key words: feedback control, MKP-1, protein–protein interaction, signal transduction.

INTRODUCTION

The mitogen-activated protein (MAP) kinases are ubiquitous signalling molecules which mediate the response of cells to a variety of extracellular stimuli, ranging from growth factors to cellular stresses [1]. To date, three major MAP kinase subfamilies have been well characterized in mammalian cells: extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase and p38 [1,2]. The activity of all MAP kinases is regulated through the reversible phosphorylation of conserved threonine and tyrosine residues in their kinase subdomain VIII by specific MAP kinase kinases (MKKs) and protein phosphatases. Once activated, these MAP kinases can translocate from the cytoplasm to the nucleus, leading to the phosphorylation of a multitude of transcription factors and altered gene transcription [3,4]. Since MAP kinase pathways play an important role in regulating a broad range of cellular processes [1], including apoptosis [5], cell cycle control [6,7] and the production of inflammatory cytokines [8], the precise regulation of these signalling proteins is crucial for maintaining the integrity of the signal transduction process.

In mammalian cells, inactivation of MAP kinases is achieved mainly by a family of dual-specificity MAP kinase phosphatases that are capable of targeting the two regulatory phosphorylation sites of these kinases [9]. So far, nine distinct mammalian MAP kinase phosphatase family members have been identified. According to their patterns of transcriptional regulation and subcellular localization, these phosphatases can be roughly divided into two groups [9]. The first group includes MAP kinase phosphatase-3 (MKP-3)/Pyst1, Pyst2, MKP-4, MKP-5 and M3/6, which are localized predominantly in the cytosol, and are not encoded by immediate-early genes. The second group of enzymes includes MKP-1 (CL100/3CH134), MKP-2, PAC-1 (phosphatase of activated cells-1) and B23, which are localized primarily in the nuclear compartment. Encoded by immediate-early genes, these nuclear MAP kinase phosphatases are rapidly and highly inducible by many of the stimuli that activate MAP kinases. For this reason, it has been suggested that these MAP kinase phosphatases play an important role in the feedback control of MAP kinase signalling in the nucleus [9–12].

Recently it has been demonstrated that catalytic activation of several cytosolic MAP kinase phosphatases can be triggered by their direct interaction with MAP kinases [13,14]. For example, MKP-3 interacts specifically with ERK MAP kinases [13,15], and the binding of ERK2 to MKP-3 dramatically enhances the catalytic activity of MKP-3 [13]. Furthermore, a point mutation in mammalian ERK2, analogous to the gain-of-function *Drosophila sevenmaker* mutant, dramatically decreases the ability of

Abbreviations used: MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; MKP-1, MAP kinase phosphatase-1; MKP-1(CS), Cys-258 → Ser mutant of MKP-1; ERK, extracellular-signal-regulated kinase; MAPKAPK, MAP kinase-activated protein kinase; MK2/3, MAP kinase-activated protein kinase 2/3; MKK, MAP kinase kinase; MKK6b(E), MKK6b in which Ser-207 and Thr-211 have been mutated to Glu; $p38^{N316}$, p38 Asp-316 → Asn mutant; PAC, phosphatase of activated cells; HA, haemagglutinin; GST, glutathione S-transferase; *p*-NPP, *p*-nitrophenyl phosphate; MBP, myelin basic protein; Hsp25, heat-shock protein of 25 kDa; UVC, short-wavelength UV light.

[1] To whom correspondence should be addressed (e-mail yusen-liu@nih.gov).

ERK2 to bind and trigger the catalytic activation of MKP-3, and substantially increases the resistance of ERK2 to a number of MAP kinase phosphatases [13,16,17]. In contrast with MKP-3, the relatively non-selective MAP kinase phosphatase MKP-4 can interact with the members of all three major MAP kinase subfamilies and become catalytically activated [13]. These results illustrate a novel mechanism to ensure the tight feedback control of MAP kinase signalling in the cytosol. However, it is still unclear whether such a regulatory mechanism applies to the inducible, nuclear MAP kinase phosphatases and serves as an additional means to ensure the feedback control of MAP kinases in the nuclear compartment.

In the present study we show that MKP-1 binds to p38 both *in vivo* and *in vitro*, and that this interaction enhances the catalytic activity of MKP-1. We further demonstrate that this interaction between p38 and MKP-1 is mediated through the p38 C-terminal domain. A *sevenmaker*-like mutation in p38 dramatically decreases its interaction with MKP-1 and substantially compromises its stimulatory effect on the catalytic activity of MKP-1. Compared with the wild-type p38 protein, this mutant p38 exhibits significantly increased resistance to inactivation by MKP-1. Our studies provide the first example of the catalytic activation of an inducible nuclear MAP kinase phosphatase by its binding to a MAP kinase, suggesting that the catalytic activation of nuclear MAP kinase phosphatases through interaction with MAP kinases may constitute an important part of the feedback control mechanism for MAP kinase signalling in the nuclear compartment.

EXPERIMENTAL

Cell culture and treatments

HeLa and 293T cells were grown in a 37 °C humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, U.S.A.). HeLa cells were transfected using LIPOFECTAMINE® (Life Technologies) as previously described [12]. 293T cells were transfected using FuGENE^{J}6 transfection reagent (Boehringer Mannheim, Indianapolis, IN, U.S.A.) according to the manufacturer's specifications. The DNA content in the transfection cocktails was kept constant using an empty vector. At 48 h after transfection, cells were irradiated with short-wavelength UV light (UVC) (100 J/m^2) as previously described [12]. At 30 min after UVC treatment, cells were harvested in the following lysis buffer: 10 mM Hepes, pH 7.4, 50 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM dithiothreitol, 10 mM NaF, 1 mM sodium orthovanadate, 2 μM leupeptin, 2 μM aprotinin and 1 mM PMSF.

Plasmids

Haemagglutinin (HA)-tagged JNK1 plasmid (pSRα-HA–JNK1) was kindly provided by M. Karin (Department of Pharmacology, University of California, San Diego, La Jolla, CA, U.S.A.) [18]. The JNK1 coding sequence was replaced with the mouse p38α coding sequence PCR-amplified from pCMV5-Flag–p38 [19] to create pSRα-HA–p38. Three Myc epitope tags were inserted after the HA coding sequence to create pSRα-HA–Myc–p38. pET41a-p38 was created by cloning the p38 open reading frame into pET41a (Novagen, Madison, WI, U.S.A.). The p38 Asp-316 → Asn mutant construct (p38^{N316}) was created with a site-directed mutagenesis kit (QuickChange; Stratagene, La Jolla, CA, U.S.A.). The pSRα-Flag–MKP-1 construct was created by replacing the HA–JNK1 coding sequence with a Flag–MKP-1 sequence PCR-amplified from rat MKP-1 cDNA [12]. Similarly, pSRα-Flag–MKP-2 was generated from a human EST (expressed sequence tag) clone (A.T.C.C., Manassas, VA, U.S.A.). The open reading frame of rat MKP-1 was fused to glutathione S-transferase (GST) by cloning into pEBG [20] to create pEBG-MKP-1. The Cys-258 → Ser mutant (pEBG-MKP-1CS) was created through site-directed mutagenesis (QuickChange; Stratagene). All of the constructs obtained were confirmed by sequencing reactions.

Antibodies and Western blot analysis

Monoclonal anti-GST and polyclonal anti-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal anti-phospho-p38 was purchased from New England Biolabs (Beverly, MA, U.S.A.). Monoclonal anti-HA (12CA5) was purchased from Boehringer Mannheim. Monoclonal anti-c-Myc (9E10) and anti-Flag (M2) antibodies, and monoclonal anti-HA (16B12) and anti-Flag (M2) affinity matrices, were obtained from BAbCo (Richmond, CA, U.S.A.). Monoclonal anti-MKK3 was obtained from Transduction Laboratories (Lexington, KY, U.S.A.), and polyclonal anti-MKK6 was purchased from Chemicon International (Temecula, CA, U.S.A.).

Western blot analysis was carried out as previously described [21]. For re-probing, blots were stripped with Re-Blot (Chemicon International).

Assessment of protein–protein interactions *in vivo*

To study the binding of Flag–MKP-1 to HA–p38, soluble lysates from transfected 293T cells were incubated with 20 μl of monoclonal anti-HA affinity matrix at 4 °C overnight with gentle rotation. The immunocomplexes were washed twice with lysis buffer and twice with lysis buffer containing 150 mM NaCl, then eluted twice with 40 μl of lysis buffer containing 0.4 μg/μl HA peptide (BAbCo). The eluted proteins were subjected to Western blot analysis with anti-HA and anti-Flag antibodies. To analyse the interaction between GST–MKP-1CS and HA–p38 or HA–p38^{N316}, soluble lysates from transfected cells were incubated with glutathione–Sepharose beads (Pharmacia Biotech, Piscataway, NJ, U.S.A.) at 4 °C for 1 h. Following incubation, the beads were washed as described above and used for Western blot analysis with anti-Flag and anti-GST antibodies.

Assessment of protein–protein interactions *in vitro*

Flag–MKP-1 protein was prepared by immunoprecipitation using the anti-Flag affinity matrix from 293T cells transfected with pSRα-Flag–MKP-1, and subsequent elution with Flag peptide (BAbCo). Recombinant GST, GST–p38 and GST–p38^{N316} proteins were produced in *Escherichia coli* using the pET41a-based expression system (Novagen) and purified by glutathione–Sepharose chromatography according to the manufacturer's specifications (Pharmacia). Immobilized GST fusion proteins were incubated overnight at 4 °C with purified Flag–MKP-1 protein or with 200 μg of soluble lysates from untransfected cells or from cells transfected with plasmids for either Flag–MKP-1 or Flag–MKP-2. The beads were washed as previously described and subjected to Western blot analysis.

Assessment of the catalytic activity of MKP-1

Recombinant GST–MKP-1 fusion protein was produced in 293T cells through the transient transfection of pEBG-MKP-1.

The fusion protein was then isolated from the cell lysates using glutathione–Sepharose beads. After washing three times each with lysis buffer supplemented with 200 mM NaCl, PBS and reaction buffer (50 mM Tris/HCl, pH 7.4, 2 mM dithiothreitol and 5 mM $MgCl_2$), the fusion protein was eluted into the reaction buffer containing 10 mM GSH. Typically, this procedure can yield 200 μg of full-length GST–MKP-1 protein from twenty 150 mm dishes of 293T cells with a purity of about 60 %, as judged by Coomassie Blue staining and Western blot analysis with anti-GST antibody. The catalytic activity of this phosphatase was measured in 96-well plates in 200 μl of 50 mM Tris/HCl (pH 7.4) containing 5 mM dithiothreitol, 20 mM *p*-nitrophenyl phosphate (*p*-NPP), 3.5 μg of GST–MKP-1 and the indicated amounts of purified wild-type or mutant p38 MAP kinase. Reaction rates were measured at 405 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

MAPKAPK2/3 (MAP kinase-activated protein kinase 2/3) kinase assay

MAPKAPK2 and MAPKAPK3 are two highly similar protein kinases that are regulated by p38 MAP kinase. Since a rabbit polyclonal antiserum generated against MAPKAPK2 recognizes both kinases, the combined activity of the two kinases (referred to as MK2/3) can be assessed through immunocomplex kinase assays [22]. Briefly, endogenous MK2/3 was immunoprecipitated from 500 μg of HeLa cell lysates with 3 μg of a rabbit polyclonal antiserum kindly provided by J. Huot (Centre de Recherche en Cancerologie de l'Universite Laval, L'Hotel-Dieu de Quebec, Quebec G1R 2J6, Canada) and Protein A–Sepharose (Pharmacia) as previously described [22]. The kinase activities of the MK2/3 immunocomplexes were assayed using recombinant Hsp25 (heat-shock protein of 25 kDa) (Stressgen, Vancouver, Canada) as a substrate.

Activation of recombinant p38 *in vitro*

Constitutively active human MKK6b(E) (MKK6b in which Ser-207 and Thr-211 have been mutated to Glu) was expressed in *E. coli* as a $(His)_6$-tagged protein with the plasmid pET14-MKK6b(E), kindly provided by J. Han (Department of Immunology, The Scripps Research Institute, La Jolla, CA, U.S.A.) [23]. Recombinant $(His)_6$–MKK6b(E) was purified with Ni^{2+}-nitrilotriacetate agarose and eluted with 300 mM imidazole according to the manufacturer's specifications (Novagen). GST–p38 or GST–$p38^{N316}$ (0.5 μg) was incubated with 0.1 μg of $(His)_6$–MKK6b(E) and 6 μg of myelin basic protein (MBP) (Sigma, St. Louis, MO, U.S.A.) at 30 °C for 30 min in 35 μl of reaction buffer containing 50 mM Tris/HCl (pH 7.4), 2 mM dithiothreitol, 10 mM $MgCl_2$, 10 μM ATP and 15 μCi of [γ-^{32}P]ATP. Proteins in the reaction mixtures were then resolved by SDS/PAGE (15 % gel) and subjected to autoradiography.

RESULTS

MKP-1 interacts with p38 MAP kinase *in vivo*

To determine whether MKP-1 and p38 can form complexes *in vivo*, Flag–MKP-1 or HA–p38 proteins were transiently expressed alone or co-expressed in 293T cells. The transiently transfected cells were either irradiated with UVC to activate p38 or left untreated. HA–p38 was immunoprecipitated from the cell lysates, eluted with HA peptide and then subjected to Western blot analysis (Figure 1, upper two panels). Despite the fact that all of the transfected cells expressed relatively high levels of the epitope-tagged proteins (Figure 1, lower two panels), and HA–p38 was immunoprecipitated from these cells with comparable efficiency (Figure 1, second panel), Flag–MKP-1 was only detected in the samples from cells expressing both proteins (Figure 1, top panel). These results indicated that Flag–MKP-1 was able to form complexes with HA–p38 in transfected cells. Interestingly, comparable amounts of Flag–MKP-1 protein were co-precipitated with HA–p38 from both untreated and UVC-treated cells, suggesting that the interaction between p38 and MKP-1 may not depend on the kinase activity of p38.

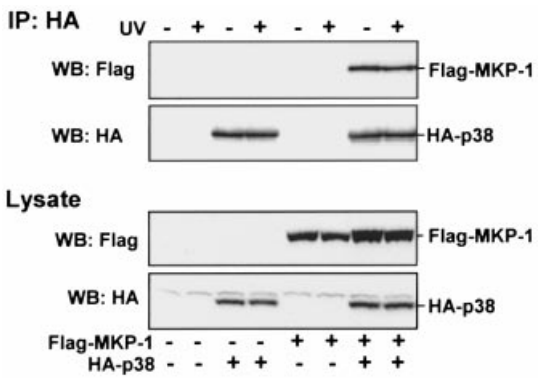

Figure 1 p38 interacts with MKP-1 *in vivo*

293T cells were transiently transfected with 4 μg of pSRα-HA–p38 (lanes 3 and 4), 6 μg of pSRα-Flag–MKP-1 (lanes 5 and 6) or both plasmids (lanes 7 and 8). Cells were irradiated with UVC or left untreated, and harvested 30 min later. HA–p38 was immunoprecipitated (IP) from 2 mg of total cell lysates with the anti-HA matrix. Samples in the first two lanes were from untransfected cells. The immunoprecipitated proteins were eluted from the matrix and analysed by Western blot (WB) analysis with anti-Flag antibody (top panel). The same membrane was stripped and re-probed with anti-HA antibody (second panel). Flag–MKP-1 (third panel) and HA–p38 (bottom panel) proteins in the lysates were detected by Western blot analysis.

p38 MAP kinase stimulates the catalytic activity of MKP-1

To examine the effect of p38 on the catalytic activity of MKP-1, recombinant MKP-1 was expressed in 293T cells as a GST fusion protein, and purified using glutathione–Sepharose beads. After extensive washing with buffers containing 200 mM NaCl, the fusion proteins were eluted with glutathione. GST–MKP-1 was enriched to a purity of approx. 60 %, as judged by Coomassie Blue staining (Figure 2A, left panel). Western blot analysis of the enriched proteins with anti-GST antibody revealed a single band of approx. 66 kDa (Figure 2A, right panel), consistent with the notion that GST–MKP-1 was expressed and enriched as a full-length protein. Re-probing this membrane with various antibodies against MAP kinase did not detect any contamination of the endogenous MAP kinase proteins (results not shown). The catalytically inactive GST–MKP-1CS mutant was also expressed and purified in a similar fashion. The catalytic activity of the recombinant MKP-1 proteins was measured as hydrolysis of *p*-NPP (Figure 2B). Consistent with a previous report [24], recombinant MKP-1 protein alone exhibited low but significant activity to hydrolyse *p*-NPP, while the MKP-1CS protein had no effect on *p*-NPP (Figure 2B). Furthermore, incubation of GST–MKP-1 with recombinant p38 protein resulted in a dose-dependent increase in the catalytic activity of GST–MKP-1 towards *p*-NPP (Figure 2B). While 32 μg of GST–p38 alone had no effect on *p*-NPP hydrolysis, it increased the catalytic activity of 3.5 μg of GST–MKP-1 protein by approx. 7-fold (Figure 2B).

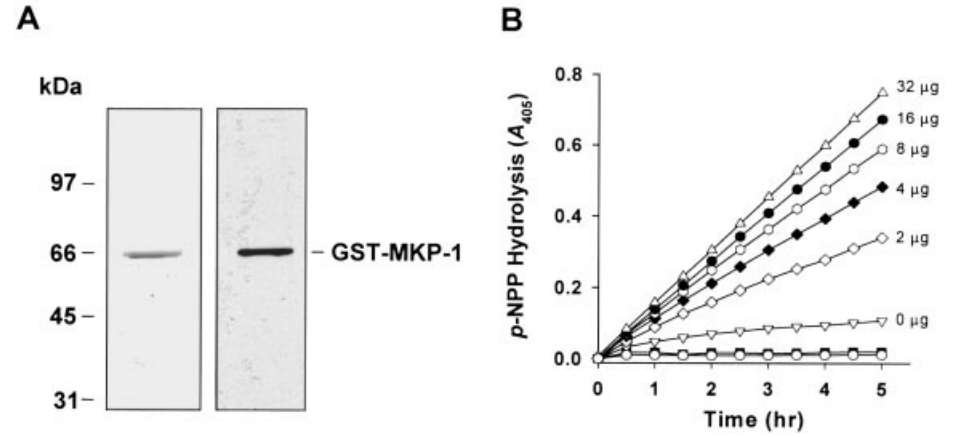

Figure 2 Catalytic activation of MKP-1 by p38 MAP kinase

GST–MKP-1 was expressed in 293T cells and purified using glutathione–Sepharose beads. GST–p38 was expressed in *E. coli* and purified by glutathione–Sepharose chromatography. The fusion proteins were eluted with 10 mM glutathione. Phosphatase activity of GST–MKP-1 was measured as *p*-NPP hydrolysis at 25 °C, monitored as absorbance at 405 nm (A_{405}). (**A**) Purified GST–MKP-1. Left panel: Coomassie Blue staining of GST–MKP-1 protein. Molecular masses of the protein markers are indicated on the left. Right panel: immunoblot of GST–MKP-1 fusion protein with anti-GST antibody. (**B**) Time course of *p*-NPP hydrolysis by 3.5 μg of GST–MKP-1 in the presence of the indicated amount of GST–p38. The hydrolysis of *p*-NPP by 3.5 μg of catalytically inactive GST–MKP-1CS (■) or 32 μg of GST–p38 (○) is presented in the same panel.

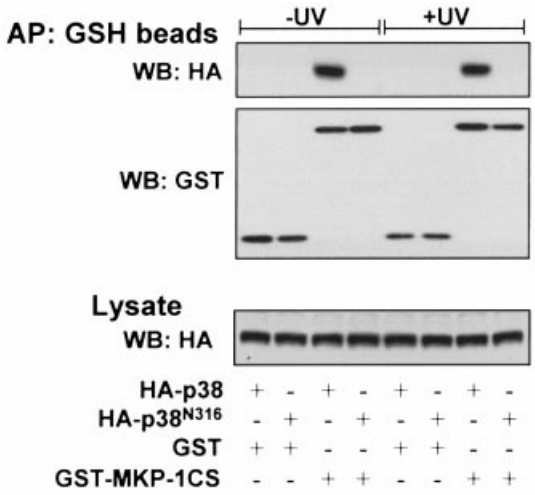

Figure 3 Point mutation of aspartic acid to asparagine at position 316 of p38 (p38^{N316}) reduces the affinity of the kinase for MKP-1

HeLa cells were transiently transfected with 2 μg of pEBG or pEBG-MKP-1CS and 1 μg of pSRα-HA–p38 or pSRα-HA–p38^{N316}, as indicated. Cells were then treated with UVC or were left untreated. GST and GST–MKP-1CS were affinity-purified (AP) with glutathione–Sepharose beads and subjected to Western blot (WB) analysis with anti-HA antibody (top panel). This membrane was stripped and re-probed with anti-GST antibody (middle panel). Total cell lysates from the above samples were used for Western blot analysis with anti-HA antibody (bottom panel).

A *sevenmaker*-like mutation in p38 substantially reduces its affinity for MKP-1 and significantly compromises its stimulatory effect on MKP-1 catalytic activity

A *sevenmaker* mutation in the C-terminus of ERK2 (ERK2 D319N) results in enhanced kinase activity, which is due to resistance to inactivation by phosphatases [13,16,17,25,26]. p38 has a very similar C-terminal region, in which the aspartic acid residue is conserved. To determine if this site of p38 is involved in the catalytic activation of MKP-1 and/or the negative regulation by this phosphatase, a *sevenmaker*-like mutation was created in p38 by site-directed mutagenesis to change the aspartic acid to asparagine at amino acid position 316. To ascertain the affinity of this mutant protein for MKP-1, a construct expressing this mutant p38 (HA–p38^{N316}) or a vector expressing wild-type HA–p38 was co-transfected into HeLa cells with a construct expressing either GST or GST–MKP-1CS. GST–MKP-1CS has a Cys-258 → Ser mutation in the catalytic domain of MKP-1, which renders it catalytically inactive and considerably more stable than the wild-type protein (results not shown). Following transfection, the cells were either irradiated with UVC or left untreated. GST and GST–MKP-1CS were affinity purified from the lysates, and the co-purification of HA–p38 or HA–p38^{N316} was analysed by Western blot analysis using anti-HA antibody. Binding of HA–p38^{N316} to GST–MKP-1CS was virtually undetectable, whereas the wild-type HA–p38 was able to bind to GST–MKP-1CS under both control and UVC-stimulated conditions (Figure 3, top panel). Levels of HA–p38 and HA–p38^{N316} in the cell lysates (Figure 3, bottom panel), as well as those of GST–MKP-1CS (Figure 3, middle panel), were comparable among samples.

The binding affinity of wild-type MKP-1 for p38 and p38^{N316} was also examined *in vitro*. Recombinant GST, GST–p38 and GST–p38^{N316} fusion proteins were immobilized on glutathione–Sepharose beads, and incubated with lysates from cells transfected with the Flag–MKP-1 expression vector. Flag–MKP-1 protein in the HeLa cell lysate was able to form complexes with GST–p38, but not with GST alone (Figure 4A). The binding of Flag–MKP-1 to GST–p38^{N316} was reduced to be barely detectable. To investigate if this reduced affinity for p38^{N316} was specific for MKP-1, the binding of Flag–MKP-2 to wild-type and mutant p38 fusion proteins was also tested. Like MKP-1, MKP-2 can also be rapidly induced by extracellular stimuli and localized exclusively in the nucleus [27,28]. As shown in Figure 4(B), Flag–MKP-2 bound to GST–p38, but not to GST–p38^{N316} or GST alone. These results indicate that the C-terminal domain of p38 serves as a common binding site for both MKP-1 and MKP-2.

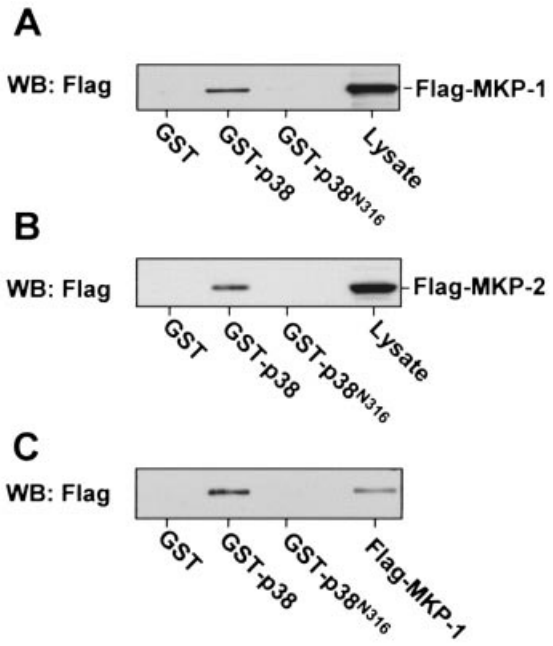

Figure 4 p38^{N316} mutant protein exhibits a substantially reduced interaction with MKP-1 and MKP-2 *in vitro*

(**A**) A portion of 4 μg of purified GST, GST–p38 or GST–p38^{N316} bound to glutathione–Sepharose beads was incubated with 200 μg of lysates from HeLa cells transfected with pSRα-Flag–MKP-1. Flag–MKP-1 bound to the purified proteins was detected by Western blot (WB) analysis with anti-Flag antibody. (**B**) A portion of 4 μg of purified GST, GST–p38 or GST–p38^{N316} bound to glutathione–Sepharose beads was incubated with 200 μg of lysates from HeLa cells transfected with pSRα-Flag–MKP-2. Flag–MKP-2 bound to the purified proteins was detected by Western blot analysis with anti-Flag antibody. (**C**) Purified Flag–MKP-1 protein was incubated with 4 μg of purified GST, GST–p38 or GST–p38^{N316} bound to glutathione–Sepharose beads. The beads were then washed as described and subjected to Western blot analysis using anti-Flag antibody. In these experiments, lysates containing the appropriate Flag-tagged phosphatase (**A** and **B**) or purified Flag–MKP-1 protein (**C**) were included in the last lane as a positive control.

In order to determine whether the interaction between MKP-1 and p38 was direct or dependent upon scaffolding proteins present in the cell lysates, the interaction between GST–p38 protein and purified Flag–MKP-1 protein was examined. Flag–MKP-1 was expressed in 293T cells, affinity-purified using an immobilized monoclonal antibody against the Flag tag, and then eluted from the immunocomplexes. Similar to the observation using the Flag–MKP-1-containing lysates, the purified Flag–MKP-1 protein bound to GST–p38, but not to GST–p38^{N316} (Figure 4C).

The influence of this p38 mutation on the p38-mediated catalytic activation of MKP-1 was examined using *p*-NPP hydrolysis assays (Figure 5). Consistent with the observation that the p38^{N316} mutant displays a severe defect in its ability to bind to MKP-1, it also exhibited significantly reduced efficiency in the catalytic activation of GST–MKP-1. Approximately 4 times more p38^{N316} protein was required to achieve the same stimulatory effect as with wild-type p38 (Figure 5A). The deficient ability of p38^{N316} to trigger MKP-1 activation does not reflect misfolding of GST–p38^{N316}, since GST–p38^{N316} was phosphorylated as effectively as GST–p38 by constitutively active MKK6b protein, and exhibited intrinsic kinase activity towards MBP comparable with that of GST–p38 (Figure 5B). Taken together, these results demonstrate that MKP-1 interacts with p38 through the C-terminal domain of p38. The *sevenmaker*-like mutation severely decreases the interaction of p38 with both MKP-1 and MKP-2, and compromises the stimulatory effect of p38 on the catalytic activity of MKP-1.

p38^{N316} displays increased resistance to dephosphorylation by MKP-1

The effect of the *sevenmaker*-like mutation on the MKP-1-mediated dephosphorylation of p38 was examined in the following transient transfection experiments. First, either Myc–p38 or Myc–p38^{N316} was transiently expressed in HeLa cells along

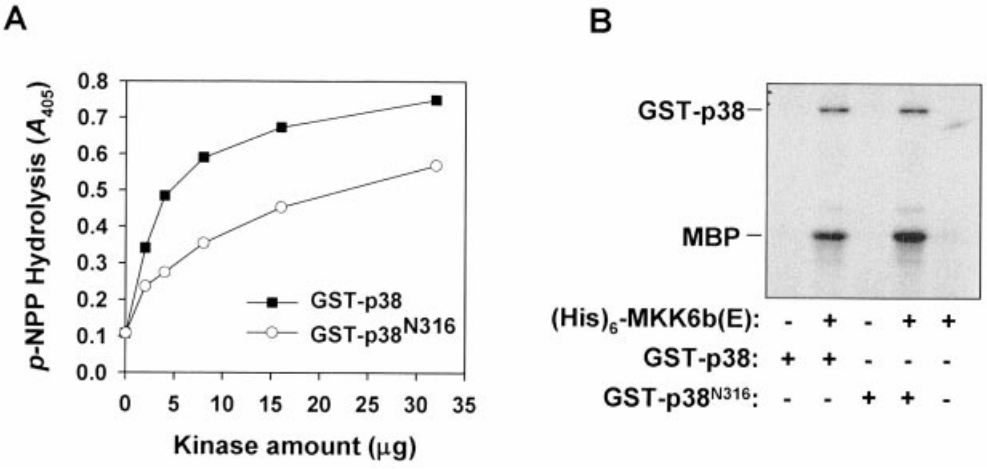

Figure 5 p38^{N316} exhibits a substantially reduced ability to catalytically activate MKP-1

(**A**) The phosphatase activity of 3.5 μg of GST–MKP-1 was measured as *p*-NPP hydrolysis, monitored as absorbance at 405 nm (A_{405}), in the presence of the increasing amounts of GST–p38 (■) or GST–p38^{N316} (○) fusion proteins. GST–p38^{N316} was expressed in *E. coli* and purified by glutathione–Sepharose chromatography. *p*-NPP hydrolysis at the 5 h time point is presented. (**B**) Activation of GST–p38 and GST–p38^{N316} by constitutively active MKK6b. A 0.5 μg portion of GST–p38 or GST–p38^{N316} was incubated with [γ-^{32}P]ATP and 6 μg of MBP in the absence or presence of 0.1 μg of (His)$_6$–MKK6b(E). Protein phosphorylation was detected by autoradiography after separation of the reaction by electrophoresis. Phosphorylation of MBP by MKK6b(E) alone was included in the last lane as a control.

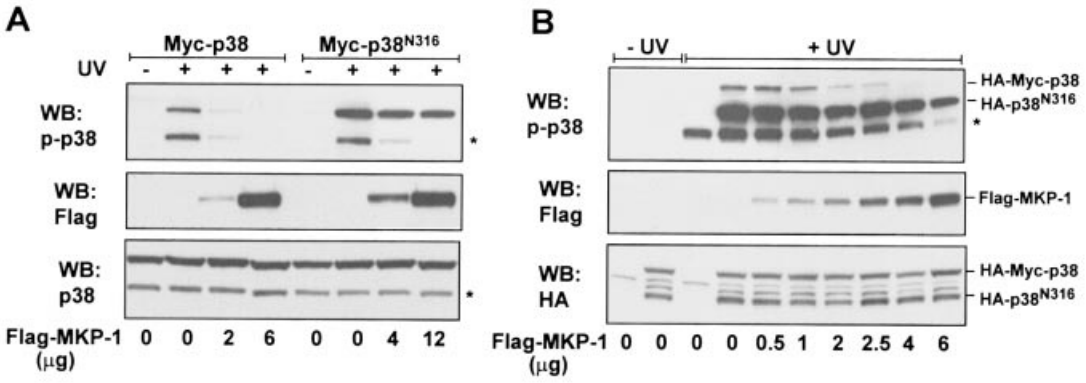

Figure 6 p38^{N316} displays increased resistance to dephosphorylation by MKP-1

(**A**) HeLa cells were transiently transfected with 1 μg of either pSRα-Myc–p38 or pSRα-Myc–p38^{N316}, along with the indicated amounts of pSRα-Flag–MKP-1. Cells were either treated with UVC or were left untreated, and total cell lysates were used for Western blot (WB) analysis with anti-phospho-p38 antibody. The membrane was stripped and re-probed with both anti-Flag and anti-p38 antibodies. (**B**) HeLa cells were transfected with a mixture of 1.5 μg of pSRα-HA–Myc–p38 and 1 μg of pSRα-HA–p38^{N316}, and the indicated amounts of pSRα-Flag–MKP-1. Cells were either treated with UVC or were left untreated, and total cell lysates were used for Western blot analysis with the anti-phospho-p38 antibody. Samples in the first and third lanes are from untransfected cells. The membrane was stripped and re-probed with anti-Flag and anti-HA antibodies. The position at which endogenous p38 migrates is indicated by *.

with increasing amounts of Flag–MKP-1 plasmid (Figure 6A, middle panel). Following UVC stimulation, the ability of MKP-1 to dephosphorylate p38 and p38^{N316} was evaluated by Western blot analysis using an antibody specific to phospho-p38. The overall levels of p38 and p38^{N316} were comparable among the samples (Figure 6A, bottom panel). In the absence of Flag–MKP-1, both Myc–p38 and Myc–p38^{N316}, as well as the endogenous p38 proteins, were phosphorylated following UV treatment. In cells expressing Myc–p38, UV-induced phosphorylation of both Myc–p38 and endogenous p38 was potently inhibited by Flag–MKP-1 in a dose-dependent manner (Figure 6A, top panel). In cells expressing Myc–p38^{N316}, although Flag–MKP-1 effectively inhibited UV-induced phosphorylation of endogenous p38, even the highest dose of Flag–MKP-1 had little effect on the phosphorylation of Myc–p38^{N316} (Figure 6A, top panel).

To ensure that these striking differences between the wild-type and mutant p38 proteins were not due to secondary effects resulting from the transfected plasmids, an alternative experimental design was employed. In order to distinguish HA–p38 from HA–p38^{N316} so that their sensitivities to MKP-1-mediated inactivation could be compared directly within the same cells, the HA–p38 expression vector was engineered to add three tandem repeated Myc tags between the HA-coding and p38-coding sequences (HA–Myc–p38). A mixture of the two plasmids, expressing either HA–p38^{N316} or HA–Myc–p38, were transiently transfected into HeLa cells together with increasing amounts of Flag–MKP-1 plasmid (Figure 6B). Expression levels of HA–p38^{N316} and HA–Myc–p38 were comparable among all transfected samples (Figure 6B, bottom panel). No appreciable difference in the basal levels of phosphorylation between HA–Myc–p38 and HA–p38^{N316} was detected. However, upon UVC irradiation, HA–p38^{N316} was phosphorylated to a much greater degree than HA–Myc–p38. Additionally, in comparison with HA–Myc–p38, HA–p38^{N316} displayed significantly stronger resistance to dephosphorylation by MKP-1. The strikingly higher phosphorylation of HA–p38^{N316} compared with HA–Myc–p38 in the absence of Flag–MKP-1 suggests that p38^{N316} is also significantly more resistant to dephosphorylation by endogenous phosphatases, probably including MKP-1.

The effect of the increased resistance of p38^{N316} to dephosphorylation by MKP-1 on downstream targets was assessed

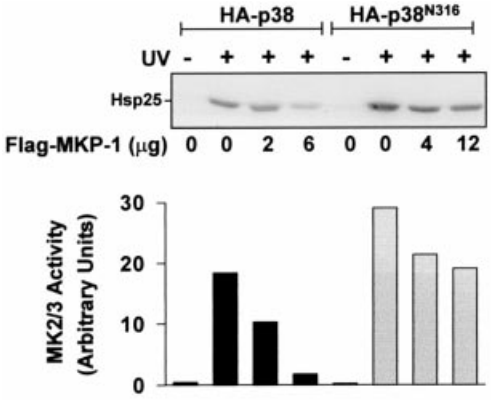

Figure 7 Resistance of p38^{N316} to inactivation by MKP-1 is reflected in an increase in UVC-stimulated MK2/3 activity

HeLa cells were transiently transfected with 1 μg of either pSRα-HA–p38 or pSRα-HA–p38^{N316}, and the indicated amounts of pSRα-Flag–MKP-1. Cells were either treated with UVC or were left untreated, and MK2/3 kinase activity was assessed by immunocomplex kinase assays using Hsp25. The incorporated ^{32}P was quantified using the ImageQuant program (Molecular Dynamics). Bars are placed directly underneath the lanes that they represent.

by examining the activity of the p38-regulated protein kinases MK2/3. The almost complete inhibition of the phosphorylation of endogenous p38 by the transfection of higher amounts of Flag–MKP-1 suggests that nearly all cells were transfected (Figure 6A). Therefore endogenous MK2/3 can serve as a downstream reporter of p38 activity. HeLa cells were transfected with either HA–p38 or HA–p38^{N316} expression vector and the indicated amounts of Flag–MKP-1 (Figure 7). The activity of MK2/3 was assessed through immunocomplex kinase assays. As indicated in Figure 7, without UVC treatment, MK2/3 activity was low but comparable in cells transfected with either HA–p38 or HA–p38^{N316} expression plasmids. Upon UVC treatment, MK2/3 was activated to a higher level in cells expressing HA–p38^{N316} than in cells expressing HA–p38. Most importantly, increased MKP-1 expression potently inhibited UVC-induced

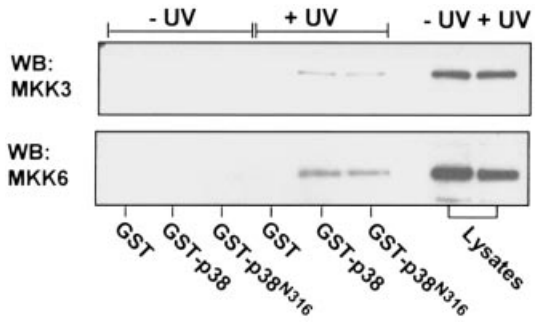

Figure 8 Mutation of Asp-316 to Asn in p38 does not affect its interaction with either MKK3 or MKK6

Portions of 200 μg of lysate proteins from control or UVC-treated HeLa cells were incubated overnight at 4 °C with 4 μg of purified GST, GST–p38 or GST–p38^{N316} bound to glutathione–Sepharose beads. The beads were washed as described and the bound proteins were analysed by Western blot (WB) analysis using anti-MKK3 (upper panel) and anti-MKK6 (lower panel) antibodies. Total cell lysates containing 20 μg of lysate proteins from both UVC-stimulated and control cells were included in the end lanes as positive controls.

MK2/3 activation, while expression of MKP-1 had only a minor effect on UVC-induced MK2/3 activation in cells expressing HA–p38^{N316}.

Mutation of Asp-316 to Asn does not affect the interaction between p38 and either MKK3 or MKK6

Very recently, it has been demonstrated that the interaction between p38 and its upstream kinase MKK6 occurs through the same domain that is responsible for its interaction with MKP-5 [29]. To determine if the Asp-316 → Asn mutation interferes with the binding of this protein to upstream kinases, the interaction between p38 or p38^{N316} and MKK3 or MKK6 was analysed (Figure 8). Lysate proteins from control or UVC-treated HeLa cells were incubated with GST, GST–p38 or GST–p38^{N316} immobilized on glutathione–Sepharose beads. MKK3 or MKK6 pulled down by the GST fusion proteins was detected by Western blot analysis. As indicated in Figure 8, GST alone did not bind either MKK3 or MKK6 in any of the lysates. The amount of MKK3 or MKK6 pulled down by either GST–p38 or GST–p38^{N316} from control lysates was barely detectable. However, both GST–p38 and GST–p38^{N316} (Figure 8, upper panel) pulled down comparable amounts of MKK3 from lysates of UVC-treated HeLa cells. Similarly, the amounts of MKK6 pulled out by GST–p38 and GST–p38^{N316} from lysates of UVC-irradiated cells were also comparable. Western blot analysis verified that lysates obtained from control and UVC-treated cells contained equal amounts of MKK proteins (either MKK3 or MKK6) (Figure 8). Therefore these results indicate that this Asp-316 → Asn mutation does not interfere with the ability of p38 to interact with its direct upstream kinases. Our results also suggest that p38 displays a higher affinity for the activated (relative to inactive) forms of MKK3 or MKK6 proteins.

DISCUSSION

Interaction between MKP-1 and p38

Previous studies have indicated that a number of predominantly cytosolic MAP kinase phosphatases and at least two cytosolic tyrosine phosphatases can interact with and effectively inactivate p38 [13,25,30,31]. In addition to these predominantly cytosolic phosphatases, at least two nuclear MAP kinase phosphatases, MKP-1 and the haematopoietic PAC-1, have also been demonstrated to inactivate p38 efficiently [17,19,32,33]. However, the interaction between p38 and these nuclear MAP kinase phosphatases has not been investigated. In the present study, we have demonstrated a direct interaction between MKP-1 and p38. Unlike ERK2, which only binds to a catalytically inactive MKP-1 mutant [10,34], p38 interacts with both the wild-type and catalytically inactive phosphatases (Figures 1 and 3). Similar to the interaction between MKP-3 and ERK MAP kinases [13,15], the interaction between MKP-1 and p38 does not seem to depend on the catalytic activity of the kinase, since this interaction did not differ between control and UVC-treated cells (Figure 1). The association between MKP-1 and p38 appears to be mediated primarily through the C-terminal domain of the kinase. Previously, this p38 domain has been implicated in the interaction of p38 with both MKP-5 [29] and the cytoplasmic tyrosine phosphatase LC-PTP [31]. A point mutation in p38 which changes the acidic residue Asp-316 into Asn, analogous to the ERK2 *sevenmaker* mutant [13], greatly reduces its ability to bind to MKP-1 and severely compromises its stimulatory effect on the catalytic activity of this phosphatase (Figures 4–6). The substantially compromised, but still evident, stimulatory effect of p38^{N316} on the catalytic activity of MKP-1 suggests that other residues, in addition to Asp-316, may also be involved in the interaction between these two molecules. Very recently, Tanoue et al. [29] have demonstrated that mutating Asp-313 and Asp-315 to Asn in the C-terminal domain of p38 decreases the interaction between p38 and MKK6. Along with these two mutations, mutating a third acidic residue, Asp-316, to Asn completely abolishes the interaction between p38 and MKP-5. Therefore it is likely that, in addition to Asp-316, these two residues (Asp-313 and Asp-315) also play a role in the interaction between p38 and MKP-1, and are responsible for the stimulatory effect of the p38^{N316} mutant on MKP-1 catalytic activation.

Interestingly, the p38 triple mutant used by Tanoue et al. [29] binds neither MKP-5 nor MKK6. We found that, unlike that triple mutant, mutation of Asp-316 alone (p38^{N316}) resulted in decreased interaction with MKP-1, but had little effect on binding to either MKK3 or MKK6. The observation that p38^{N316} becomes potently phosphorylated upon UVC treatment is more consistent with the gain-of-function phenotype of the ERK *sevenmaker* mutant. One obvious explanation for the different binding properties of the two distinct mutants is that perhaps the defect of the triple mutant used by Tanoue et al. [29] is more severe than that of the single mutant we used. It is possible that primary residues involved in the interaction between p38 and MKK3 or MKK6 are different from those involved in the interaction between p38 and MKP-1. That is, Asp-316 is the principal residue responsible for the interaction of p38 with MKP-1, while it plays only a minor role in the interaction with MKK3 or MKK6. Thorough quantitative analysis of the interactions between these kinases and phosphatases may provide important insight into the complex regulation of MAP kinase cascades.

Catalytic activation of MKP-1 by p38

A number of dual-specificity MAP kinase phosphatases encoded by immediate-early genes have been identified in the last few years, and have been demonstrated to localize predominantly in the nucleus [9,11,27,35]. Since genes encoding these phosphatases are highly inducible by conditions that also activate MAP kinase pathways, it has been suggested that they may play a crucial role in the feedback control of MAP kinase-regulated gene tran-

scription [9–12]. In the present paper we provide the first example of catalytic activation of an inducible nuclear MAP kinase phosphatase through interaction with a MAP kinase. Our results suggest that catalytic activation of the nuclear phosphatases through interaction with MAP kinases may serve as an additional feedback control mechanism to regulate MAP kinase-regulated gene transcription.

Despite the fact that catalytic activation by binding to MAP kinases has been demonstrated for several cytosolic MAP kinase phosphatases, it is becoming clear that not all of these phosphatases undergo MAP kinase-dependent activation. For example, MKP-5 has been shown to interact with and effectively dephosphorylate p38, yet binding to p38 has little effect on the catalytic activity of MKP-5 [30]. Similarly, binding of the tyrosine phosphatase PTP-SL to ERK2 does not result in enhanced activity of PTP-SL against *p*-NPP [26]. On the other hand, MKP-3 and Pyst2 are highly activated through binding to ERK2, while MKP-1, MKP-4 [13] and to a lesser extent Pyst2 [14] are significantly activated through binding to p38. Recent studies on the crystal structure of the catalytic domain of MKP-3/Pyst1 have suggested that catalytic activation of this phosphatase by binding to ERK2 is mediated through a conformational change that moves the 'general acid loop' to the active site [36]. Given the amino acid differences in the acidic loops of these phosphatases, it is not surprising that they may display different activation profiles in response to p38 binding. A significant difference between the reported activation profile of MKP-3 by ERK2 and that of MKP-1 by p38 (Figure 5) is that a much higher p38/MKP-1 ratio appears to be required to reach the optimal catalytic activity of MKP-1. This suggests that the interaction between p38 and MKP-1 may be weaker, and, as a result, a higher p38/MKP-1 ratio is required for achieving the optimal active state of the phosphatase.

Although we have demonstrated that the nuclear MAP kinase phosphatase MKP-1 can be catalytically activated through binding to p38, it remains to be determined whether other nuclear MAP kinase phosphatases share this catalytic activation property. It is also possible that this ability to catalytically activate MKP-1 is not restricted to p38. In fact, we have obtained preliminary evidence to indicate that both ERK1 and JNK1 can stimulate the catalytic activity of MKP-1 (P. Chen, D. Hutter and Y. Liu, unpublished work). In summary, MKP-1 expression is tightly regulated both at the transcriptional level and through post-translational stabilization by MAP kinase-mediated phosphorylation [37]. Catalytic activation of MKP-1 through interaction with MAP kinases is likely to confer additional strength and versatility to the phosphatase for responding to diverse extracellular signals.

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