Protein Phosphatase 4 Is Involved in Tumor Necrosis Factor-α-induced Activation of c-Jun N-terminal Kinase*

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Protein phosphatase 4 (PP4, previously named protein phosphatase X (PPX)), a PP2A-related serine/threonine phosphatase, has been shown to be involved in essential cellular processes, such as microtubule growth and nuclear factor κB activation. We provide evidence that PP4 is involved in tumor necrosis factor (TNF)-α signaling in human embryonic kidney 293T (HEK293T) cells. Treatment of HEK293T cells with TNF-α resulted in time-dependent activation of endogenous PP4, peaking at 10 min, as well as increased serine and threonine phosphorylation of PP4. We also found that PP4 is involved in relaying the TNF-α signal to c-Jun N-terminal kinase (JNK) as indicated by the ability of PP4-RL, a dominant-negative PP4 mutant, to block TNF-α-induced JNK activation. Moreover, the response of JNK to TNF-α was inhibited in HEK293 cells stably expressing PP4-RL in comparison to parental HEK293 cells. The involvement of PP4 in JNK signaling was further demonstrated by the specific activation of JNK, but not p38 and ERK2, by PP4 in transient transfection assays. However, no direct PP4-JNK interaction was detected, suggesting that PP4 exerts its positive regulatory effect on JNK in an indirect manner. Taken together, these data indicate that PP4 is a signaling component of the JNK cascade and involved in relaying the TNF-α signal to the JNK pathway.

A major mechanism by which cells regulate protein function is to add or remove phosphate groups on serine, threonine, and tyrosine residues. The steady-state level of phosphorylation and, thus, the strength and duration of the signal transmitted are balanced by the opposing actions of protein kinases and protein phosphatases (1–3). Protein kinases, protein phosphatases, and their substrates are integrated within an elaborate signaling network (3–5). The defective or inappropriate operation of this network leads to many diseases such as cancer, diabetes, and autoimmune disorders (6).

Mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), and p38, play essential roles in many important biological processes such as the stress response, cell proliferation, apoptosis, and tumorigenesis (7–9). MAPK activation involves sequential protein kinase reactions within a three-kinase module (MAP3K-MAP2K-MAPK), whereby a MAP3K phosphorylates and activates a MAP2K, a dual-specificity kinase, that then phosphorylates and activates a MAPK (7, 8, 10). In vivo MAPK phosphorylation is a reversible process, indicating that protein phosphatases provide an additional level of regulation of MAPKs. In fact, the magnitude and duration of MAPK activation are tightly controlled by the coordinate actions of protein kinases and protein phosphatases. A large number of mammalian MAPK phosphatases have been identified, including dual-specificity phosphatases and tyrosine-specific phosphatases (11, 12). There is evidence that serine/threonine-specific phosphatases also regulate MAPKs (13, 14). MAPK phosphatases inactivate MAPKs by directly dephosphorylating both threonine and tyrosine residues of MAPKs (12). The coordinate regulation by protein kinases and phosphatases also occurs at many other points within the three-kinase module. For example, MKP-1, a dual-specificity phosphatase, inhibits ERK, but positively regulates Raf-1 and MKK in an ERK-independent manner (15). PP2A also acts on multiple components of the ERK pathway (12).

Protein phosphatase 4 (PP4, previously named protein phosphatase X (PPX)) is a novel protein serine/threonine phosphatase that is a member of the PP2A family of phosphatases (16). PP4 is highly conserved during evolution, with human and Drosophila PP4 sharing 91% amino acid identity (16). It has been shown that PP4 is localized at the centrosomes in mammalian cells and Drosophila embryos, and that PP4 is involved in the regulation of microtubule growth/organization at centrosomes (17, 18). Our previous studies showed that PP4 interacts with members of the nuclear factor κB (NF-κB) family (such as c-Rel, p50, and RelA), stimulates the DNA binding activity of c-Rel, and activates NF-κB-mediated transcription (19). The high degree of conservation of PP4 suggests that PP4 may be

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The abbreviations used are: MAPK, mitogen-activated protein kinase; TNF-α, tumor necrosis factor-α; PP4, protein phosphatase 4; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; HEK293, human embryonic kidney 293; GST, glutathione S-transferase; PKC, protein kinase C; DMEM, Dulbecco’s modified Eagle’s medium; PIPES, 1,4-piperazinediethanesulfonic acid; PCM, pericentriolar matrix; MKK, MAPK kinase; TBS, Tris-buffered saline; TBST, Tris-buffered saline plus Tween 20; DAPI, 4,6-diamino-2-phenylindole; PVDF, polyvinylidene difluoride; NF-κB, nuclear factor κB.
involved in many more essential cellular processes and is tightly controlled in vivo. It has been shown that PP4 is car-
boxymethylated (20). Furthermore, three potential regulatory
modified atmosphere of 5% CO\textsubscript{2}. HEK293T cells were plated at a density

**MATERIALS AND METHODS**

**Reagents—**\[\gamma\]\textsuperscript{32}P\textsuperscript{ATP} and [\textsuperscript{32}P]orthophosphate were purchased from ICN Biomedicals (Irvine, CA). An enhanced chemiluminescence system was purchased from Amersham Biosciences, Inc. Ser/Thr phosphatase assay kit 1 was purchased from Upstate Biotechnology, Inc. (Waltham, MA). TNF-\alpha was purchased from R&D Systems. Anti-HA antibody (12CA5) was purchased from Roche Molecular Biochemicals. Monoclonal anti-Flag (M2) and anti-\gamma-tubulin antibodies were purchased from Sigma. Monoclonal anti-PP1 and anti-c-Myc (9E10) antibodies, and goat anti-Bcl-X\textsubscript{L} antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-aldolase antibody was purchased from Biodesign (Saco, ME). Monoclonal anti-golgin-97 was purchased from Molecular Probes (Eugene, OR). Rabbit anti-GRPT8 polyc-

**Centrosome Isolation—**Centrosomes were purified from HeLa cells by a standard protocol (32, 33). Briefly, \(6 \times 10^5\) HeLa cells were incubated with 0.2 \mu M nocodazole and 1 \mu M cytochalasin D at 37 °C for 60 min. After trypsinization, the cells were pelleted and washed one time with 1× PBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) and one time with 0.1× PBS + 8% sucrose. The cells were then resuspended in 2 ml of 0.1× PBS + 8% sucrose and lysed by adding 8 ml of fractionation lysis buffer (1 mM HEPES [pH 7.2], 0.5% Nonidet P-40, 0.5 mM MgCl\textsubscript{2}, 0.1% \text{-mercaptoethanol}, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mM p-aminophenylmercuricbenzenesulfon fluoride, 1 mM Na\textsubscript{2}VO\textsubscript{4}, and 0.5 mM NaF). The lysate was spun at 10,000 × g for 15 min. The supematant was collected and spun again at 2,500 × g for 10 min. The supernatant was transferred into a new tube through a 70-\mu m nylon filter (Falcon 2530). The resulting supernatant was incubated with 10 mM HEPES and 1 mg/ml DNase on ice for 30 min, transferred to a 15-ml ultracen-

**In Vivo Binding Assays—**GST and GST-SAPK fusion protein were immobilized on glutathione-Sepharose 4B beads equilibrated in incubation buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethyl-
sulfonyl fluoride, 1 mg/ml leupeptin, and 2 mg/ml aprotinin. Cell lysates (600 \mu g) from HEK293 cells stably transfected with Flag-PP4 or GST cells transiently transfected with Myc-M3 were incubated with GST-JNK fusion protein or GST-4T-Sepharose beads in incubation buffer containing 3 mg/ml bovine serum albumin at 4 °C for 2 h. The beads were washed five times with the incubation buffer, boiled in a SDS-PAGE loading buffer for 5 min, resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and then subjected to Western blotting with an anti-Flag (M2) or an anti-Myc antibody. The membrane was then stripped with stripping buffer (62.5 mM Tris-HCl [pH 6.7], 10 mM 2-mercaptoethanol, 2% SDS) and reprobed with an anti-GST antibody.

**Immunofluorescence—**As described in detail (34), cells were grown on coverslips and the coverslips were washed with 0.5% Triton X-100 for 2 min and fixed in 4% ultrapure formaldehyde (Polysciences, Inc.) in PEM buffer (80 mM K-PIPES [pH 7.0], 5 mM EGTA, 2 mM MgCl\textsubscript{2}) for 10–20 min. For the immunofluorescence of \beta-tubulin, 4% polyethylene glycol was added to PEM buffer during the permeabilization and fixation steps. After they were fixed, the coverslips were washed with PEM buffer and permeabilized in 0.5% Triton X-100 in PEM buffer for 30 min. Coverslips were then washed with PEM buffer and blocked in 2.5% nonfat dry milk in TBST buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 0.1% Tween 20) overnight. The next day, the coverslips were incubated for 1 h at 37 °C with primary antibodies diluted in TBST, washed in TBST, and incubated for 1 h at 37 °C with secondary antibodies diluted in 1:200 in TBST. After washing in TBST, coverslips were counterstained with 0.4 \mu g/ml 4,6-diamino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR) in TBST and mounted with

**Phosphatase Assays—**HEK293T cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA, 1 mM dithiothreitol, 50 \mu M \text{p-aminophenylmercuri}-

**Coimmunoprecipitation, Immunocomplex Kinase Assays, and Western Blot Analysis—**Coimmunoprecipitation and immunocomplex kinase assays were performed as described previously (28–31). Western blot analysis was performed using an enhanced chemiluminescence detection kit according to the manufacturer's protocols (Amersham Biosciences, Inc.).
FIG. 1. Characterization of a PP4-specific antibody, Ab104. A, the anti-PP4 antibody, Ab104, specifically recognizes PP4, but not PP2A and PP6. HEK293T cells were transfected with 2 μg of empty vector (lanes 1), 2 μg of Flag-PP4 (lanes 2), 2 μg of Flag-PP2A (lanes 3), or 2 μg of Flag-PP6 (lanes 4). Cells were harvested 36 h after transfection and subjected to SDS-PAGE. Western blotting was performed with 1 μg/ml Ab104. The experiments were repeated four times with similar results. B, PP4 co-purifies with centrosomes. Centrosomes were prepared from 6 × 10⁷ HeLa cells and purified on a discontinuous sucrose gradient. 10% of protein recovered from each fraction and 5 μg of HeLa whole cell lysate (W) were Western-blotted for the presence of PP4 (Ab104) and subcellular compartment markers: γ-tubulin (centrosome), aldolase (cytosol), lamin B1 (nucleus), GRP78 (endoplasmic reticulum), golgin-97 (Golgi), and Bel₁X₁ (mitochondria). C, PP4 is a component of the centrosome. HeLa cells were grown on polylysine-coated coverslips, extracted in 0.5% Triton X-100 for 2 min, and fixed in 4% formaldehyde. Fixed cells were incubated with DAPI DNA stain (DAPI; blue), human autoimmune serum 4171 (red), and the peptide-purified antibody PP4 antibody Ab104 (PP4; green; panels a–d) or normal preimmune serum from the same rabbit used to generate Ab104, before immunization with peptide (a., green; panels e–h). Panels PCM, PP4, and DAPI were merged (merged; panel d), to identify areas of colocalization of PP4 and PCM staining (yellow). Arrows indicate position of centrosomes. The experiments were repeated at least three times with similar results.

RESULTS

PP4 Is Activated by TNF-α—In an effort to investigate which signaling pathway(s) PP4 may be involved in, we examined the effect of TNF-α on PP4 phosphatase activity. We first generated an anti-PP4 antibody, Ab104, which recognizes the C-terminal region of PP4. Western blot analysis indicated that Ab104 specifically recognized PP4, but not the most highly homologous phosphatases PP2A and PP6 (Fig. 1A). Previously, PP4 had been shown to localize to the centrosomes via immunofluorescence staining (17, 18). To confirm the specificity of Ab104, we isolated centrosomes from HeLa cells and performed Western blotting with antibodies to PP4 (Ab104), as well as markers for various subcellular compartments. PP4 localized to centrosome fractions, and these fractions were shown to be free of contamination from other subcellular compartments (Fig. 1B). We noticed that PP4 did not peak with γ-tubulin. Considering that γ-tubulin is a component of the centrosomes and that PP4 has been previously reported to be a component of the pericentriolar matrix of the centrosomes (17), the slight difference in the Western blot detection may be the result of slight differences in the densities of the two centrosomal structures. The association of PP4 with the centrosome was further confirmed by immunofluorescence staining using a peptide purified anti-PP4 antibody (Ab104). As shown in Fig. 1C, PP4 co-localized with proteins of the pericentriolar matrix (PCM). Taken together, these data show that PP4 is a component of the centrosome.

We then measured the phosphatase activity of PP4 before
cells were treated with TNF-α). The amounts of PP4 immunoprecipitated as a substrate (upper panel) PP4 was immunoprecipitated with an anti-PP4 antibody (Ab104). The JNK phosphatase activity was determined by using GST-c-Jun-(1-79) as a substrate. The experiments were repeated three times with similar results.

The experiments were repeated at least two times with similar results.

and after TNF-α treatment. PP4 phosphatase assays were established by using a synthetic peptide substrate, KTpIRR, as a substrate. As shown in Fig. 2C, JNK was activated by TNF-α (10 ng/ml) for the period of time indicated. Flag-PP4 was immunoprecipitated with an anti-Flag antibody (M2) and subjected to SDS-PAGE. The separated proteins were transferred to PVDF, and autoradiography was performed. B, TNF-α-induced phosphorylation occurs on serine and threonine residues of PP4. The corresponding PP4 bands were cut from the PVDF membrane (A) and subjected to phosphaamino acid analysis. The experiments were repeated at least two times with similar results.

FIG. 2. TNF-α activates both PP4 and JNK in HEK293T cells. A, establishment of PP4 phosphatase assays. 800 μg of HEK293 cell lysate was immunoprecipitated with either anti-PP4 antibody (Ab104) or protein A beads alone. The immunoprecipitates were washed and incubated with assay buffer and KTpIRR peptide at 30 °C for various times, from 0 to 120 min, as indicated (upper panel). 200, 400, 600, or 800 μg of HEK293 cell lysate was immunoprecipitated with either anti-PP4 antibody (Ab104) or beads alone. The immunoprecipitates were washed and incubated with assay buffer and KTpIRR peptide at 30 °C for 30 min (lower panel). The phosphatase assays were read at 650 nm. The readings are the average and standard deviation of three separate immunoprecipitations (PP4) or two separate immunoprecipitations (beads). B, TNF-α activates PP4 phosphatase activity. HEK293T cells were seeded at a density of 3.5 × 10⁶ cells/100-mm dish. After 24 h, the cells were treated with TNF-α (10 ng/ml) for various times as indicated. PP4 was immunoprecipitated with an anti-PP4 antibody (Ab104). The PP4 phosphatase activity was determined by using a synthetic peptide, KTpIRR, as a substrate (upper panel). The amounts of PP4 immunoprecipitated were monitored by Western blotting using an anti-PP4 antibody (Ab101; lower panel). The experiments were repeated at least three times with similar results. C, TNF-α activates JNK kinase activity. HEK293T cells were seeded at a density of 3.5 × 10⁶ cells/100-mm dish. After 24 h, the cells were treated with TNF-α (10 ng/ml) for various times as indicated. JNK was immunoprecipitated with an anti-JNK antibody (Ab101). The JNK phosphatase activity was determined by using GST-c-Jun(1-79) as a substrate. The experiments were repeated three times with similar results.

and after TNF-α treatment. PP4 phosphatase assays were established by using a synthetic peptide substrate, KTpIRR. We first wanted to ensure that the PP4 phosphatase assay is able to measure PP4 phosphatase activity. Thus, we tested the assay to determine the linear range of the assay and to show that increasing amounts of PP4 correlate with increasing PP4 activity. PP4 showed a time-dependent increase in its phosphatase activity in a time period of 1–50 min of incubation of PP4 with the peptide substrate (Fig. 2A, upper panel). Within this time frame, PP4 activity increased with increased amounts of PP4 (Fig. 2A, lower panel). HEK293T cells were treated with TNF-α (10 ng/ml), and endogenous PP4 was immunoprecipitated from the cells with the PP4-specific antibody, Ab104. The PP4 phosphatase activity was measured by incubating the immunoprecipitated PP4 with the peptide substrate, KTpIRR, for 30 min. PP4 phosphatase activity was increased following TNF-α stimulation in a time-dependent fashion, peaking at 10 min (Fig. 2B, upper panel). PP4 activity was decreased after 10 min, indicating that TNF-α-induced PP4 activation was a transient event. The increased phosphatase activity was not caused by variation in levels of PP4 because the amounts of PP4 immunoprecipitated were comparable (Fig. 2B, lower panel). Therefore, PP4 was activated in response to TNF-α in HEK293T cells.

It is known that TNF-α is a potent activator of the JNK pathway. To establish a possible link between PP4 and the JNK pathway in response to TNF-α, endogenous JNK was immunoprecipitated with an anti-JNK1 antibody (Ab101) from HEK293T cells, and its kinase activity was determined by an immunocomplex kinase assay using GST-c-Jun(1-79) as substrate. As shown in Fig. 2C, JNK was activated by TNF-α with kinetics similar to that of PP4 in HEK293T cells. Thus, PP4 was activated concomitantly with JNK activation in response to TNF-α in HEK293T cells.

TNF-α Induces Serine and Threonine Phosphorylation of PP4—To further confirm the involvement of PP4 in TNF-α signaling, we examined the effect of TNF-α on the phosphorylation state of PP4, because PP2A, the phosphatase most homologous to PP4, is regulated by phosphorylation. HEK293T cells were transfected with Flag-PP4, labeled in vivo with [32P]orthophosphate, and treated with TNF-α (10 ng/ml). Flag-PP4 was then immunoprecipitated with an anti-Flag antibody

FIG. 3. TNF-α induces serine and threonine phosphorylation of PP4. A, TNF-α induces PP4 phosphorylation. HEK293T cells (1 × 10⁶ cells in 100-mm dish) were transfected with 5 μg of Flag-PP4. After 40 h, the cells were labeled in phosphate-free DMEM supplemented with 5% of dialyzed serum and 100 μCi/ml [32P]orthophosphate for 4 h at 37 °C and treated with TNF-α (10 ng/ml) for the period of time indicated. Flag-PP4 was immunoprecipitated with an anti-Flag antibody (M2) and subjected to SDS-PAGE. The separated proteins were transferred to PVDF, and autoradiography was performed. B, TNF-α-induced phosphorylation occurs on serine and threonine residues of PP4. The corresponding PP4 bands were cut from the PVDF membrane (A) and subjected to phosphaamino acid analysis. The experiments were repeated at least two times with similar results.
We found that TNF-α/H9251 treatment induced phosphorylation of PP4 in a time-dependent manner, peaking at 5 min (Fig. 3A). Phosphoamino acid analysis showed that TNF-α/H9251-induced phosphorylation of PP4 occurred on serine and threonine residues (Fig. 3B). These results indicate that PP4 is inducibly phosphorylated in response to TNF-α/H9251 in HEK293T cells.

JNK Activation by TNF-α Is Blocked by PP4-RL—To investigate the functional involvement of PP4 in the TNF-α signaling, we examined the contribution of PP4 to TNF-α-induced JNK activation. We first constructed a PP4 mutant, PP4-RL, in which the replacement of arginine 236 with leucine resulted in the loss of its phosphatase activity (Fig. 4B). We then examined the effect of PP4-RL on JNK activation by TNF-α. HEK293T cells were transfected with HA-JNK1 alone or HA-JNK1 plus PP4-RL (2 µg). Empty vector was used to normalize the amount of transfected DNA. 36 h after transfection, the cells were treated with TNF-α (10 ng/ml) for 10 min. Cell lysates were prepared, HA-JNK1 was immunoprecipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate (top panel). Expression levels of HA-JNK and HA-PP4-RL were monitored by immunoblotting using an anti-HA antibody (12CA5, lower panel). The experiments were repeated three times with similar results.

We also established a HEK293 cell clone, called HEK293-PP4-RL, that stably expresses HA-PP4-RL (Fig. 4C, right panel). HEK293-PP4-RL cells were treated with TNF-α (10 ng/ml) for various times. Cell lysates were prepared, JNK1 was immunoprecipitated with an anti-JNK1 antibody (Ab101), and immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate (left panel). Cell lysates from parental HEK293 cells and a HA-PP4-RL stably transfected clone, HEK293-PP4-RL, were subjected to SDS-PAGE and Western blotting with an anti-PP4 antibody (Ab104) or an anti-HA antibody (12CA5, right panel).

We found that TNF-α treatment induced phosphorylation of PP4 in a time-dependent manner, peaking at 5 min (Fig. 3A). Phosphoamino acid analysis showed that TNF-α-induced phosphorylation of PP4 occurred on serine and threonine residues (Fig. 3B). These results indicate that PP4 is inducibly phosphorylated in response to TNF-α in HEK293T cells.

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We also established a HEK293 cell clone, called HEK293-PP4-RL, that stably expresses HA-PP4-RL (Fig. 4C, right panel). HEK293-PP4-RL cells were treated with TNF-α (10 ng/ml) for various times (0 to 60 min), and endogenous JNK was immunoprecipitated from the cells with an anti-JNK antibody (Ab101), and immunocomplex kinase assays were performed using GST-c-Jun-(1–79) as a substrate (left panel). As shown in Fig. 4C (left panel), a decrease in JNK activation by TNF-α in HEK293-PP4-RL cells was detected, in comparison to the parental HEK293 cells. Although JNK activation by TNF-α peaked at 10 min in HEK293T cells (Fig. 2C), TNF-α-induced JNK activation peaked at 20 min in HEK293 cells (Fig. 4C, left panel). This kinetic difference between HEK293 and HEK293T cells may be the result of the presence of SV40 large T antigen in HEK293T cells. Taken together, these data indicate that PP4 is required for transducing TNF-α signals to the JNK pathway.
PP4 Specifically Activates JNK, but Not p38 and ERK2—To confirm the involvement of PP4 in the JNK signaling pathway, we tested whether expression of PP4 had any effect on the activity of JNK. Hemagglutinin (HA)-tagged JNK1 was co-transfected in HEK293T cells with PP4, PP1, another serine/threonine phosphatase, or M3/6, a dual-specificity MAPK phosphatase. HA-JNK1 was immunoprecipitated, and its kinase activity was determined in vitro using GST-c-Jun (1–79) as a substrate. Cotransfection of PP4 resulted in activation of JNK1 (Fig. 5, lanes 1 and 2), whereas, PP1 and M3/6 had no such effect on JNK1 (Fig. 5, lanes 1, 3, and 4). M3/6 is a known JNK-inactivating dual-specificity phosphatase, which dephosphorylates the TPY motif of JNK (27, 37). Transiently transfected JNK is somehow partially activated. Therefore, cotransfection of M3/6 with JNK resulted in inhibition of JNK activity, as expected. The nature of the inhibition of JNK by PP1 is not known at this point. It is likely that PP1 dephosphorylates the threonine residue of the TPY motif of JNK and thus inhibits JNK activity. These data indicate that PP4 exerted a positive regulatory effect on JNK1.

To determine whether PP4’s effect on JNK1 is specific, we also examined the effect of PP4 on p38 and ERK2. HEK293T cells were transfected with various amounts of the PP4 expression plasmid together with the HA-tagged MAPK constructs, HA-JNK1, HA-p38, and HA-ERK2. HA-tagged MAPKs were immunoprecipitated, and their kinase activities were determined in vitro using the appropriate substrates (GST-c-Jun for JNK1, GST-ATF2 for p38, and myelin basic protein for ERK2). JNK was activated by PP4 in a dose-dependent manner by PP4 (Fig. 6A). In contrast, PP4 had no significant effect on the activities of either p38 (Fig. 6B) or ERK2 (Fig. 6C). These data indicate that PP4 serves as a specific positive regulator for the JNK signaling pathway. We also found that PP4-RL had no effect on PKC-ζ-induced ERK and MKK6-induced p38 activation (data not shown).

We next wanted to determine whether PP4 and JNK1 interact directly with each other. We incubated GST-JNK fusion protein with cell lysates from untreated or TNF-α treated HEK293 cells stably expressing Flag-PP4. The potential PP4-JNK interaction was analyzed by SDS-PAGE and Western blotting using an anti-Flag antibody (M2). Similar to transient transfected Flag-PP4 in HEK293T cells (Fig. 3), stably expressed Flag-PP4 in HEK293 cells was also inducibly phosphorylated after 5-min treatment of TNF-α (Fig. 7A, lower panel). Association of PP4 with GST-JNK was not detectable (Fig. 7A, upper panel) in the absence or presence of TNF-α. We also

FIG. 5. PP4 activates JNK in transient transfection assay. HEK293T cells (1.5 × 10⁵ cells in 35-mm wells) were transfected with HA-JNK1 (0.1 μg) alone or with 2 μg of PP4, PP1, or Myc-M3/6. Empty vector was used to normalize the amount of transfected DNA. 36 h after transfection, the cell lysates were prepared. HA-JNK1 was immunoprecipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using GST-c-Jun (1–79) as a substrate. Expression levels of HA-JNK1, PP4, PP1, and Myc-M3/6 were monitored by immunoblotting using anti-HA (12CA5), anti-PP4 (Ab104), anti-PP4, and anti-Myc antibodies, respectively (bottom panels). The experiments were repeated at least 10 times with similar results.

FIG. 6. PP4 specifically activates JNK, but not p38 and ERK2. A, HEK293T cells (1.5 × 10⁵ cells in 35-mm wells) were transfected with HA-JNK1 (0.5 μg) alone or HA-JNK1 plus various amounts of PP4 as indicated. Empty vector was used to normalize the amount of transfected DNA. 36 h after transfection, the cell lysates were prepared. HA-JNK1 was immunoprecipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using GST-c-Jun (1–79) as a substrate. Expression levels of HA-JNK1 and PP4 were monitored by immunoblotting using anti-HA (12CA5) and anti-PP4 antibodies, respectively (bottom panels). The experiments were repeated at least 10 times with similar results. B, HEK293T cells (1.5 × 10⁵ cells in 35-mm wells) were transfected with HA-p38 (1 μg) alone, HA-p38 plus various amounts of PP4, or HA-p38 plus 2 μg of HA-MKK6, as indicated. Empty vector was used to normalize the amount of transfected DNA. 36 h after transfection, the cell lysates were prepared. HA-p38 was precipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using GST-ATF2 (1–96) as a substrate. Expression levels of HA-p38, HA-MKK6, and PP4 were monitored by immunoblotting using anti-HA (12CA5) and anti-PP4 antibodies, respectively (bottom panels). C, HEK293T cells (1.5 × 10⁵ cells in 35-mm wells) were transfected with HA-ERK2 (1 μg) alone, HA-ERK2 plus various amounts of PP4, or HA-ERK2 plus 1 μg of HA-PKC-ζ, as indicated. Empty vector was used to normalize the amount of transfected DNA. 36 h after transfection, the cell lysates were prepared. HA-ERK2 was precipitated with an anti-HA antibody (12CA5), and immunocomplex kinase assays were performed using myelin basic protein as a substrate. Expression levels of HA-ERK2, HA-PKC-ζ, and PP4 were monitored by immunoblotting using anti-HA (12CA5) and anti-PP4 (Ab104) antibodies, respectively (bottom panels).
Positive Regulation of the JNK Pathway by PP4

found that PP4 had no phosphatase activity toward in vitro phosphorylated GST-JNK (data not shown). Under the same conditions, however, M3/6, a dual-specificity phosphatase known to target JNK directly, interacted with GST-JNK (Fig. 7B). Taken together, these data suggest that PP4 affects the JNK pathway in an indirect manner.

DISCUSSION

TNF-α is an important effector cytokine for inflammatory and immune responses and is involved in many important cellular processes, such as proliferation, differentiation, and apoptosis (38). A variety of protein phosphatases have been implicated in TNF-α signaling. For example, calcineurin, a calcium-dependent serine/threonine phosphatase, participates in TNF-α-mediated apoptosis in rat hepatoma cells (39) and SHP-2, a Src homology 2-containing phosphotyrosine phosphatase, mediates the induction of interleukin-6 by TNF-α through modulation of the NF-κB pathway (40). Another phosphotyrosine phosphatase, SHP-1, has been shown to mediate TNF-α’s inhibitory effect on vascular endothelial cell growth factor-induced endothelial cell proliferation (41). PP2A has also been shown to be involved in many TNF-α-induced cellular processes (42–45). However, many of these studies relied on the use of okadaic acid, an inhibitor for PP1 and PP2A. Because okadaic acid inhibits PP4 with an IC50 comparable with that of PP2A (17), it is necessary to reexamine some of the functions assigned to PP2A. We provide evidence here that PP4, a novel member of the PP2A family, was activated by TNF-α in HEK293T cells, as indicated by increased phosphatase activity, and increased serine and threonine phosphorylation of PP4 itself. The involvement of PP4 in TNF-α signaling was further demonstrated by the observation that a PP4 mutant blocked TNF-α-induced JNK activation. Demonstration of the involvement of PP4 in TNF-α signaling will help in exploring the molecular mechanism by which TNF-α regulates cellular processes.

We found that the activation of PP4 by TNF-α was accompanied by an increase in the serine and threonine phosphorylation of PP4. These results indicate the novel finding that a member of the PP2A family is subject to regulation by serine phosphorylation. It has been known that the catalytic subunit of PP2A is subject to phosphorylation of a conserved tyrosine and an as yet unidentified threonine (46–48), and that phosphorylation of either the tyrosine or the threonine site inhibits phosphatase activity of PP2A in vitro. However, in human hepatoma Hep3B cells, interleukin-6 induced an increase in both the phosphorylation and phosphatase activity of PP2A (39). The nature of PP4 serine and threonine phosphorylation in response to TNF-α remains unknown at this point. We noted that PP4 phosphorylation preceded PP4 activation in response to TNF-α (5 min versus 10 min). Considering the existence of multiple potential phosphorylation sites on PP4, we speculate that PP4 may be subject to multiple phosphorylation in response to TNF-α, and it is the phosphorylation that occurred at 10 min, but not at 5 min, that contributes to activation of PP4. Further study, including identification of the phosphorylation site(s) and characterization of site-directed mutants of PP4, is required to understand the relationship between the phosphorylation, which occurred at 5 min, and PP4 activation. Alternatively, we cannot exclude the possibility that PP4 phosphorylation precedes PP4 activation by inducing conformational change(s) and/or recruiting some regulatory subunits required for the activation of PP4.

Phosphorylation-dependent inactivation is characteristic of many types of protein kinases, such as DNA-dependent protein kinase (49), phosphoinositide 3 kinase (50), Raf-1 (51–53), and CLK1 (54). It has been shown that PP2A dephosphorylates the inhibitory phosphoserine residue 259 of Raf-1 and thus serves as a positive regulator for Raf-1, an upstream activating kinase for the ERK pathway (55). Raf-1 and MEK1/2, another upstream activating kinase for the ERK pathway, are positively regulated by MAPK phosphatase 1, a dual-specificity phosphatase, in an ERK-independent manner (15). We provide evidence here that PP4 acts as a specific positive regulator for the JNK pathway. However, we did not detect a direct interaction between PP4 and JNK1, strongly suggesting that PP4 exerts its positive regulatory effect on the JNK pathway in an indirect

Fig. 7. PP4 does not interact with JNK in vitro. A, no PP4-JNK association was detected in vitro. HEK293 cells stably transfected with Flag-PP4 (10F1 clone) were seeded at 4 × 10^5 cells/100-mm dish and treated with TNF-α (10 ng/ml) for 5 min the next day. 600 μg of lysate from either TNF-α-treated or untreated 10F1 HEK293 cells was incubated with GST or GST-JNK fusion protein immobilized onto glutathione-agarose beads for 2 h at 4 °C. The PP4-JNK interaction was analyzed by immunoblotting with an anti-Flag antibody (M2) to detect Flag-PP4 bound to GST-JNK after SDS-PAGE (upper panel). The GST and GST-JNK were monitored by immunoblotting with an anti-GST antibody (middle panel). The experiments were repeated three times with similar results. To assure Flag-PP4 was in a phosphorylated state, HEK293 cells stably transfected with Flag-PP4 (10F1 clone) were seeded at 4 × 10^5 cells/100-mm dish and treated with TNF-α (10 ng/ml) for 5 min. Flag-PP4 was immunoprecipitated with an anti-Flag antibody (M2) and subjected to SDS-PAGE and autoradiography. The experiments were repeated two times with similar results. B, M3/6 associates with JNK in vitro. GST or GST-JNK fusion protein was immobilized on glutathione-agarose beads and incubated with 600 μg of lysate from HEK293T cells transiently transfected with Myc-M3/6 for 2 h at 4 °C. The M3/6-JNK interaction was analyzed by immunoblotting with an anti-Myc antibody to detect Myc-M3/6 bound to GST-SAPK after SDS-PAGE (upper panel). The GST and GST-SAPK were monitored by immunoblotting with an anti-GST antibody (lower panel).
manner. Given the fact that the core of the JNK signaling pathway is a multiple-kinase module that is assembled by scaffold proteins to act as a stimulus-specific signaling complex (7–9), and that the magnitude and duration of JNK activation are tightly controlled by the coordinate actions of protein kinases and protein phosphatases (12), we speculate that PP4 may target and activate the JNK upstream activating kinase(s), which is negatively regulated by phosphorylation, and subsequently leads to JNK activation. The target for PP4 could be a kinase at one or multiple levels of the JNK signaling cascade.

In addition to regulation of upstream activating kinases, we cannot exclude the possibility that PP4 may target a phosphatase which inhibits JNK, and thus exert an indirect positive effect on the JNK pathway. This putative JNK phosphatase may be activated by phosphorylation, and hence inactivated by dephosphorylation. Because only JNK, but not p38 or ERK, is activated by PP4, the putative phosphatase should also be JNK-specific. Inhibition of this JNK-specific phosphatase by PP4-mediated dephosphorylation would then lead to JNK activation. Therefore, some JNK-specific, dual-specificity phosphatases, such as M3/6 (37), may be good candidates for PP4 targets.

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