Activation of MAP kinase family members triggered by TPA or ionomycin occurs via the protein phosphatase 4 pathway in Jurkat leukemia T cells

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Abstract. Protein phosphatase 4 (PP4) is a protein phosphatase 2A (PP2A)-related, okadaic acid-sensitive, serine/threonine protein phosphatase that shares 65% amino acid identity with PP2A. Numerous studies have shown that protein phosphatase is involved in the regulation of T cell signaling and activation. In this study, we investigated the effect of overexpression of PP4 on the expression of members of the MAP kinase family in Jurkat leukemia T cells, which had previously been stimulated with UV, 12-O-tetradecanoylphorbol-13-acetate (TPA), ionomycin and okadaic acid. We found that the overexpression of PP4 expressed relatively low activity in the absence of any kind of stimulation. However, TPA, UV or ionomycin treatment strongly increased the activity of PP4. In addition, Jurkat T cells, transfected with various expression plasmids and/or stimulated with TPA, UV or ionomycin strongly induced the c-Jun N-terminal kinase (JNK) and p38, whereas the extracellular signal-regulated kinase (ERK)-1/2 kinase pathway was weekly activated. Treatment of Jurkat T cells with okadaic acid, an inhibitor of PP2, also inhibited the increase of JNK and p38 activity induced by PP4. The effect of okadaic acid on the activity of PP4 was similar to that observed in Jurkat T cells treated with a dominant negative c-Jun (dn-jun). These results indicate that the activation of JNK and p38, but not ERKs, is a target for the PP4 activity in Jurkat leukemia T cells.

Introduction

Protein phosphatase 4 (PP4; also known as PPX), a novel serine/threonine phosphatase, appears to play a key role in the regulation of c-Jun N-terminal kinase (JNK)-1 expression (1,2). PP4 is structurally related to the protein phosphatase 2A (PP2A) family of enzymes (with 65% amino acid sequence identity) and contains putative okadaic acid and microcystin-LR binding domains; it has been highly conserved during evolution (1,3). It is known that the phosphorylation of MAP kinases (MAPKs) *in vivo* is a reversible process, indicating that protein phosphatases (PPs) provide an additional level of regulation of MAPK functions (4). The MAPK family consists of a group of three different kinase pathways: the extracellular signal-regulated kinases 1 and 2 (ERK-1/2), also referred to as p44/42 (5); the JNKs, also known as the stress-activated protein kinases (SAPK) (6); and the p38 kinases, which are usually activated by environmental stresses and correlated with programmed cell death (7-10). JNK-1 is activated by the dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues within a Thr-Pro-Tyr motif located in kinase sub-domain VIII (11,12). Once activated, JNK-1 upregulates gene expression by phosphorylating the activating motif of transcription factors, such as ATF2 (9,13), and the Ets domain of transcription factors Elk-1 and Sap-1 (14,15). The activation of the transcription factor activating protein-1 (AP-1) mediated by JNK-1, by means of the phosphorylation of c-Jun, has been implicated in oncogenic transformation (16-19).

Transcription factor phosphorylation by protein kinases has received a great deal of attention with regard to prostate cancer. By contrast, very little is known about the implications of PPs and de-phosphorylation in this type of cancer. The phosphorylation of MAPKs *in vivo* is a reversible process, indicating that PPs provide an additional level of regulation of MAPK functions (4,20). A group of dual-specificity MAP kinase phosphatases has been identified to inactivate MAPKs through de-phosphorylation of both Thr and Tyr residues (21). There is growing evidence suggesting that PP is implicated in various types of cancer (20,22). PP4, a serine/Thr phosphatase, appears to play a major role in the regulation of JNK-1 expression (23). Since PP4 is a PP2A-related serine/Thr phosphatase, at a 50% inhibitory concentration (IC_{50}), comparable to that of PP2A, it raises the possibility that certain cellular functions of PP2A, determined in T cells using okadaic acid, may in fact be those of PP4 (24,25). This high degree of conservation suggests that PP4 is involved in critical cellular events. As PP4 is found predominantly in centrosomes, microtubule nucleation could be one such event (2,25). PP4 also interacts with and down-regulates insulin receptor substrate 4, following tumor necrosis factor α (TNF-α) stimulation (20,26), and is involved in the TNF-α-induced activation of the JNK signaling pathway (27).

On the other hand, okadaic acid, a specific inhibitor of the PP2 phosphatase family, also inhibits PP4. Previous studies

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have indicated that PP4 has various cellular functions that are distinct from PP2A activity (28). PP4 interacts with c-Rel/RelA, stimulates the DNA-binding activity of c-Rel and activates nuclear factor-κB (NF-κB)-mediated transcription (29).

In view of this information, activation of JNK-1 by 12-O-tetradecanoylphorbol-13-acetate (TPA), UV or ionomycin, appears to rely on a delicate balance in which PPs, such as PP4, play a crucial role counteracting the affects of protein phosphorylation. In this study, we demonstrated that the strong activation of JNK-1, p38 and EGR1 in Jurkat leukemia T cells treated with TPA, UV and ionomycin, was dependent on PP4, and this resulted in the induction of DNA binding and transcription activities of nuclear factor AP-1 and EGR-1. The findings presented here suggest that PP4 may play an important role in Jurkat leukemia T cells through the positive regulation of JNK-1, p38 and EGR1 activities.

Materials and methods

Reagents. The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin and bestatin were purchased from Roche (Indianapolis, IN, USA); T4 polynucleotide kinase and poly $(dI\text{-}dC)_2$ were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Tris-Borate-EDTA buffer and acrylamide-bisacrylamide (29:1) were obtained from Bio-Rad (Richmond, CA, USA). Luciferase assay reagent, lysis buffer and the pGL-2 luciferase vector were obtained from Promega (Madison, WI, USA). ATP and ionomycin were purchased from Sigma (St. Louis, MO, USA). Anti-HA antibody (12CA5) was purchased from Roche Molecular Biochemicals. Monoclonal anti-Flag (M2) was purchased from Sigma. Anti-PP4, anti JNK, p38 and ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Myelin basic protein (MBP) was purchased from Stratagene (La Jolla, CA, USA). Luciferase assay reagent, lysis buffer and the pGL-2/pGL-3 luciferase vector were obtained from Promega (Madison, WI, USA). GST-cJun (1-79) and GST-ATF-2 (1-96) were a gift from Dr Roger Davis (Howard Hughes Medical Institute, MA, USA). Production and purification of GST-cJun and GST-ATF-2 proteins were performed as previously described (29).

Plasmids. Human JNK-1 was a gift from Dr Dan Mercola (Sydney Kimmel Cancer Center). The dn-c-Jun was obtained from Dr Michael Karin (University of California, San Diego, CA, USA). The PP4 cDNA was cloned from the HeLa λgt11 cDNA library. The cDNA, which encompassed the complete open reading frame, was blunt end ligated into *Eco*RV-digested pBluescript (Stratagene) to create pKS/PP4 and sequenced. The resulting plasmids were prepared by two rounds of cesium chloride centrifugation. The constructs for GST-Jun (1-79) and GST-ATF-2 (1-96) were gifts from Dr Roger David (29,30).

Cell culture and transfection. Jurkat leukemia T cells were obtained from ATCC. All experiments were carried out using the human T cell, Jurkat, which responds with a pattern of IL-2 production similar to that of B7-1 and LFA-3 co stimulation as normal human T cells (29). The human Jurkat leukemia T cells were maintained in RPMI-1640 culture medium supplemented with 2 mM glutamine and 10% FBS (complete medium). All tests on the Jurkat T cells were performed at a concentration of $1x10^7$ cells/ml in complete medium.

Plasmid construction and preparation of nuclear extracts. The AP-1-luciferase reporter plasmid driven by the rat prolactin minimal promoter (-36 to +37) under the control of four copies of the human AP-1 site (49) was kindly provided by M. Rincon and R.A. Flavell (Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, USA). Plasmids containing multimers of the recognition sites for NF-κB were constructed and linked to the pLuc-prolactin minimal promoter plasmid (15). The orientation of each element was confirmed by restriction enzyme cleavage: i) four copies of the AP-1, TPA, 5'-TCGATTGAGTCAGGGTAA3'; ii) two copies of the NF-κB-binding site of the human Ig κ light chain enhancer 5'-GGGACTTTCC-3'.

Nuclear extracts were prepared as previously described (30). The cells were grown at 37˚C in a humidified atmosphere of 10% CO_2. Cells ($2x10^7$), incubated with 0.1% DMSO (control) or 30 nM TPA or 1 mM ionomycin, were collected and washed with ice-cold phosphate-buffered saline (PBS), washed again in buffer A (10 mM HEPES, pH 7.9, 15 mM KCl, 2 mM $MgCl_2$, 6 mM DTT, 0.1 mM EDTA and 1 mM PMSF) and lysed in buffer A with 0.2% Nonidet P-40. The pelleted nuclei were re-suspended in buffer B [50 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol] in the presence of 0.3 M $(NH_4)_2SO_4$ (pH 7.9), and rocked for 30 min at 4˚C. The broken nuclei were centrifuged for 10 min at 100,000 g. A 125-μl aliquot of supernatant was transferred to a second tube and more $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M, followed by a second centrifugation of 50,000 g for 5 min. The supernatant was removed and the pellet was re-suspended in 50 μl of buffer B and stored at 70˚C. Protein concentration was estimated using the Bio-Rad stain protein assay kit with bovine albumin as the standard.

DNA transfection and luciferase activity analysis. Transfection of Jurkat cells was conducted by electroporation, using an electro cell manipulator 600 (BTX, San Diego, CA, USA) with 130 V/1700 μF capacitance. Briefly, $8x10^6$ cells were transfected with 10 μg of luciferase reporter plasmid and 5 μg of each expression plasmid, and the mixture was incubated for 24 h. Transfected cells were cultured in complete medium for 24 h and stimulated with TPA or ionomycin for another 8 h. Cells were harvested 32 h post-transfection, washed twice in PBS and treated with lysis buffer (luciferase assay; Promega) for 5-10 min on ice. Lysates were spun down for 1 min and all supernatants were analyzed using luciferase reagent (Promega) and measured as a duplicate in a luminometer (MicroLumat LB 96 P; Berthold) for 5 sec. Background measurement was subtracted from each duplicate and experimental values were expressed either as recorded light units of luciferase activity or as relative activity compared to extracts from unstimulated cells (29,30).

Western blotting. Jurkat leukemia T cells were plated at a density of $2x10^7$ per 35 mM dish, 24 h prior to treatment. All cultures were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4,

150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS). Lysates were heated at 100˚C for 10 min. Equal volumes of extract were separated by SDS/PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated with SAP/JNK, p38 or ERK-1/2 antibody (dilution 1:1,000) and PP4 antibody (dilution 1:1,000) (Santa Cruz Biotechnology). Primary incubation was followed by incubations with HRP-conjugated secondary antibody (1:2,000; NEB), and detection was made with super signal chemiluminescent substrate (31-34).

MAP kinase assay. For the ERK activity assay, cells were incubated in the absence of serum for 16 h and then treated with various agents. They were washed twice with PBS and lysed in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF). The extracts were centrifuged to remove cellular debris, and the protein content of the supernatants was determined using the Bio-Rad protein assay reagent. Protein (300 μg) from the lysate samples was used for immunoprecipitation by treatment with ERK-2 rabbit polyclonal antibody at 4˚C for 2 h. The immunoprecipitated products were washed once in lysis buffer; twice in 0.5 M LiCl, 0.1 M Tris, pH 8.0; and twice in kinase assay buffer (25 mM HEPES, pH 7.2-7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1 mM dithiothreitol). The samples were then re-suspended in 30 μl of kinase assay buffer containing 10 μg of myelin basic protein and 40 μM [γ-32P] ATP (1 μCi), as previously described (20). The kinase reaction was allowed to proceed at room temperature for 5 min and stopped by the addition of Laemmli's SDS sample buffer. Reaction products were separated by 12% SDS-PAGE (20,30,31).

For JNK-1 and p38, the samples were incubated at 4˚C overnight with the N-terminal c-Jun (1-79) and ATF-2-glutathione S-transferase fusion protein, respectively, bound to glutathione-Sepharose beads in order to selectively precipitate JNK-1 and p38 from cell lysates. Subsequently, the beads were washed to remove non-specifically bound proteins. The kinase reaction was then carried out in the presence of cold ATP, and samples were resolved on 12% SDS-gel electrophoresis followed by western blotting with phosphor specific c-Jun and p38 antibodies. This antibody specifically recognizes JNK-1-induced phosphorylation of c-Jun at Ser63, a site important for c-Jun-dependent transcriptional activity (30,31).

Results

TPA (30 nM) and ionomycin (1 µM) strongly activate JNK-1 and p38 pathways in Jurkat leukemia T cell lines. C-jun-NH2 kinases (JNK) and p38 are among the UV-activated protein kinases that play an important role in cellular stress response via the phosphorylation of c-Jun, ATF2 and p53.

JNK, p38 and ERK-2 activity in Jurkat leukemia T cells treated with various stimuli. To determine the activity of JNK, p38 and ERK-2 in Jurkat leukemia T cell lines, we treated the cells with FCS 0.5%, FCS 10% and/or TPA, ionomycin and UV. As shown in Fig. 1A, the protein expression of JNK, p38 and ERK-2 was assessed by western blot analysis. The cells were treated with various stimuli. TPA (30 nM), ionomycin (1 μM) and UV strongly activated JNK and p38 expression. By contrast, the effect was only marginal in the activation of ERK.

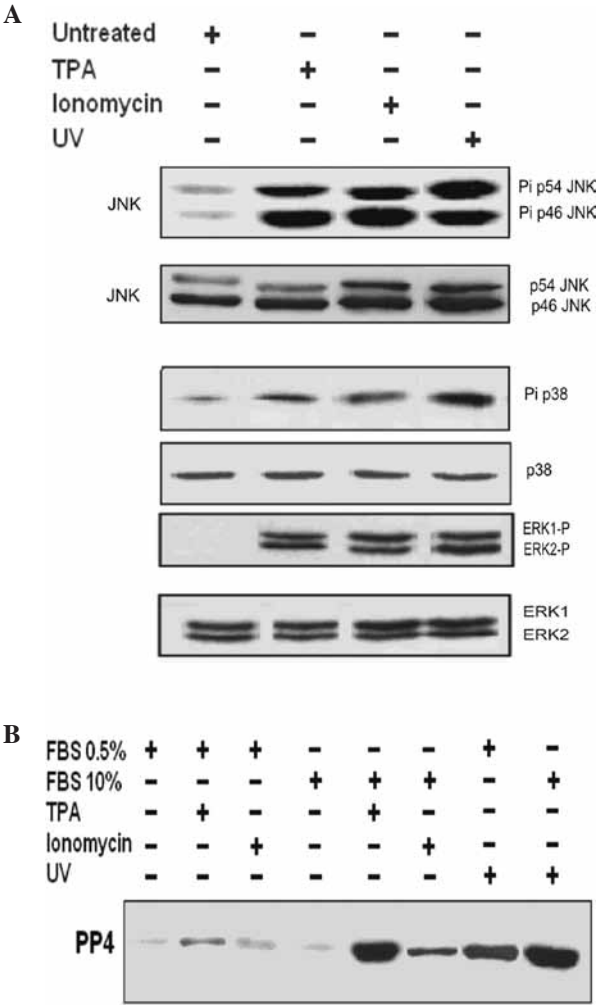

Figure 1. Western blot analysis of the protein levels of JNK-1/2, p38, ERK-1/2 and PP4 in Jurkat leukemia T cells. (A) Cells were cultured in 10% PBS and treated with TPA, ionomycin or UV. Protein levels of JNK1/2, p38 and ERK-1/2 were analyzed. (B) Protein levels of PP4 in Jurkat cells. Cells were cultured in 0.5 or 10% PBS and treated with TPA (30 nM) or ionomycin (1 μM). The expression levels of PP4 were analyzed. One of two similar experiments is shown. TPA, 12-O-tetradecanoylphorbol-13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

Serum increases the induction of PP4 expression in Jurkat leukemia T cells. PP4 is structurally related to PP2A. PP2A acts as a tumor suppressor that is involved in human breast carcinogenesis and contains a binding domain of okadaic acid, a tumor promoter. We sought to ascertain the levels of endogenous PP4 in Jurkat leukemia T cell lines grown in medium containing different concentrations of serum and following treatment with TPA, ionomycin or UV radiation as the control. We investigated the effect of serum on the ability of TPA, ionomycin and UV to induce PP4 expression. Cells were pre-incubated or not with medium containing 0.5% FBS for 16 h and were later treated with TPA (30 nM) or ionomycin (1 μM) or UV (100 J/m^2). Cytosol extracts were prepared at the indicated period of time and the expression of PP4 protein

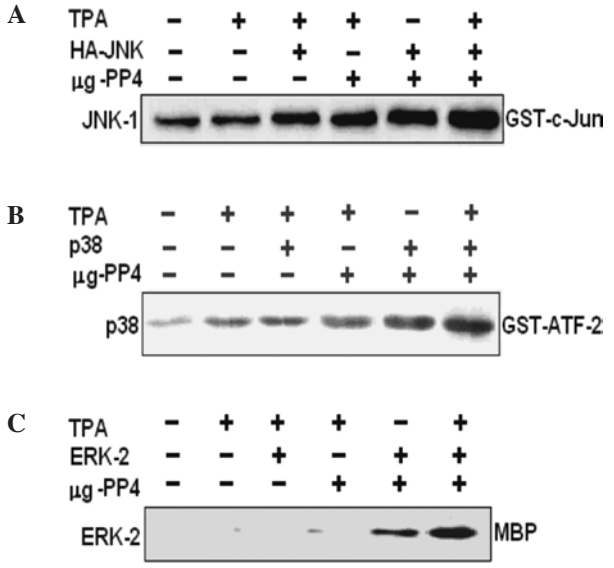

Figure 2. PP4 stimulated the expression of JNK-1/2 and p38, but not ERK. Expression plasmid (3 μg) for (A) HA-JNK-1 or (B) p38 or (C) ERK-2 was co-transfected with a PP4 (3 μg) expression plasmid in Jurkat leukemia T cells. Cell lysates were prepared 48 h post-transfection and JNK-1, p38 and ERK-2 kinase activities were assayed as described in Materials and methods. (A) JNK-1 kinase activity was assayed using GST-cJun as the substrate (1-79). (B) p38 kinase activity was assayed using GST-ATF-2 as the substrate. (C) ERK-2 activity was assayed using MBP as the substrate. One of three similar experiments is shown. TPA, 12-O-tetradecanoylphorbol-13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PP4, protein phosphatase 4; MBP, myelin basic protein.

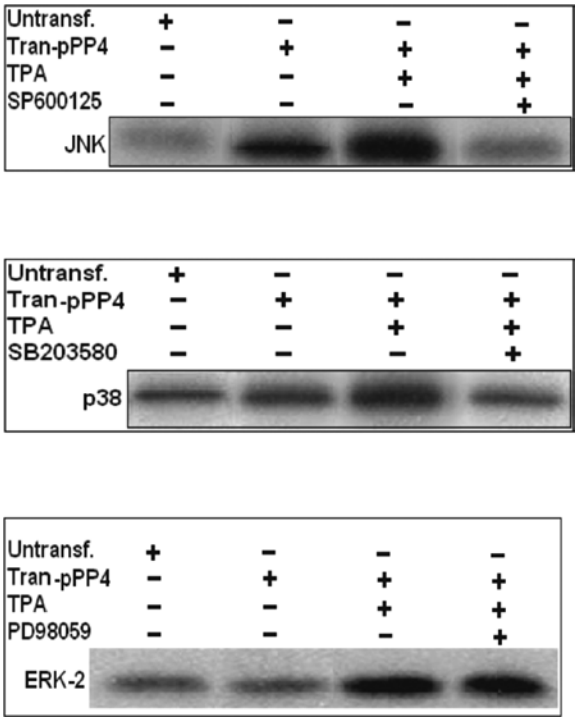

Figure 3. Jurkat cells transfected with PP4 were pre-incubated for 90 min with SP600125 (50 μM) or SB203580 (30 μM) or PD98059 (10 μM) prior to stimulation with TPA (30 nM) for an additional 90 min. (A) Induction of JNK-1 activity by PP4 or PP4/TPA was completely prevented in the presence of SP600125. (B) TPA-mediated activation of p38 was not completely blocked by the kinase inhibitor, SB203580. (C) TPA-mediated activation of ERK-2 was not blocked by the kinase inhibitor, PD98059. One of two similar experiments is shown. TPA, 12-O-tetradecanoylphorbol-13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PP4, protein phosphatase 4.

activity was subsequently measured by western blotting. The induction of PP4 activity was clearly dependent on the serum concentration. The expression of PP4 was notably higher when the cells were cultured in 10% serum concentration, compared to cells cultured in 0.5% FBS TPA, ionomycin and UV, further increasing the expression of PP4 (Fig. 1B).

PP4 specifically activates JNK-1 and indirectly activates EGR1. To demonstrate that PP4 specifically activates JNK-1, we compared its effect on JNK-1 activity with other members of the MAPK family, such as p38 and ERK. To this end, we co-transfected different amounts of expression plasmid for JNK-1, or p38, or ERK, combined with an established amount of PP4 or empty vector into Jurkat cells. Cell lysates were prepared 24 h after transfection. The cells transfected with HA-JNK were immunoprecipited with an anti-HA antibody (12CA5) and the activity of JNK-1 was examined by a kinase assay using GST-c-Jun (1-79) as the substrate (Fig. 2A). To elucidate the specificity of the effect of PP4 on p38 and ERK activity, Jurkat cells were co-transfected with an expression plasmid for PP4 and p38 (Fig. 2B) or ERK (Fig. 2C), respectively. In both cases, kinases were immunoprecipited and assayed over the substrates GST-ATF2 for p38 and MBP for ERK-2. Notably, while PP4 increased JNK-1 and p38 activities (Fig. 2A and B), it had no effects on ERK-2 activity (Fig. 2C). These results indicate that PP4 is a specific positive regulator for the JNK-1 and p38 pathways, but not for ERK-2.

Pre-incubation of Jurkat T cells (transfected with PP4) with SP600125 for JNK, but not SB203580 for p38 or PD98059 for ERK-2, prevents PP4- or TPA/PP4-induced expression of proteins, respectively. Jurkat cells transfected with PP4, were pre-incubated for 90 min with SP600125 (50 μM), SB203580 (30 μM), or PD98059 (10 μM) prior to stimulation with TPA (30 nM) for an additional 90 min. Cell extracts were prepared and subsequently analyzed for JNK-1, p38 and ERK-2 activities. Whole cell extracts were prepared and subsequently assayed for JNK, p38 and ERK-2 protein activity using western blot analysis. Induction of JNK-1 activity by PP4 or PP4/TPA was completely prevented in the presence of SP600125 (Fig. 3A). Notably, TPA-mediated activation of p38 was not completely blocked by the kinase inhibitor SB203580 (Fig. 3B). By contrast, PP4 was unable to increase the activity of ERK-2, and TPA-mediated activation of ERK-2 was not blocked by the kinase inhibitor PD98059 (Fig. 3C). However, the inhibition for JNK-1 was more efficient than the inhibition for p38, suggesting that PP4 could be a specific phosphatase for the regulation of the JNK pathway.

Okadaic acid treatment blocks the effect of PP4 transcriptional activity and the expression of the trans-dominant inhibitor of c-Jun, cJun-Ala (63,73) inhibits the activity of AP-1 reporter gene. We have attempted to determine whether or not okadaic acid affects the activity of PP4 using a luciferase assay. The results of this experiment, shown in Fig. 4A, demonstrate that co-transfection of PP4 with a luciferase responsive reporter gene, carrying a tandem sequence for AP-1 (4xAP-1), increases the

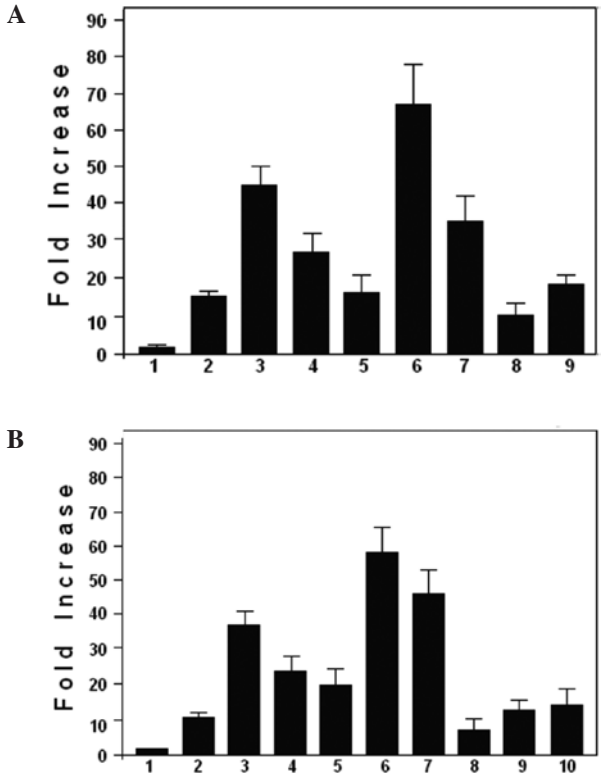

Figure 4. Blocking of PP4 induced the activation of MAP kinase members (JNK-1, p38 and ERK-2) by okadaic acid in Jurkat cells, assayed by a 4xAP-1 luciferase vector, and altered the transcriptional activity of the nuclear factor AP-1, as demonstrated in a luciferase assay. A similar assay was performed using dn-cJun (Luciferase assay carrying 4xAP-1). One of three individual experiments is shown. (A) 1, Untransfected cells; 2, cells transfected with 4xAP-1; 3, cells transfected with 4xAP-1 + ATP; 4, cells transfected with 4xAP-1 + ionomycin; 5, cells transfected with 4xAP-1 + PP4; 6, cells transfected with 4xAP-1 + PP4 + TPA; 7, cells transfected with 4xAP-1 + PP4 + ionomycin; 8, cells transfected with 4xAP-1 + PP4 + okadaic acid; 9, cells transfected with 4xAP-1 + PP4 + okadaic acid + TPA. (B) 1, Untransfected cells; 2, cells transfected with 4xAP-1; 3, cells transfected with 4xAP-1 + ATP; 4, cells transfected with 4xAP-1 + ionomycin; 5, cells transfected with 4xAP-1 + cJun; 6, cells transfected with 4xAP-1 + cJun + TPA; 7, cells transfected with 4xAP-1 + cJun + ionomycin; 8, cells transfected with 4xAP-1 + dn-cJun; 9, cells transfected with 4xAP-1 + dn-cJun + TPA; 10, cells transfected with 4xAP-1 + dn-cJun + ionomycin. One of two similar experiments is shown. TPA, 12-O-tetradecanoylphorbol-13-acetate; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; PP4, protein phosphatase 4.

luciferase activity 2-fold, compared to cells transfected with the luciferase vector alone. The activity of the AP-1 reporter gene was further increased with the addition of TPA (30 nM) (8-fold compared to the AP-1 reporter gene alone), as shown in Fig. 4A. However, treatment of cells with okadaic acid (2 nM), strongly decreased the activity of the reporter gene induced by TPA or ionomycin, as shown in Fig. 4A (bars 6 and 7, respectively).

To compare the effect of okadaic acid on the expression of the AP-1 luciferase reporter gene, we assayed the inhibition of the reporter gene AP-1, co-transfecting a dominant negative c-Jun with the reporter gene. Expression of this reporter in Jurkat leukemia T cells leads to low activity of the reporter gene. The addition of TPA and ionomycin strongly increased the activity of this reporter gene. Co-expression with a known activator of the JNK-1 pathway, an expression vector for the wild-type c-Jun, leads to substantial activation of the 6xAP-1 reporter. Moreover, the transfection of an expression vector carrying a dominant negative c-Jun (dn-c-Jun Ala 63,73), but not the wild-type c-Jun, leads to readily detectable inhibition of the 6xAP-1 activity in Jurkat leukemia T cells (Fig. 4B).

Discussion

The results from the present study showed that PP4 stimulated the activity and expression of both JNK and p38 in an *in vitro* assay using Jurkat leukemia T cells as a model for T cell response, whereas ERK-2 showed differential response. For JNK and p38, the phosphate PP4 is a critical player in mediating expression and transcriptional response to TPA or ionomycin in stimulated Jurkat cells and strongly influencing basal transcriptional activity. By contrast, blocking the JNK and p38 pathways reduced basal and the magnitude of responses to TPA-stimulated Jurkat cells. However, the ERK levels of stimulated TPA Jurkat cells remained unchanged.

At the expression level, JNK (SP) and/or p38 (SB) blockade strongly decreased the expression levels of JNK and p38. Nevertheless, ERK (PD) blockade had no effect on the expression of ERK induced by TPA. Of note, the pattern of ERK-2 secretory responses to PD blocking in cells stimulated by TPA was similar to that of cells transfected with PP4 and stimulated with TPA. This suggests that the ERK kinase pathway is not a target for PP4. It appears unlikely that the reduction in JNK and p38 release in the presence of the JNK (SP) or p38 (SB) blockers is due to a cytotoxic effect over time, because the ERK activity was not reduced. It is known that the induction of IL-2 activity by phorbol myristate acetate and ionomycin is inhibited by okadaic acid, suggesting that an okadaic acid-sensitive serine/Thr phosphatase is required for IL-2 gene activation in T cells (34). AP-1, a transcription factor involved in T cell growth and gene regulation, is regulated by PP2A in dividing human T lymphocytes (35).

Furthermore, okadaic acid-sensitive PPs have been implicated in the upregulation of STAT3 activity and IL-1 gene expression in T cells (36). It has been shown that okadaic acid also inhibits PP4 with a 50% inhibitory concentration (0.01 nM *in vitro*) comparable to that of PP2A (38). Therefore, it is plausible that some of these identified functions assigned to PP2A using okadaic acid may in fact be functions of PP4 (20). PP4 interacts with and downregulates insulin receptor substrate 4 following TNF-α stimulation (38), and is involved in TNF-α-induced activation of the JNK protein signaling pathway (24).

It has also been demonstrated that PP4 interacts with and regulates HPK1, a mitogen-activated protein kinase upstream regulator, in a T cell receptor-dependent manner (39), indicating that PP4 may be a novel serine/Thr phosphatase involved in T cell signaling. This suggestion was supported by the fact that co-transfection of PP4 with a luciferase reporter carrying a tandem repeated of AP-1, in Jurkat T cells previously treated with okadaic acid, was unable to increase the transcriptional activity of AP-1-luciferase response. This result was compared to the response of Jurkat cells co-transfected with a wild-type c-Jun or a dominant negative c-Jun. Both okadaic acid and dn-cJun were able to decrease the transcriptional activity of the luciferase-AP-1 induced by PP4 and TPA.

Finally, based on our findings, we assert that the upregulation of JNK-1 by PP4, resulting from TPA and ionomycin treatment in Jurkat leukemia T cells, results in the estab-

lishment of an anti-apoptotic state, as has previously been published in the case of JNK, but not of PP4 (40). Our study supports the existence of a causative relationship between PP4 and JNK/p38, but not with ERK. It appears that PP4 acts in synergy with signals induced by TPA and ionomycin, triggering an overexpression of JNK-1 and p38 in Jurkat leukemia T cells.

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