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The Serine/Threonine Phosphatase, PP2A: Endogenous Regulator of Inflammatory Cell Signaling

Thomas P. Shanley, Niti Vasi, Alvin Denenberg, and Hector R. Wong

We have investigated the regulation of kinases and phosphatases in early gene activation in monocytes because these cells are implicated in the pathogenesis of acute inflammatory states, such as sepsis and acute lung injury. One early gene up-regulated by endotoxin is c-Jun, a member of the activating protein (AP) family. C-Jun is phosphorylated by c-Jun N-terminal kinase (JNK) and associates with c-Fos to form the AP-1 transcriptional activation complex that can drive cytokine expression. Inhibition of the serine/threonine phosphatase, PP2A, with okadaic acid resulted in a significant increase in JNK activity. This finding was associated with increased phosphorylation of c-Jun, AP-1 transcriptional activity, and IL-1β expression. Activation of PP2A inhibited JNK activity and JNK coprecipitated with the regulatory subunit, PP2A-β, supporting the conclusion that PP2A is a key regulator of JNK in the context of an inflammatory stimulus.

The inflammatory process initiated by Gram-negative bacteria involves the activation of monocytes/macrophages by the LPS component of the bacterial cell wall. At physiologic concentrations, this activation requires association of LPS with the serum protein, LPS-binding protein, and subsequent binding of this complex to CD14 expressed on the monocyte/macrophage cell surface (1–3). This binding induces the transcription of several genes encoding proinflammatory mediators. LPS-activated gene induction has been shown to be mediated, in part, through induction of the AP-1 family of transcriptional activation factors (4, 5). AP-1 is composed of homodimers and heterodimers of the Jun (e.g., c-Jun), Fos (e.g., c-Fos), or activating transcription factor (e.g., activating transcription factor-2) proteins (6, 7). In one of the most well studied examples of AP-1 activity, c-Jun/c-Fos proteins combine as a heterodimer to form an active AP-1 complex (8). This complex is able to recognize the AP-1 consensus sequence or TPA-response element on the promoters of many genes to activate transcription. An example of this important activation process in acute inflammation is the transcriptional activation of cytokines (e.g., IL-1β; Ref. 9) and adhesion molecules (e.g., ICAM-1; Ref. 10).

There exist various regulatory mechanisms of AP-1 activity including transcriptional activation of subunits (to increase the abundance of AP-1 proteins), as well as posttranscriptional modification (e.g., phosphorylation of the proteins) to increase their activity (6). Although c-Jun is constitutively present in cells in an inactive form, activation of the cell by LPS induces phosphorylation of two serine residues (serine 63 and 73) within the transactivation domain of c-Jun (11). This phosphorylation is mediated by p46 and p54 isoforms of the c-Jun N-terminal kinase (JNK)β, a member of the mitogen-activated protein (MAP) kinase family, and serves to increase both the stability (12) and transcriptional activity (13) of c-Jun via phosphorylation.

It has long been postulated that the regulation of any phosphorylated protein is in part balanced by the activity of kinases and phosphatases. Recent work in TCR signaling has supported the simple but eloquent hypothesis that phosphatases directly regulate associated kinases. For example, the prolactin-enriched protein tyrosine phosphatase was shown to dephosphorylate and inactivate the Src-related kinases after T cell activation (14). Similarly, the 1xB kinase was shown to be regulated by the serine phosphatase, PP2A (15). We hypothesized that in the context of an inflammatory stimulus, such as LPS, JNK might similarly be a target of regulation by phosphatases. In these studies, it is demonstrated that inhibition of serine/threonine phosphatases by okadaic acid (OA) resulted in increased JNK kinase activity that was associated with increased phosphorylation of c-Jun, increased nuclear translocation of AP-1 and AP-1-driven transcriptional activity, as well as increased IL-1β expression. In coculture studies, the regulatory subunit of PP2A, PP2A-β, coprecipitated with JNK. Specific pharmacologic inhibition of PP1 by phosphatidic acid (PA) had no effect on JNK activity, whereas activation of PP2A by a high dose of the same agent decreased JNK activity. Together these data support the hypothesis that PP2A is a key endogenous regulator of AP-1 and as such may be a valid therapeutic target in the setting of acute inflammation.

Materials and Methods

Cells

The human acute monocytic leukemia cell line, THP-1, was purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, kanamycin, 2-ME, and 2% glutamine pH 7.35 at a density of 0.5–1 × 10⁶ cells/ml at 37°C in humidified 5% CO₂ in air. Studies were performed in T25 flasks (Becton Dickinson, Mountain View, CA) at a density of 5–10 × 10⁶ cells/ml after a 3-h differentiation step with IPN–γ (100 U/ml). Stimulation was performed with 1 μg/ml LPS (Escherichia coli, serotype O55: B5; Sigma, St. Louis, MO). The pretreatment time for OA (Sigma) or PA (1,2-dioleoyl-sn-glycero-3-phosphate; Avanti, Alabaster, AL) was 60 min in all experiments. PA required dissolving in 1 μg/ml LS (Escherichia coli, serotype O55: B5; Sigma, St. Louis, MO). The pretreatment time for OA (Sigma) or PA (1,2-dioleoyl-sn-glycero-3-phosphate; Avanti, Alabaster, AL) was 60 min in all experiments. PA required dissolving in a 1:1 mixture of 100% ethanol/sterile water with 30 min of ultrasonication. At the highest dose used (5 mM), the dilution of PA into media was 1:1000. Unstimulated and
LPS-stimulated cells received a similar amount of 1:1 EtOH/H₂O for drug vehicle control (1:1000).

In vitro kinase assay for JNK activity
JNK activity was determined as previously reported (16). Approximately 5–10 × 10⁶ cells in suspension were washed with 0.9% NaCl, centrifuged for 5 min at 3000 rpm at 4°C, and the cell pellet was lysed at 4°C with a lysis buffer containing 50 mM Tris, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1% Triton X-100, 0.5% Nonidet P-40, 10% glycerol, 2 mM DTT, 1 mM PMSF, 0.1 mM sodium orthovanadate, 2 mM para-nitrophenylphosphate, and 0.3 U/ml aprotinin. The cell lysate was centrifuged for 10 min at 10,000 × g at 4°C and stored at −80°C for kinase assay. Measurement of supernatant protein concentration was performed by the Bradford assay using a Bio-Rad protein assay kit. For immunoprecipitation, 800 ng of sample protein, adjusted to a final volume of 300 µl with lysis buffer, was mixed on a rocker at 4°C for 60 min with either 5 µl polyclonal anti-JNK Ab (cat. no. sc-474; Santa Cruz Biotechnology, Santa Cruz, CA) or 5 µl anti-MKP-2 (Santa Cruz). After the sample-Ab mixing, the NaCl concentration was adjusted to 400 mM with 1 M NaCl, and 30 µl of protein G-Sepharose beads (Amersham-Pharmacia, Piscataway, NJ), equilibrated with lysis buffer, were mixed at 4°C overnight. The pellet was washed twice with the lysis buffer, followed by washing with a kinase assay buffer containing 25 mM HEPES, 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerocephosphate, 0.1 mM Na₃VO₄, 2 µg/ml leupeptin, 1 mM PMSF, 0.3 U/ml aprotinin, and 2 mM para-nitrophenylphosphate, and the pellet was resuspended in kinase assay buffer. For the JNK kinase activity, the resuspended pellet was mixed with 20 µg GST-c-Jun or GST protein devoid of c-Jun as a result of not placing a nonradioactive, malachite green-based phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY) were used to estimate the size of the immunoreactive bands. Phosphatase activity determination
A nonradioactive, malachite green-based phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY) were used to estimate the size of the immunoreactive bands. Western blot analysis
Cytoplasmic and nuclear extracts and determination of AP-1 transcriptional activity by EMSA EMSA was performed as previously reported (17). After stimulation with LPS in the presence or absence of OA, cells were collected on ice by gentle agitation and centrifuged (1500 × g, 10 min, 4°C). Cell pellets were washed twice with ice-cold PBS and then treated with 50 µl ice-cold buffer A (10 mM HEPES, pH 7.9; 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF) and centrifuged (1500 × g, 10 min, 4°C). The supernatant was aspirated off and the pellet resuspended in 50 µl buffer A + 0.1% Nonidet P-40. This suspension was incubated on ice for 5 min, then centrifuged (14,000 rpm, 10 min at 4°C). The supernatant containing the cytoplasmic portion was saved. The crude nuclear pellet was resuspended in 200 µl buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 25% glycerol, v/v) and incubated on ice for 15 min. This fraction was centrifuged (14,000 rpm, 15 min at 4°C) and the supernatant containing the nuclear protein extracts was collected. Protein concentrations were determined using a Bio-Rad Bradford assay mentioned above. Proteins were assessed either by EMSA (nuclear) or by Western blot analysis (cytoplasmic). Purity of nuclear extracts was confirmed by absence of cytoplasmic protein contamination (data not shown).

EMSA used a double-stranded AP-1 consensus oligonucleotide probe (5’-CCG TTG ATG AGT CCG GAA-3’) (Promega, Madison, WI) that was end-labeled with [γ-32P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham, Arlington Heights, IL). Binding reactions containing 35 fmol (∼1 × 10⁹ dpm) of oligonucleotide and 1 µg of nuclear protein, were performed for 30 min at room temperature in binding buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2.5 mM DTT, 20% glycerol (v/v), and 0.5 µg poly(dI:dC) (Pharmacia)). Reaction volumes were held constant at 10 µl. Unlabeled competitor oligonucleotide (AP-1) or irrelevant oligonucleotide (Oct-1) was added 30 min before the addition of radiolabeled probe. The binding reactions were stopped after 1 h with the addition of 1 µl 10× gel loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, 40% glycerol (v/v)). The reaction was run on a non-denaturing, 4% polyacrylamide electrophoretic gel in 0.5× TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA) at 100 V/15 mA for ~2 h. The EMSA gel was vacuum dried and visualized with Kodak X-OMAT film (Rochester, NY) exposed to the gel at ~70°C for ~3 h.

Determination of AP-1 transcriptional activity
To determine the transcriptional activity of AP-1, transient transfections of THP-1s were performed using a luciferase-linked, tandem AP-1 promoter construct, 3xAP1Luc (19), provided by Dr. Roland Schmid (University of Ulm, Ulm, Germany). Transient transfections were performed as previously reported (20, Brieﬂy, cells were centrifuged (1100 rpm, 10 min) and washed once in warmed sterile PBS buffer. Cells were then resuspended in STBS buffer in a concentration of 2 × 10⁶/ml followed by the addition of 10 µg/ml DEAE dextran (Sigma). Cells were separated into 6-ml aliquots, and 6 µg of the 3xAP1Luc construct with a β-galactosidase/Luc reporter construct were added. Cell/dextran/DNA mixtures were incubated at 37°C for 10–15 min until trypan blue inclusion reached ~20%. At this time, transfections were centrifuged (1000 rpm, 10 min) washed once in warmed STBS, and then resuspended in 10 ml fresh RPMI 1640 media and placed in T25 flasks (48 h, 37°C, 5% CO₂) before being subjected to experimental conditions. For transient transfection assays, because no differentiation step occurred, cells were stimulated with 10 µg/ml of LPS as previously reported (20). Pretreatment with OA or PA was for 60 min before LPS stimulation. Cell lysates were harvested in 100 µl luciferase lysis buffer (Promega), subjected to a freeze-thaw cycle, and centrifuged (4000 rpm, 5 min) before measuring luciferase activity according to the manufacturer’s instructions (Promega) using a Berthold AutoLumat LB953 luminometer. Luciferase activity is reported as light units corrected for total cellular protein.
Measurement of IL-1β

Cell culture supernatant levels of IL-1β were measured by ELISA (BioSource International, Camarillo, CA) according to the manufacturer’s instructions. The minimal detectable level for IL-1β was 1 pg/ml. Samples values were determined in duplicate from four to eight wells per condition.

Statistics

Values are expressed as mean ± SEM. Intergroup comparisons were made using unpaired, two-tailed Student’s t test. Differences were accepted as significant for p < 0.05.

Results

Activation of JNK

THP-1 cells were differentiated by pretreatment with IFN-γ for 30 min and stimulated with LPS (1 µg/ml media). Cell extracts were harvested over time and subjected to in vitro kinase activity determination. As shown in Fig. 1A, JNK activity increased significantly over time with peak activity at 60 min. Control lanes (e, empty GST-vector) were run in the absence of c-Jun-GST to confirm the specificity of the kinase reactions. For subsequent studies, the 60-min time point was used. Similarly, a dose-response curve for LPS activation of JNK at 60 min was performed as shown in Fig. 1B. Although as little as 10 ng/ml LPS stimulated JNK activity compared with control cells (0 ng/ml lane), the highest baseline-to-signal ratio was achieved with 1 µg/ml LPS (1000 ng/ml lane). Thus, this dose was used for subsequent studies. Activation of JNK was associated with a similar increase over time of c-Jun expression (Fig. 1C) via a well described, autoamplification process (21, 22) supporting the biological relevance of the measured kinase activity. These results established a working in vitro model of inflammation for examining the regulation of JNK by phosphatases.

Regulation of JNK by phosphatase inhibition

Because JNK is phosphorylated on a threonine residue (6), we aimed to determine whether JNK was regulated by serine/threonine phosphatases using the inhibitor, OA. Treatment with OA before LPS stimulation resulted in a dose-dependent and significant increase in JNK activity compared with LPS-stimulated cells (Fig. 2A). Of note, OA added directly to the kinase reaction after immunoprecipitation did not affect JNK activity (data not shown), suggesting that there was no direct effect of OA on JNK. Interestingly, OA alone was sufficient to augment JNK activity as shown in Fig. 2B, although there remained a significant effect on LPS-mediated JNK activation (compare lanes 2 and 3 to lanes 6 and 7, Fig. 2B). This OA-induced increase in JNK activity correlated to a dose-dependent inhibition of serine/threonine phosphatase activity (Fig. 3). The effect of increased JNK activity induced by OA was also associated with hyperphosphorylation of c-Jun as demonstrated by Western blot analysis (Fig. 4). These results demonstrated that OA inhibition of serine/threonine phosphatases substantially increased activation of JNK resulting in c-Jun hyperphosphorylation.

AP-1 transcriptional activity

The biological relevance of OA-induced activation of JNK was supported by the associated findings of increased AP-1 nuclear translocation and binding as detected by EMSA (Fig. 5A). In this study, pretreatment with escalating doses of OA resulted in a significant increase in the signal for AP-1. This signal was competed off by nonradiolabeled AP-1 but not Oct-1. To test the functional consequence of this finding, AP-1 promoter-driven expression of

FIGURE 1. A, Time course of LPS-induced JNK activity as measured by in vitro kinase assay. The amount of phosphorylated c-Jun-GST protein (j) increases over time with a peak signal at 60 min after stimulation (1 µg/ml media). Alternate wells are shown with an empty GST vector (e). Blot is representative of three separate experiments. B, Dose-response curve for LPS activation of JNK as determined by kinase assay at 60 min. LPS stimulation from 10 to 1000 ng/ml are compared with control cells (0 ng/ml lane), the highest baseline-to-signal ratio was achieved with 1 µg/ml LPS (1000 ng/ml lane). Thus, this dose was used for subsequent studies. Activation of JNK was associated with a similar increase over time of c-Jun expression (Fig. 1C) via a well described, autoamplification process.

FIGURE 2. A, Effect of OA on JNK activity as measured by in vitro kinase assay 60 min after LPS stimulation. A dose-response relationship between OA and phosphorylation of c-Jun-GST was found. Blot is representative of four separate experiments. B, Comparison of the effect of OA on JNK activity either alone (lanes 1–4) or before LPS stimulation (60 min) (lanes 5–7). Although OA alone was able to activate JNK, its effect remained more pronounced after LPS activation of JNK (compare lane 2 with 6 and lane 3 with 7). Blot is representative of two separate determinations.
Luciferase was determined using the 3xAP1Luc construct in transient transfection assays (Fig. 5B). These results showed that OA treatment resulted in a dose-dependent augmentation of AP-1-driven luciferase expression measured 5 h after LPS stimulation. The finding of decreased luciferase expression at the highest dose (500 nM) was likely a result of cytotoxicity from OA. In preliminary toxicity studies, cell viability remained >95% (by MTT assay) at 2 h after 1000 nM OA and there was minimal LDH release, whereas by 4 h cell viability decreased by 50% and LDH was detected in cell culture supernatants (data not shown). The presence of a signal for AP-1 and a high level of constitutive AP-1 luciferase activity in unstimulated cells was likely the result of serum activation of this pathway as has been demonstrated by others (23, 24).

Because AP-1 drives expression of IL-1β after LPS stimulation in human monocytes (9), we measured cell culture supernatant levels of IL-1β in our model system. Supernatants were harvested 4 h after stimulation with LPS either with (1 h pretreatment) or without OA. As shown in Fig. 6, OA before LPS resulted in a significant increase in IL-1β at the 500- and 1000-nM doses. In unstimulated cells and those treated with 10, 100, or 500 nM OA alone, no IL-1β was detected (data not shown). However, 1000 nM OA alone did result in an increase in IL-1β expression from THP-1 cells independent of LPS stimulation, supporting the observation that OA likely effects the serum-induced activation of AP-1. Taken together, these data supported the hypothesis that serine/threonine phosphatase inhibition by OA results in a biologically relevant activation of JNK resulting in increased proinflammatory cytokine expression.

Coprecipitation of PP2A-A/α with JNK

Because previous data had demonstrated physical associations between unrelated phosphatases and the kinases that they regulated, we hypothesized that there may be a similar association between JNK and one of the serine/threonine phosphatases. Western blot analysis of whole-cell lysates showed the presence of all regulatory subunits for PP2A whose levels were generally unaffected during the 30 min after LPS stimulation (Fig. 7A). To test the hypothesis that one of the regulatory subunits of PP2A was physically associated with JNK, lysates from either unstimulated or LPS-stimulated cells were immunoprecipitated with either anti-JNK Ab or an irrelevant, isotype-matched α-MKP-2 Ab. PP2A-A/α (molecular mass = 65 kDa) was the only subunit that demonstrated a specific interaction in the anti-JNK immunoprecipitated product (Fig. 7B), and suggested that this PP2A subunit shared a physical association with JNK. It was also observed that LPS stimulation consistently resulted in a diminished PP2A-A/α signal after JNK immunoprecipitation (Fig. 7B, lane 4 vs 6) that was confirmed by an analysis of the kinetics of this after LPS stimulation (Fig. 7C). Although it is intriguing to speculate that the inflammatory stimulus somehow dissociates PP2A-A/α from JNK thus favoring kinase activity, this hypothesis remains to be fully tested.
are expressed as mean ± SEM (n = 6 wells per condition). Note the logarithmic scale for IL-1β levels (γ-axis). Values were determined in triplicate and are expressed as mean ± SEM (n = 6 wells per condition).

**FIGURE 6.** Effect of OA on IL-1β expression. LPS stimulation resulted in a substantial increase in IL-1β expression at 4 h. (IL-1β was undetected in unstimulated cells). OA pretreatment resulted in a dose-dependent increase in IL-1β. The highest dose of OA used (1000 nM) alone caused a significant increase in IL-1β. Note the logarithmic scale for IL-1β levels (γ-axis). Values were determined in triplicate and are expressed as mean ± SEM (n = 6 wells per condition).

**Discussion**

Intracellular signal transduction pathways are key regulators of a number of cellular functions. Their role in the cellular response to inflammatory stimuli that results in cytokine expression has been increasingly investigated. A process crucial to such pathways is the reversible phosphorylation of participating proteins. This process seems to be regulated in part by a balance between kinase and phosphatase activity (26). An important example of a kinase that participates in transcriptional activation of so-called “early” inflammatory genes is JNK. This kinase phosphorylates c-Jun facilitating its binding to c-Fos to form the activation complex, AP-1, and direct transcription of such genes as IL-1, IL-8, and ICAM-1 (6–11). Because JNK is phosphorylated by upstream members of the MAP kinase pathway on a threonine residue, we hypothesized that a member of the serine/threonine phosphatases might regulate its activity. The serine/threonine phosphatases are a class of enzymes that dephosphorylate proteins and have traditionally been divided into two classes on the basis of their sensitivity to two heat-stable inhibitors (27, 28). However, a phylogenetic schema has suggested classifying these enzymes into two families: family I including PP1, PP2A, and PP2B and family II including PP2C (29). Although phosphatase inhibition has resulted in increased AP-1 activity, what has remained unknown in this signal cascade is whether a member of the serine/threonine class of phosphatases might have any direct regulatory effects on JNK activity.

The data presented provide evidence that JNK is regulated by PP2A in the context of an inflammatory stimulus. Pharmacological inhibition of PP2A by OA resulted in substantial increases in JNK activity. Although thought to be a nonspecific PP1/PP2A inhibitor, others had demonstrated that the doses of OA used only inhibited PP2A similar to our findings (30). Nevertheless, the availability of...
a PP1 selective inhibitor, a PA derivative, allowed us to confirm the specificity of these findings (25). PA had no effect on JNK until a higher dose, which also activates PP2A, was used. This dose resulted in an inhibition of JNK activity in a manner consistent with our hypothesis. The data presented are also consistent with previous observations that described an effect of OA on inducing IL-1β expression via a mechanism related to the presence of an AP-1-sensitive binding site on a chloramphenicol acetyltransferase reporter plasmid (31). Induction of increased chloramphenicol acetyltransferase activity was hypothesized to involve the effect of OA on protein phosphorylation. This effect of OA had also been observed to be related to increased transcriptional activation of the jun proto-oncogene in PMA-stimulated Jurkat cells (32). This study concluded that the protein phosphatases may have been negative regulators of the AP-1 transcription factor. Our data are consistent with both these previous studies and support the hypothesis that OA regulation of AP-1 activity is related to a negative regulatory effect of PP2A on JNK activity.

To study the regulation of JNK activity, we used an in vitro model of macrophage immunostimulation with LPS as was used by others (33). Subsequent to this observation, there have been several levels of kinase-dependent cell signaling that have been implicated that result in JNK activation by immunostimulation. Various models have demonstrated activation of protein tyrosine kinases (33, 34), stress-activated protein kinase kinases (35), and the MAP kinases (eg., extracellular signal-related kinase (ERK) 1/2; Ref. 36). Based on our data, we are unable to exclude the possibility of an effect of PP2A inhibition on an upstream kinase that is necessary for JNK activation. Although PP2A has been demonstrated to inhibit constitutive protein kinase C (37) and ERK2 (38), these studies focused on regulation of cellular entry into the growth cycle, whereas our focus was on the state of JNK regulation in the context of an inflammatory stimulus. The methodology that could be used to define this direct regulation would include co-overexpression of JNK with a biologically active form of PP2A, or more specifically, PP2A-Aα, which has proven to be technically difficult (39). Successful overexpression of the regulatory elements of PP2A has been limited to the catalytic subunit (40), although this molecular strategy remains a focus of our ongoing investigations.

The strongest evidence for PP2A directly regulating JNK is derived from the immunoprecipitation studies, which clearly demonstrate a physical association between PP2A-Aα and JNK. This association consistently seemed to be less after LPS treatment and raises the intriguing possibility that immunostimulation somehow results in the physical dissociation of PP2A-Aα from JNK, although this hypothesis remains to be tested. There exist other examples of regulated phosphatase-kinase pairs that share a physical association. For example, ERK2 has been shown to bind the MAP kinase phosphatase-3 (MKP-3) resulting in regulation of MAP kinase activation (41), whereas PP1 has been shown to bind the A-kinase anchoring protein AKAP220 (42). Physical associations between various subunits of the ubiquitous PP2A and kinase species do not seem unique to JNK because these have been described for the catalytic subunit, PP2A-C, binding to both the calmodulin-dependent protein kinase IV (43), the 70-kDa S6 kinase (44), and
the p21-activated kinase (45). Alternatively, the regulatory subunit of PP2A may simply serve as a scaffolding protein for a larger signal transduction complex comprised of JNK and other upstream members of the MAP kinase family that could be regulated by other, OA-sensitive phosphatases. Determination of such protein-protein interactions remains the focus of ongoing studies. Together with our data, these observations remain consistent with the hypothesis that regulation of key phosphorylation events is governed by the proximity of kinases and phosphatases close to their substrate (26). Therefore, phosphatases may be a target of therapeutic interventions in an attempt to regulate inflammatory cell signaling.

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