The Calcium/Calmodulin-dependent Protein Phosphatase Calcineurin Is the Major Elk-1 Phosphatase*

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The transcription factor Elk-1 is a component of ternary complex factor and regulates gene expression in response to a wide variety of extracellular stimuli. Phosphorylation of the C-terminal domain of Elk-1, especially at serine 383, is important for its transactivation activity. Recently, mitogen-activated protein kinases, such as extracellular signal-regulated kinase, stress-activated protein kinase, and p38 mitogen-activated protein kinase have been demonstrated to be Elk-1 kinases. However, negative regulators of Elk-1, such as protein phosphatases, still remain to be identified. Here we report that COS cell lysates were able to dephosphorylate an extracellular signal-regulated kinase-phosphorylated glutathione S-transferase-Elk c fusion protein, including serine 383. The phosphatase activity was inhibited by cyclosporin A (a calcineurin inhibitor) but not by okadaic acid (a PP1 and PP2A inhibitor). Purified calcineurin also could efficiently dephosphorylate glutathione S-transferase-Elk c in vitro. Pretreatment of COS cells with cyclosporin A significantly enhanced epidermal growth factor-induced serine 383 Elk-1 phosphorylation whereas ionomycin inhibited the Elk-1 phosphorylation. These data provide both in vitro and in vivo evidence that calcineurin is the major Elk-1 phosphatase and plays a critical role in Elk-1 regulation. The identification of calcineurin as the major Elk-1 phosphatase may provide a mechanism for Elk-1 regulation by Ca2+ signals as well as a possible biochemical basis for the neurotoxicity and nephrotoxicity of the immunosuppressant drug cyclosporin A.

Activation of the mitogen-activated protein kinase (MAP1 kinase, also known as the extracellular signal-regulated ki-
ERK1 in kinase buffer (18 mm HEPES, pH 7.5, 10 mm magnesium acetate, 50 μM ATP) for 30 min at 30 °C. 32P-Labeled GST-Elkc was prepared in the presence of [γ-32P]ATP in the same reaction mixture described above. Phosphorylated GST-Elkc was purified with glutathione-agarose (Sigma) and eluted in buffer (10 mM Tris-Cl, pH 7.5) containing 5 mM glutathione.

Phosphatase Assays—Starved COS cells were washed with ice-cold phosphate-buffered saline twice and lysed by scraping in buffer A (50 mm HEPES, pH 7.5, 150 mm NaCl, 8 mm β-mercaptoethanol, 10 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride) supplemented with 1% Triton X-100. The cell lysates were clarified by centrifugation at 12,000 × g for 20 min at 4 °C, and the supernatants were used for in vitro dephosphorylation assays.

In vitro phosphatase assays were performed using phosphorylated GST-Elkc (GST-pElkc) as a substrate. GST-pElkc (1 μg) was incubated with the indicated amount of cell lysates in a total volume of 30 μl of buffer A containing various phosphatase inhibitors as indicated. The reactions were allowed to proceed for 30 min at 30 °C and then stopped by adding SDS-polyacrylamide gel electrophoresis sample buffer.

Assays using purified calcineurin were performed by incubating 1 μg of GST-pElkc with the indicated amount of calcineurin (Sigma) in reaction buffer, which contained 50 mm HEPES, pH 7.5, 1 mg/ml bovine serum albumin (Sigma), 1 mM dithiothreitol, 1 mM CaCl2, and 1 μM calmodulin (Calbiochem) in a total volume of 30 μl for 15 min at 30 °C. A portion of the reactions (10 μl) was separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P (Millipore), and subjected to autoradiography or immunoblot analysis (probed with anti-pElk-1 antibody, New England Biolabs) to monitor dephosphorylation of GST-pElkc.

Transfections—COS cells in 6-cm dishes were transfected with pcDNA3-Elk-1 (0.5 μg) by the DEAE-dextran method essentially as described (26). Twenty-four hours after transfection, cells were starved for another 24 h by reducing serum concentration to 0.1%. Transfected cells were stimulated with EGF (50 ng/ml, Calbiochem) for the indicated period. Cells were lysed directly by addition of 100 μl of SDS sample buffer and subjected to immunoblot as described above.

RESULTS

Elk-1 Phosphatase Activity in COS Cell Lysates Is Sensitive to Cyclosporin A—We first examined Elk-1 phosphatase activity in COS cell lysates using phospho-GST-Elkc. This recombinant GST-Elkc consists of the murine Elk-1 C-terminal transactivation domain fused to GST. 32P-Labeled phospho-GST-Elkc was efficiently dephosphorylated in a lysate-dependent manner (Fig. 1A). To determine which subfamily of serine/threonine phosphatases was responsible for this activity we exploited the availability of inhibitors specific for each class of phosphatase: PP1, PP2A, PP2B (calcineurin), and PP2C. As can be seen in Fig. 1A, inclusion of a general serine/threonine phosphatase inhibitor, NaF (10 mM), in the reaction blocked Elk-1 dephosphorylation. Addition of EGTA and EDTA also efficiently inhibited phospho-GST-Elkc phosphatase activity. These data indicate that the primary Elk-1 phosphatase in COS cell lysates requires the presence of a divalent cation for activity. This is indicative of either calcineurin or PP2C as having Elk phosphatase activity. Addition of Ca2+ reversed the inhibitory effect of EDTA, suggesting that the phosphatase activity in the lysate is Ca2+-dependent (data not shown). OA (1 μM), a selective inhibitor of PP1 and PP2A, had no significant effect on this phosphatase activity (Fig. 1A). In contrast, the immunosuppressant CsA (5 μM), which inhibits calcineurin phosphatase activity via association with immunophilins, dramatically reduced phospho-GST-Elkc phosphatase activity in COS cell lysates (Fig. 1A). This result suggests that the primary Elk-1 phosphatase is cyclosporin A-sensitive and, therefore, most likely calcineurin.

Earlier reports have established that phosphorylation of serine residue 383 of Elk-1 is important for its transactivation activity (5–10). Therefore, in an attempt to precisely monitor the dephosphorylation of GST-Elkc with respect to a functionally important phospho-amino acid residue, we performed immunoblot analysis using an anti-phospho Elk-1 antibody, which only recognizes the phosphoserine 383 and, therefore, active form of Elk-1. Fig. 1B demonstrates that COS cell lysates also could dephosphorylate serine 383 of the phosphorylated GST-Elkc, and this phosphatase activity was significantly inhibited by NaF, chelators of divalent cations, and CsA but not OA, consistent with the results using 32P-labeled GST-Elkc.

We performed experiments to determine whether the Elk-1 phosphatase activity in COS lysates was inhibitable in a dose-dependent manner. CsA effectively inhibited Elk-1 phosphatase activity in a dose-dependent fashion (Fig. 1C), whereas only extremely high concentrations of OA (10 μM) would begin to show any inhibition in identical experimental conditions (Fig. 1D). It is important to note that this concentration of OA is 103–104-fold higher than necessary to inhibit PP1 and PP2A (IC50 of OA for PP2A, PP1, and calcineurin is 0.1, 20, and 5000 nM, respectively). Similarly, preincubation of COS cells with CsA was also able to inhibit Elk-1 phosphatase activity of crude cell lysates in a dose-dependent manner (Fig. 2, A and B).
Calcineurin Is an Elk-1 Phosphatase

Calcineurin bands are indicated by an arrow. The phosphatase activity (Fig. 2) was readily detected a 61-kDa band (Fig. 2). Consistent with this hypothesis, Western blotting of COS cell lysates is due to calcineurin. Phosphorylation of Elk-1 (serine 383) was detected by immunoblotting. Since calcineurin activity is regulated in vivo, we examined the effects of CsA on Elk-1 phosphorylation in intact COS cells. We first transfected an expression vector encoding mouse Elk-1 cDNA into COS cells and stimulated the transfected cells with EGF and monitored Elk-1 serine 383 phosphorylation by immunoblot with the anti-pElk-1 antibody. As shown in Fig. 3A, treatment with CsA significantly enhanced the phosphorylation of Elk-1 when compared with untreated cells. CsA increased both the basal as well as the stimulated Elk-1 phosphorylation. In contrast, OA did not increase Elk-1 phosphorylation (data not shown). Since calcineurin activity is regulated by Ca2+, we examined the effects of a Ca2+ ionophore on Elk-1 phosphorylation in vivo. As shown in Fig. 4B, the presence of a Ca2+ ionophore (ionomycin) dramatically inhibited EGF-stimulated Elk-1 phosphorylation in intact COS cells. Cells were treated with 1 μM ionomycin for 5 min before stimulation with EGF (50 ng/ml) for 10 min. Immunoblot analysis was performed with the indicated antibody.

**Fig. 2.** Elk-1 phosphatase activities inhibited by pretreatment with CsA but not OA. A, COS cells were first treated with OA (1 μM) or CsA (5 μM) for 30 min. The cells were washed and cell lysates were prepared as described under "Experimental Procedures." Phosphorylation of Elk-1 (serine 383) was detected by immunoblotting. Lane 1 contains no lysate. Lanes 2–4 contain lysates from cells pretreated with CsA, OA, or untreated control, respectively. B, dose-dependent inhibition. COS cells were treated with various concentrations of CsA as indicated (lanes 2–6). The assay was performed as described for A. Lane 1 is a control containing no cell lysate. C, expression of calcineurin in COS cells. Cell lysates (50 μg) of COS cells (lane 1) or HeLa cells (lane 2) were probed with anti-calcineurin antibody (Transduction Laboratories). The calcineurin bands are indicated by an arrow.

**Fig. 3.** Dephosphorylation of Elk-1 by calcineurin. A, dephosphorylation of Elk-1 by calcineurin is Ca2+-calmodulin-dependent. Phosphorylated GST-Elk-1 was incubated with purified calcineurin (CN, 0.2 μM) in the presence or absence of Ca2+-calmodulin (CaM*) as indicated. Phosphorylation of Elk-1 (serine 383) was detected by immunoblotting. B, dose-dependent dephosphorylation of Elk-1 by calcineurin.

**Fig. 4.** CaA enhances EGF-induced Elk-1 phosphorylation in vitro. A, phosphorylation of Elk-1 but not ERK is enhanced by treatment of CaA. COS cells were transfected with 0.5 μg of pcDNA3-Elk-1 as described under "Experimental Procedures." The transfected cells were untreated or treated with 5 μM CaA for 30 min and stimulated with EGF (50 ng/ml) for the indicated times. The phosphorylation of Elk-1 and ERK was determined by immunoblotting. B, ionomycin inhibits EGF-induced Elk-1 phosphorylation. Elk-1 was transfected into COS cells. Cells were treated with 1 μM ionomycin for 5 min before stimulation with EGF (50 ng/ml) for 10 min. Immunoblot analysis was performed with the indicated antibody.
kinases in response to a wide range of extracellular stimuli and essential for Elk-1 activation.

**DISCUSSION**

The TCF family of transcription factors is tightly regulated by phosphorylation and dephosphorylation (5–13, 27). Extensive studies have demonstrated that members of the MAP kinases, including ERK, stress-activated protein kinase, and p38, are responsible for the phosphorylation and activation of TCFs. Since many target genes of the TCF are expressed transiently upon stimulation, it therefore may be important to dephosphorylate and inactivate TCF transcription factors. We have demonstrated that calcineurin is the major Elk-1 phosphatase based on results from experiments performed both *in vitro* and *in vivo*. In this report, we showed that COS cell lysates can efficiently dephosphorylate ERK1-phosphorylated GST-Elk, including serine 383, whose phosphorylation is critical for Elk-1 activity. This phosphatase activity in the lysate is inhibited by EGTA and EDTA and also highly sensitive to inhibition by CsA but not by OA. We also showed that purified calcineurin contains Elk-1 phosphatase activity and that pre-treatment of CsA enhanced EGF-induced serine 383 Elk-1 phosphorylation in intact cells. Identification of calcineurin as the major Elk-1 phosphatase clearly will shed light on our understanding of the regulation of Elk-1.

Zink et al. (27) reported previously that OA can increase the TCF activity as determined by a gel shift assay. OA increased the basal and EGF-stimulated TCF activity. They subsequently showed that OA activates MAP kinase (28). No direct dephosphorylation of Elk-1 was examined. Therefore, whether OA directly blocks TCF dephosphorylation or enhances phosphorylation via activation of ERK is not clear. Our data do not support that PP2A is the major Elk-1 phosphatase. It is widely observed that the treatment of OA can result in ERK activation (29–31). A likely explanation for the enhancement of TCF by OA is due to the indirect effect of ERK activation.

Calcineurin is a Ca$^{2+}$ and calmodulin-dependent serine/threonine protein phosphatase (17). It is interesting to note that activation of growth factor receptors results in activation of the Ras-MAP kinase pathway as well as activation of phospholipase Cγ and Ca$^{2+}$ signals (32). Activation of Ras-MAP kinase leads to numerous cellular responses including activation of TCF. The Ca$^{2+}$ signal, via the activation of calcineurin, may serve as a built-in mechanism to down-regulate TCF after its activation. Another potential physiological significance of calcineurin in TCF regulation is that calcineurin may play an important role in cross-talk linking different signaling pathways. For example, activation of calcineurin by one hormone may specifically prevent the activation of TCF by growth factors but not affect the other targets of the Ras-MAP kinase pathways and, therefore, alter cellular responses.

Calcineurin appears to control various cellular events through regulation of the phosphorylation states of its targets. Previous reports have demonstrated that the inhibitors of PP1, NF-AT, N-methyl-b-aspartate receptor, and IP$_3$ receptors are physiological substrates of calcineurin (18, 19, 33–35). For example, NF-ATs are transcriptional factors, known to play a key role in the regulation of cytokine gene transcription during immune response (36). Calcineurin has been shown as the target of immunosuppressive drugs, CsA and FK506 (20). These drugs also have a wide range of side effects, such as nephrotoxicity and neurotoxicity (22), and these toxic effects correlate with the ability of the drugs to inhibit calcineurin activity, suggesting that substrates other than NF-AT are targets of calcineurin in kidney and brain. In this study we have shown that another transcriptional factor, Elk-1, is also a target of calcineurin in COS cells. One cannot help but think that the effect of immunosuppressants on TCF activity may have a role for the toxic effects of these drugs. In conclusion, we reported here that Elk-1 is a novel substrate of calcineurin, which dephosphorylates and inactivates Elk-1.

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