Stimulation of Elk1 Transcriptional Activity by Mitogen-activated Protein Kinases Is Negatively Regulated by Protein Phosphatase 2B (Calcineurin)*

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Cellular calcium (Ca\(^{2+}\)) and the Ca\(^{2+}\)-binding protein calmodulin (CaM) regulate the activities of Ca\(^{2+}\)/CaM-dependent protein kinases and protein phosphatase 2B (calcineurin). Functional interactions between CaM kinases and mitogen-activated protein (MAP) kinases were described. In this report, we describe cross-talk between calcineurin and mitogen-activated protein kinase signaling. Calcineurin was found to specifically down-regulate the transcriptional activity of transcription factor Elk1, following stimulation of this activity by the ERK, Jun N-terminal kinase, or p38 MAP kinase pathways. Expression of constitutively activated calcineurin or activation of endogenous calcineurin by Ca\(^{2+}\) ionophore decreased the phosphorylation of Elk1 at sites that positively regulate its transcriptional activity. Calcineurin specifically dephosphorylates Elk1 at phosphoserine 383, a site whose phosphorylation by MAP kinases makes a critical contribution to the enhanced transcriptional activity of Elk1. The cross-talk between calcineurin and MAP kinases is of physiological significance as low doses of Ca\(^{2+}\) ionophore which by themselves are insufficient for c-fos induction can actually inhibit induction of c-fos expression by activators of MAP kinases. Thus through the effect of calcineurin on Elk1 phosphorylation, Ca\(^{2+}\) can have a negative effect on expression of Elk1 target genes. This mechanism explains why different levels of intracellular Ca\(^{2+}\) can result in very different effects on gene expression.

The second messenger Ca\(^{2+}\) plays a critical regulatory role in a variety of physiological processes in many different cell types (1, 2). One of the most important mediators of Ca\(^{2+}\) signaling is the Ca\(^{2+}\)-binding protein, calmodulin (CaM)\(^1\) (3).

*This research was supported in part by grants from the National Institutes of Health (to M. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a postdoctoral fellowship from the Leukemia Research Foundation.

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\(^1\) The abbreviations used are: CaM, calmodulin; CaM-K, Ca\(^{2+}\)/CaM-dependent protein kinases; MAP, mitogen-activated protein; MAPK, MAP kinase; CsA, cyclosporin A; CREB, cAMP-responsive element binding protein; SRF, serum response factor; GST, glutathione S-transferase; WCE, whole cell extract; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin; NE, nuclear extract; TCF, ternary complex factors; RT-PCR, reverse transcriptase-polymerase chain reaction; JNK, Jun N-terminal kinase; CRE, cAMP response element; MEK, MAP kinases; ATF, activating transcription factor; EGF, epidermal growth factor; SRE, serum response element.
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(25–27). Therefore, there must be additional transcription factors whose activity is modulated by calcineurin.

An additional target for calcineurin that is involved in T cell activation is a yet-to-be identified component of the signaling pathway that leads to activation of JNK (Jun N-terminal kinase) in T cells (28). Stimulation of T cells with a phorbol ester and Ca$^{2+}$ ionophore results in synergistic JNK activation, which is blocked by CsA or FK506 (29). Recent studies have shown that the target for calcineurin is a T cell-specific component of the JNK pathway that acts in synergy with protein kinase C0 (28). However, in other cell types calcineurin activation or treatment with CsA has no effect on JNK activity (28).

In addition to JNK, which belongs to the MAP kinase (MAPK) family, mammalian cells express two other MAPK cascades that link a variety of extracellular stimuli to changes in gene expression (30). The most well characterized MAPK cascade leading to ERK activation is stimulated most efficiently by mitogens and growth factors, which activate receptor tyrosine kinases (31). Like all MAPKs, the ERKs are phosphorylated and activated by specific MAPK kinases (MAPKK), the MEKs, which themselves are activated by MAPKK kinases (MAPKKK), one of which is Raf-1 (30, 32). The MAPK pathways that lead to JNK and p38 MAPK activation are most potently triggered by physical and chemical stresses (UV irradiation, osmotic shock, and heat shock) and proinflammatory cytokines (33, 34). JNK and p38 are also activated by specific MAPKs, JNK1 (MKK4) (35, 36), and JNK2 (MKK7) (37–39) for JNK, and MKK3, and MKK6 for p38 (40–42). These MAPKKs are in turn activated by several MAPKKKs, including MEKK1 (43), MEKK2, and -3 (44), TAK1 (45), ASK1 (46), and MLK3 (47). The three components (MAPK, MAPKK, and MAPKKK) of each cascade are engaged in sequential phosphorylation reactions in the cytoplasm (30). Activated MAPKs are then redistributed in the cell, with a portion entering the nucleus (48), where they phosphorylate several transcription factors. The ERKs are known to phosphorylate Elk1 (49), ETS2 (50), c-Myc (31), and c-Myb (31). The JNKs phosphorylate c-Jun (51), ATF2 (52), and Elk1 (48). The p38s phosphorylate ATF2 (52), Elk1 (49), CHOP (53), and MECP2 (54). In many of these cases, the transactivating potential of the affected transcription factor is augmented upon its phosphorylation, leading to changes in the expression profile of target genes (33, 51). One target gene for all three MAPK cascades is the c-fos proto-oncogene (49). The c-fos promoter contains a composite binding site recognized by SRF and ternary complex factors (TCFs) (55). The activity of the TCF protein Elk1 is stimulated by all three MAPK cascades through phosphorylation of sites within its C-terminal activation domain (49).

Interaction between Ca$^{2+}$ signals and three MAPK cascades have been reported. In several cell types, elevated intracellular Ca$^{2+}$ can stimulate MAPK activity (29, 56). Specifically, CaM-Ks may activate MAPK cascades in fibroblasts (57). As mentioned above, in T cells, calcineurin can participate in JNK activation through synergy with protein kinase C0 (28). In this report, we investigated cross-talk between calcineurin and MAPK pathways in cell lines of either fibroblast or lymphoid origin. We found that calcineurin specifically dephosphorylates one substrate that is common to all three MAPKs, the transcription factor Elk1, thus negatively regulating its transcriptional activity. This mechanism explains the regulatory versatility of intracellular Ca$^{2+}$, which can exert very different effects on gene expression depending on its level.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—COST cells and Jurkat cells were maintained in Dulbecco’s modified Eagle’s medium and RPMI 1640, respectively, supplemented with glutamate (1 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (Life Technologies, Inc.).

**Plasmids**—Mammalian expression vectors (Raf-1, MEKK1, JNK2, MEKK6, MEK, JNK1, ERK2, p38, CnA(CaM-Al), CnB, Gp-A-c-Jun, GAL4-Elk1, and GAL4-ATF2) and vectors for GST fusion proteins (GST-c, -c-1, -c-2, -c-3, GST-EZK1, and GST-ATF2) were prepared (15.5 μg) (28, 39, 41, 43, 58, 59). GST-ETS2 vector encoding the human ETS2 (21–226) was constructed by PCR and subcloned into pGEX-4T-2 (Amersham Pharmacia Biotech) (50).

**Transfection**—COST cells were seeded at 2 × 10$^5$ cells/well in 6-well plate (35-mm diameter) the day before transfection. For reporter assay, cells were transfected with Superfectamine (Qiagen) according to the manufacturer’s instructions. 5×GAL4-LUC vector (1.5 μg), and mammalian expression vectors of Raf-1, MEKK1, JNK2, MEKK6, MEK1, p38, GAL4-c-Jun, GAL4-Elk1, GAL4-ATF2 (5 ng of each, alone or in combination as indicated), and CnAL(CaM-Al) expression vector (200 ng, as indicated) were transfected into the cells. In each transfection, 50 ng of pActin-β-gal was cotransfected to monitor the transfection efficiency. The total amount of DNA was kept at 2 μg with empty p5Sre vector. The cells were incubated with DNA for 2 h and then incubated with regular media (10% fetal bovine serum) for 1 h. The cells were then incubated with media without serum. 24 h later, the cells were harvested and lysed. The luciferase and β-galactosidase activities were measured as described before (38). For immunoblotting analysis, COS7 cells were transfected with Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s instructions. The HA-tagged proteins (CnA and CnB) were detected with anti-HA monoclonal antibody (Bio-Rad). Protein samples (50 μg of each) were separated by SDS-PAGE (10% polyacrylamide), transferred to nylon membrane (Immobilon-P, Millipore), and blotted according to the manufacturer’s instructions. The HA-tagged proteins (CnA and CnB) were detected with anti-HA monoclonal antibody (Santa Cruz Biotechnology, Inc.). The subsequent procedures were performed with the enhanced chemiluminescence detection kit (ECL, Amersham Pharmacia Biotech). In the case of Jurkat cells, nuclear extract (NE) was prepared as described (22). NE samples (200 μg of protein each) were separated by SDS-PAGE and blotted with anti-Elk1. NE samples (4 μg of each) were also incubated with a DNA affinity resin prepared by coupling SRE (DNA-binding site for SRF and TCFs) to cyagenic bromide-activated Sepharose (Amersham Pharmacia Biotech). Bound proteins were eluted with SDS loading buffer, separated by SDS-PAGE, and blotted with anti-phospho-Elk1.

**Immunoprecipitation and In Vitro Phosphorylation**—After 24 h of transfection, transfected cells (HA-ERK2) were stimulated with 150 nM 12-O-tetradecanoylphorbol-13-acetate (Sigma) for 15 min, and transfected cells (HA-JNK1) were treated with UV light at 40 J/cm$^2$ for 20 s as described previously (36, 39). The WCE was incubated with HA monoclonal antibody (12CA5) and 20 μl of protein A-Sepharose (Amersham Pharmacia Biotech). In the case of Jurkat cells, nuclear extract (NE) was prepared as described (22). NE samples (200 μg of protein each) were separated by SDS-PAGE and blotted with anti-Elk1. NE samples (4 μg of each) were also incubated with a DNA affinity resin prepared by coupling SRE (DNA-binding site for SRF and TCFs) to cyagenic bromide-activated Sepharose (Amersham Pharmacia Biotech). Bound proteins were eluted with SDS loading buffer, separated by SDS-PAGE, and blotted with anti-phospho-Elk1.

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**RNA Analysis**—Total RNA from Jurkat cells was prepared with the RNeasy mini kit (Qiagen). Reverse transcriptase (RT)-polymerase chain reaction (PCR) was conducted with the Superscript One-Step RT-PCR system (Life Technology, Inc.) according to the manufacturer’s
instructions. For c-fos, 20 ng of RNA was used. The sequences of the two primers were 5'-CACCAGACCTTGGCAAAGATC-3' and 5'-CTGTCAGAGGCTCCCGAGTC-3'. For gapdh, 1 ng of RNA was used. The sequences of the two primers were 5-ACCAGATCTGATGCCCATCAC-3' and 5'-TCCACCAACCTTGCTTGTA-3'. The PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. The optical density of the bands was quantified by using Alphaimager (Alpha Innotech). A standard curve was made by RT-PCR under the same conditions with a serial dilution of RNA prepared from stimulated Jurkat cells.

Phosphatase Assay—The purified substrates were incubated with calcineurin and calmodulin (Sigma) in phosphatase buffer (20 mM Tris, pH 7.5, 1 mM CaCl2, 50 mM NaCl, 0.02% Triton X-100, 1 mg/ml bovine serum albumin). The reactions were carried out at 30 °C for 20 min and terminated by addition of SDS loading buffer. Phospho-substrates and free phosphate were separated by SDS-PAGE, and the results were obtained by autoradiography and quantified by PhosphorImager analysis.

RESULTS

A Constitutively Active Calcineurin Mutant Blocks Elk1 but Not c-Jun or ATF2 Activation—To investigate specifically the effect of calcineurin on different MAPK pathways, the constitutively active, Ca2+-independent, CnA mutant (ΔCaM-AI) was used. Cotransfection with an expression vector encoding a constitutively active form of MEKK1 enhances the ability of a fusion protein between the GAL4 DNA binding domain and the c-Jun activation domain (GAL4-c-Jun) to activate a reporter plasmid whose promoter contains five GAL4 DNA-binding sites (Fig. 1A). In previous experiments, the enhancement of GAL4-c-Jun transcriptional activity by cotransfected MEKK1 was shown to depend on phosphorylation of the c-Jun activation domain at serines 63 and 73 (36, 43). Coexpression of CnA(ΔCaM-AI) with MEKK1 had no effect on stimulation of GAL4-c-Jun activity (Fig. 1A). To examine the effect of CnA(ΔCaM-AI) on the ability of p38 to stimulate transcription, we used an activated MKK6 expression vector (40) to stimulate the activity of a GAL4-ATF2 fusion protein, which contains the ATF2 activation domain. The potentiation of GAL4-ATF2 activity by MKK6 was not significantly reduced by coexpression of CnA(ΔCaM-AI) (Fig. 1B). To examine the effect on ERK-mediated signaling, a chimeric GAL-Elk1 activator, which contains the Elk1 activation domain (58), was cotransfected with the 5×GAL4-LUC reporter construct (1.5 ng of DNA/plate) together with expression vectors for truncated MEKK1, activated Raf-1 (BXB), activated MKK6, p38, GAL-c-Jun, GAL4-ATF2 and GAL4-Elk1 (5 ng each), as indicated, in the absence or presence of a CnA(ΔCaM-AI) expression vector (200 ng). The total amount of DNA was kept at 2 μg. β-galactosidase expression. Shown are the average (± S.D.) of three separate experiments.

Calcineurin Has No Effect on the Phosphorylation and Activity of ERK—Upon cell-surface receptor stimulation, such as treatment with epidermal growth factor (EGF), or transient expression of constitutively active Raf-1, the ERKs are activated through phosphorylation at the conserved sites, threonine 202 and tyrosine 204, by the MEKs (60). We examined the effect of CnA(ΔCaM-AI) on ERK activation by cotransfecting it with an expression vector for epitope-tagged ERK2. Immuno blotting the transiently expressed ERK2 with an antibody specific for activated ERK (60) revealed that, as expected, ERK2 phosphorylation was induced by EGF treatment (Fig. 2A). This phosphorylation was not inhibited upon coexpression of CnA(ΔCaM-AI).

ERK2 activity was also measured by an immunocomplex kinase assay. Transiently expressed HA-ERK2 was immunoprecipitated, and the immunocomplexes were incubated with GST-Elk1 and γ32P/ATP in kinase buffer. EGF treatment stimulated ERK2 activity by about 5-fold (Fig. 2B). Coexpression with CnA(ΔCaM-AI) had no effect on the activation of ERK2 and its ability to phosphorylate GST-Elk1 in vitro.

Elk1 Is the Target for Calcineurin Activation—Transcription

FIG. 1. Constitutively active calcineurin blocks Elk1 but not c-Jun or ATF2 activation. COS7 cells were transfected with 5×GAL4-LUC reporter construct (1.5 ng of DNA/plate) together with expression vectors for truncated MEKK1, activated Raf-1 (BXB), activated MKK6, p38, GAL-c-Jun, GAL4-ATF2 and GAL4-Elk1 (5 ng each), as indicated, in the absence or presence of a CnA(ΔCaM-AI) expression vector (200 ng). The total amount of DNA was kept at 2 μg. β-galactosidase expression. Shown are the average (± S.D.) of three separate experiments.
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COS7 cells were transfected with pSR-ERK2 (50 ng/plate) with or without pSR-pCnA(ΔCaM-AI) and pSR-pCnB (475 ng each). Total amounts of DNA were kept constant at 1 μg. After 20 h, the cells were serum-starved for 3 h prior to EGF treatment (50 ng/ml for 15 min). The cells were then harvested and lysed. A, protein samples (50 μg) were analyzed by SDS-PAGE and analyzed by immunoblotting with an antibody specific for ERK phosphorylated at Tyr-204. The same blots were stripped and reprobed with an ERK2 specific antibody. B, protein samples (100 μg) were immunoprecipitated with anti-HA, and after extensive washing, the activity of HA-ERKs was assayed with purified GST-Elk1 as a substrate. The phosphorylated proteins were separated by SDS-PAGE, and the extent of GST-Elk1 phosphorylation was quantified with a PhosphorImager. C, expression of CnA was determined by immunoblotting protein samples (15 μg) using an HA antibody.

20-fold, coexpression with CnA(ΔCaM-AI) resulted in a 3-fold decrease in this stimulation (Fig. 3A). Because expression of moderate levels of MEKK1 can activate either JNK or p38 without exerting an effect on ERK activity (43, 62), we used more specific and direct JNK and p38 activators, namely JNKK2 (39) and MKK6 (40), respectively. We also used an expression vector for activated MEK1 to activate specifically the ERKs (59). As expected, coexpression with activated forms of all three MAPKKs resulted in enhanced GAL4-Elk1 transcriptional activity (Fig. 3, B–D). Regardless of the MAPKK used, coexpression of CnA(ΔCaM-AI) resulted in a severely diminished effect on GAL4-Elk1 activity.

Does calcineurin affect Elk1 abundance or phosphorylation? To answer this question we conducted immunoblotting experiments with Elk1 antibodies that recognize both non-phosphorylated and phosphorylated Elk1 or only phosphorylated Elk1 that is phosphorylated at serine 383. CnA(ΔCaM-AI) had no effect on the level of Elk1 expression, but it dramatically decreased the extent of Elk1 phosphorylation induced by EGF (Fig. 4A). Decreased Elk1 phosphorylation was also observed after treatment of EGF-stimulated cells with the Ca2+ ionophore ionomycin (Fig. 4A). As expected, coexpression of CnA(ΔCaM-AI) were coexpressed with Elk1 revealed a dose-dependent decrease in the extent of EGF-induced Elk1 phosphorylation (Fig. 4B).

**Calcineurin Activation Inhibits c-fos Induction and Elk1 Phosphorylation**—Next we examined whether activation of calcineurin has an adverse effect on one well characterized Elk1 target gene, c-fos. It is well established that elevated intracellular Ca2+ can induce c-fos transcription through CaM kinase activation which phosphorylates CREB and other related transcription factors that bind to a CRE in the c-fos promoter (64). We therefore had to find a way to stimulate calcineurin activity without activating CaM kinases. Since calcineurin is more sensitive to Ca2+ than CaM kinases (65), we first determined the minimal dose of ionomycin required for c-fos induction. As shown in Fig. 6A, treatment of Jurkat cells with more than 10 nM ionomycin can induce c-fos transcription, but treatment with 1 nM or less had no effect. We therefore examined whether treatment with 1 nM ionomycin can reduce c-fos induction by inducers that do not affect intracellular Ca2+. We chose anisomycin, a potent activator of the JNK and p38 MAP kinase pathways (48), as the inducing agent. As expected, anisomycin induced c-fos transcription (Fig. 6B). This induction was inhibited by pretreatment with 1 nM ionomycin. If this inhibitory effect is mediated by calcineurin, it should be prevented by cyclosporin A. Indeed, treatment with cyclosporin A prevented the inhibition of c-fos induction. Next we examined the effect of these treatments on the phosphorylation status of endogenous Elk1. The endogenous Elk1 protein was enriched by affinity purification on an SRE resin and its phosphorylation state determined by immunoblotting with anti-phospho-Elk1. Indeed, the effect of the different treatment on c-fos expression correlated very well with their effect on Elk1 phosphorylation at Ser-383. As the source of Elk1 in these experiments was nuclear extracts, it is very likely that the calcineurin-mediated dephosphorylation occurred in the nucleus.

**Calcineurin Directly Dephosphorylates Phospho-Elk1**—Since calcineurin is a serine/threonine protein phosphatase and its expression or activation has a marked effect on the phosphorylation of Elk1 at Ser-383 in intact cells, we examined whether Elk1 is a direct substrate for calcineurin. The substrate used in this experiment was a GST-Elk1 fusion protein that was phosphorylated in vitro by various MAPKs. As shown in Fig. 7A, calcineurin dephosphorylated phospho-Elk1 but not phospho-c-Jun or phospho-ATF2. The latter two substrates were prepared by phosphorylation of the relevant GST fusion proteins with activated JNK. Calcineurin also did not significantly dephosphorylate phospho-Ets2, even though Elk1 and Ets2 belong to the same transcription factor family and they both are substrates for ERK (50, 56). When 2.5 μM calcineurin was incubated with 0.5 μg of phospho-Elk1, 60% of the substrate was dephosphorylated within 20 min (Fig. 7B). Similar results were obtained when dephosphorylation of phospho-Elk1 was followed by immunoblotting with an antibody specific to phospho-Elk1 phosphorylated at Ser-383 (Fig. 7C). Immunoblotting with the general Elk1 antibody indicated that incubation with calcineurin reduced the phospho-Elk1 signal by dephosphorylation of phospho-Ser-383 rather than through nonspecific proteolysis.

**DISCUSSION**

Calcineurin is a ubiquitously expressed protein phosphatase that is activated upon binding of Ca2+-CaM (6, 67). So far the roles of calcineurin in signal transduction and gene regulation have been investigated only in lymphoid cells, brain, heart, and
other limited cell types (28, 68, 69). The functions of calcineurin in other cell types are poorly understood. The work described above links calcineurin to a ubiquitous sequence-specific transcription factor, Elk1, which serves as a target for all three MAPK pathways and plays an important role in induction of immediate-early gene transcription.

We found that activation of calcineurin can inhibit the stimulation of Elk1 transcriptional activity by all three MAPK cascades. MAPK activation is known to be inhibited by specific MAPK phosphatases, such as MKP-1, which directly dephosphorylate the activating phosphoacceptor sites within the T-loop regions of MAPKs (70). In the case reported here, however, the target for the phosphatase is a specific MAPK substrate rather than the MAPK themselves. However, it is likely that MAPK phosphatases and calcineurin, although structurally not related, may act synergistically in vivo. Indeed, in fission yeast, disruption of the calcineurin gene (ppb1) results in a dramatic chloride-sensitive growth arrest (71). A novel gene mmp1, which encodes a MAPK phosphatase, suppresses this defect (71). Although the biochemical mechanism accounting for this functional interaction is not known, in light of our results it is possible that calcineurin (Ppb1) dephosphorylates a MAPK substrate involved in regulation of chloride ion transport. In the absence of calcineurin, the substrate accumulates in its phosphorylated state, which prevents chloride export. One way to reverse this effect is to inhibit the activity of the MAPK responsible for the phosphorylation event.

Elk1 is the most abundant of the TCFs, first characterized as transcription factors involved in induction of c-fos and other members of its family by diverse extracellular stimuli (64). The Fos proteins are basic leucine zipper (bZIP) transcription factors, which dimerize with the Jun proteins to form stable AP-1 heterodimers (72). The proteins are involved in the regulation of many target genes. Transcription of the c-fos gene is activated by a variety of stimuli such as serum, EGF and other growth factors, UV irradiation, Ca^{2+}, cAMP, phorbol esters, and various cytokines (49, 64).

The inactivation of Elk1 by calcineurin-mediated dephosphorylation adds another layer to the complexity of c-fos promoter regulation and Ca^{2+} signaling (summarized in Fig. 8). The c-fos promoter contains two major cis elements, the CRE and the SRE (64). The CRE (cAMP response element) is recognized by the CREB/ATF group of bZIP proteins. The activity of CREB is stimulated by phosphorylation at Ser-133, which can be catalyzed by a number of protein kinases including the cAMP-dependent protein kinase A (73), the Ca^{2+}/CalM-dependent CaM kinases (74), phorbol ester-responsive protein kinase C (75), and growth factor-responsive ribosomal S6 kinases (76). The
levels were determined by immunoblotting as described in Fig. 4. EGF treatment. The cells were lysed, and phospho-Elk1 and total Elk1 separated by SDS-PAGE and immunoblotted with phospho-Elk1 and anti-Elk1 antibodies. Results were quantified by using a PhosphorImager. B, COS7 cells were transfected with pCMV-Elk1 (150 ng/plate) and increasing amounts of pSRa-CnA(ΔCaM-AI) and pSRa-CnB (1:1 ratio). After 15 min of EGF treatment, cells were harvested and lysed. Protein samples (50 μg) were separated by SDS-PAGE and immunoblotted with anti-Elk1 and total Elk1-specific antibodies.

FIG. 4. Calcineurin expression or activation reduces Elk1 phosphorylation. A, COS7 cells were transfected with pCMV-Elk1 (150 ng/plate) with or without pSRa-CnA(ΔCaM-AI) and pSRa-CnB (475 ng DNA each). The total amount of DNA was kept constant at 1 μg. After 24 h, the cells were treated with 0.2 μM ionomycin and 1 μM cyclosporin A (CsA) for 10 min prior to EGF treatment for 15 min. Cells were harvested, lysed, and analyzed by immunoblotting as described in Fig. 2, except that anti-phospho-Elk1 and anti-Elk1 antibodies were used. Results were quantified by using a PhosphorImager. B, COS7 cells were transfected with pCMV-Elk1 (150 ng/plate) and increasing amounts of pSRa-CnA(ΔCaM-AI) and pSRa-CnB (1:1 ratio). After 15 min of EGF treatment, cells were harvested and lysed. Protein samples (50 μg) were separated by SDS-PAGE and immunoblotted with phospho-Elk1 and anti-Elk1 antibodies.

activity of ATF2 is stimulated by JNK and p38-mediated phosphorylation at threonine 71 (52), whereas the mechanisms that control the activity of other ATFs are not yet known. This probably is the reason why calcineurin cannot completely block the induction of c-fos transcription by anisomycin (Fig. 6B), since ATF2 can also be phosphorylated by activated JNK and p38 and is not dephosphorylated by calcineurin (Fig. 7A). The SRE is a composite binding site for the SRFs and the TCFs (64). Several TCFs were identified, including Elk1 (49), Sap1/2 (77), and Erp/Net (78). The TCFs belong to the Ets family, characterized by a conserved Ets DNA binding domain (66). The TCFs interact with the SRE only in conjunction with binding of SRF (79). Although SRF may respond to signals that are transmitted by members of the Rho family of small G-proteins (80), or CaM kinases (11), most of the signals sensed by the SRE are received by the TCFs (49, 64). The ability of Elk1 and Sap1/2 to activate transcription is stimulated by phosphorylation of conserved sites at their C-terminal activation domain (58). Phosphorylation of these sites is mediated by various MAPKs, including ERK, p38, and JNK (49).

The most ubiquitous and prevalent TCF is Elk1. It was shown that c-fos promoter activity correlates temporally with the phosphorylation state of Elk1 (81). Thus not only Elk1 phosphorylation is an important mechanism for regulation of c-fos transcription but also its dephosphorylation is likely to be instrumental in shutting off c-fos transcription. Originally it was suggested that Elk1 might be inactivated by a constitutive phosphatase (81). Although such phosphatase may also be involved in Elk1 inactivation, the present study identified calcineurin as a physiologically relevant Elk1 phosphatase. While this manuscript was being prepared for publication, a similar conclusion was derived by Sugimoto et al. (82), although these authors documented the involvement of calcineurin only in the context of ERK-mediated Elk1 activation. Here we report that this dephosphorylation is specific to Elk1 (calcineurin does not affect c-Jun, Ets2 or ATF2) and that stimulation of Elk1 transcriptional activity by all three MAPK pathways is negatively regulated by calcineurin. Most importantly, activation of calcineurin can reduce the induction of c-fos transcription by...
inducers, such as anisomycin, which operate via MAPK pathways.

How and why Ca\textsuperscript{2+} signaling can both turn on and off c-fos transcription? One can posit several mechanisms that explain such a paradoxical effect. According to the first mechanism, the Ca\textsuperscript{2+}-responsive kinases and phosphatases have different kinetics of activation. If kinases such as CaM-Ks are activated first, they can stimulate c-fos transcription through direct phosphorylation of CREB or CREM or indirectly through stimulation of ERK, which then phosphorylates Elk1. Once calcineurin activity picks up, Elk1 is dephosphorylated and part of the Ca\textsuperscript{2+} generated stimulatory effect is attenuated. This may contribute to the transient nature of c-fos induction. A second possibility is that Ca\textsuperscript{2+}-dependent protein kinases and phosphatases have different sensitivity to Ca\textsuperscript{2+} and different affinity for CaM, and their activity is independently controlled. For example, the $K_c$ for CaM binding is 0.1 nM and 20–200 nM for calcineurin and CaM kinase II, respectively (65). Thus, a small increase in intracellular Ca\textsuperscript{2+} will have a negative effect on c-fos transcription through activation of calcineurin, whereas a large increase in Ca\textsuperscript{2+} will stimulate c-fos transcription through activation of CaM-K II and ERK. Indeed, as shown in Fig. 6, treatment of Jurkat cells with 1 nM ionomycin is not sufficient for induction of c-fos transcription presumably because it does not increase intracellular Ca\textsuperscript{2+} to a level competent for CaM kinase activation. However, that level of Ca\textsuperscript{2+} is sufficient for inhibition of c-fos transcription by inducers that work via MAPK signaling pathways, probably because it is sufficient for activation of calcineurin, as evidenced by the dephosphorylation of Elk1. Such a mechanism certainly increases the regulatory potential of Ca\textsuperscript{2+} transients.

In order to exert its regulatory effect on Elk1 bound at the c-fos promoter, calcineurin must act in the nucleus. Calcineurin is widely considered to be a cytoplasmic protein (6). It has no nuclear translocation signal (22). However, it has been detected in the nucleus of activated T cells (22). It was suggested that after calcineurin dephosphorylates inducers, such as anisomycin, which operate via MAPK pathways.

How and why Ca\textsuperscript{2+} signaling can both turn on and off c-fos transcription? One can posit several mechanisms that explain such a paradoxical effect. According to the first mechanism, the Ca\textsuperscript{2+}-responsive kinases and phosphatases have different kinetics of activation. If kinases such as CaM-Ks are activated first, they can stimulate c-fos transcription through direct phosphorylation of CREB or CREM or indirectly through stimulation of ERK, which then phosphorylates Elk1. Once calcineurin activity picks up, Elk1 is dephosphorylated and part of the Ca\textsuperscript{2+} generated stimulatory effect is attenuated. This may contribute to the transient nature of c-fos induction. A second possibility is that Ca\textsuperscript{2+}-dependent protein kinases and phosphatases have different sensitivity to Ca\textsuperscript{2+} and different affinity for CaM, and their activity is independently controlled. For example, the $K_c$ for CaM binding is 0.1 nM and 20–200 nM for calcineurin and CaM kinase II, respectively (65). Thus, a small increase in intracellular Ca\textsuperscript{2+} will have a negative effect on c-fos transcription through activation of calcineurin, whereas a large increase in Ca\textsuperscript{2+} will stimulate c-fos transcription through activation of CaM-K II and ERK. Indeed, as shown in Fig. 6, treatment of Jurkat cells with 1 nM ionomycin is not sufficient for induction of c-fos transcription presumably because it does not increase intracellular Ca\textsuperscript{2+} to a level competent for CaM kinase activation. However, that level of Ca\textsuperscript{2+} is sufficient for inhibition of c-fos transcription by inducers that work via MAPK signaling pathways, probably because it is sufficient for activation of calcineurin, as evidenced by the dephosphorylation of Elk1. Such a mechanism certainly increases the regulatory potential of Ca\textsuperscript{2+} transients.

In order to exert its regulatory effect on Elk1 bound at the c-fos or other similar promoters, calcineurin must act in the nucleus. Calcineurin is widely considered to be a cytoplasmic protein (6). It has no nuclear translocation signal (22). However, it has been detected in the nucleus of activated T cells (22). It was suggested that after calcineurin dephosphorylates inducers, such as anisomycin, which operate via MAPK pathways.

FIG. 7. Calcineurin directly dephosphorylates phospho-Elk1. A, GST-Elk1, GST-Ets2, GST-c-Jun, and GST-ATF2 fusion proteins were expressed in E. coli and purified on GSH-Sepharose. Each of the GST fusion proteins (0.5 μg each) was in vitro phosphorylated by immunoprecipitated HA-ERK2 (for GST-Elk1 and GST-Ets2) and HA-JNK1 (for GST-c-Jun and GST-ATF2) and repurified on GSH-Sepharose. The \textsuperscript{32}P-labeled substrates were incubated with the indicated concentrations of calcineurin and calmodulin purified from bovine brain in phosphatase buffer containing Ca\textsuperscript{2+} for 20 min at 30 °C. The proteins were separated by SDS-PAGE and visualized by autoradiography. B, \textsuperscript{32}P-labeled GST-Elk1 was incubated with the indicated concentrations of calcineurin, and the level of GST-Elk1 phosphorylation was determined as described above. The average (± S.D.) values of relative GST-Elk1 phosphorylation determined in three separate experiments were plotted against the concentration of calcineurin (initial level of phosphorylation was set at 100%). C, phospho-GST-Elk1 was incubated with 2.5 μM calcineurin as described above. After separation by SDS-PAGE, phospho-Elk1, and total Elk1 levels were determined by immunoblotting as described in Fig. 4.

FIG. 8. Summary of major signaling pathways that regulate the c-fos promoter. The c-fos promoter contains two major cis elements, the CRE and the SRE. The CRE is recognized by the CREB/ATF. Ser-133 of CREB can be phosphorylated by protein kinase A (PKA), CaM-Ks, and protein kinase C (PKC). Thr-71 of ATF2 can be phosphorylated by JNKs and p38s. The SRE is recognized by SRF and TCFs. Elk1, the most well characterized TCF, is a substrate for all three MAPKs, and phospho-Elk1 can be dephosphorylated by calcineurin. The phosphorylation of these transcription factors stimulates c-fos promoter activity, whereas dephosphorylation of Elk1 by calcineurin may play a role in turning off c-fos transcription. CN, calcineurin.
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NF-AT1, it accompanies it to the nucleus by virtue of its tight association with this transcription factor (22). Thus by affecting the subcellular location of calcineurin, the NF-AT proteins may modulate the ability of calcineurin to inactivate transcription factors such as Elk1.

**Acknowledgments**—We thank R. Janknecht, T. Hunter, J. Han, R. Treisman, N. G. Ahn, M. Ostrowski, Z. Wu, Y. Xia, and W. Li for providing the necessary constructs. We thank C. Hauser for critically reading the manuscript and for helpful discussion.

**REFERENCES**

1. Badin, H., Hardingham, G. E., Johnson, C. M., and Chawla, S. (1997) *Biochem. Biophys. Res. Commun.* 236, 541–543
2. Malviya, A. N., and Rogue, P. J. (1998) *J. Biol. Chem.* 273, 10759–10766
3. Treisman, N. G. Ahn, M. Ostrowski, Z. Wu, Y. Xia, and W. Li for providing the necessary constructs. We thank C. Hauser for critically reading the manuscript and for helpful discussion.

**REFERENCES**

1. Badin, H., Hardingham, G. E., Johnson, C. M., and Chawla, S. (1997) *Biochem. Biophys. Res. Commun.* 236, 541–543
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2. Malviya, A. N., and Rogue, P. J. (1998) *J. Biol. Chem.* 273, 10759–10766
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2. Malviya, A. N., and Rogue, P. J. (1998) *J. Biol. Chem.* 273, 10759–10766
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2. Malviya, A. N., and Rogue, P. J. (1998) *J. Biol. Chem.* 273, 10759–10766
3. Treisman, N. G. Ahn, M.