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c-Jun functions as a calcium-regulated transcriptional activator in the absence of JNK/SAPK1 activation

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Calcium is the principal second messenger in the control of gene expression by electrical activity in neurons. Recruitment of the coactivator CREB-binding protein, CBP, by the prototypical calcium-responsive transcription factor, CREB and stimulation of CBP activity by nuclear calcium signals is one mechanism through which calcium influx into excitable cells activates gene expression. Here we show that another CBP-interacting transcription factor, c-Jun, can mediate transcriptional activation upon activation of L-type voltage-gated calcium channels. Calcium-activated transcription mediated by c-Jun functions in the absence of stimulation of the c-Jun N-terminal protein kinase (JNK/SAPK1) signalling pathway and does not require c-Jun amino acid residues Ser63 and Ser73, the two major phosphorylation sites that regulate c-Jun activity in response to stress signals. Similar to CREB-mediated transcription, activation of c-Jun-mediated transcription by calcium signals requires calcium/calmodulin-dependent protein kinases and is dependent on CBP function. These results identify c-Jun as a calcium-regulated transcriptional activator and suggest that control of coactivator function (i.e. recruitment of CBP and stimulation of CBP activity) is a general mechanism for gene regulation by calcium signals.
Keywords: c-Jun/calcium/CBP/JNK/SAPK

Introduction

Calcium ions are versatile second messengers which control many cellular processes (Clapham, 1995). The intracellular concentration of free calcium is normally very low (<100 nM) but can increase dramatically in response to extracellular signals by either calcium entry into the cell or by calcium release from intracellular stores (Berridge, 1998). These, often short-lasting, calcium transients can activate signalling molecules such as protein kinases or protein phosphatases, mainly via the intracellular calcium receptor, calmodulin (Means, 1988; Means *et al*., 1991), that govern the physiological response of the cells. In the mammalian central nervous system, calcium is the primary mediator of electrical activity-dependent changes in gene expression (Lerea *et al*., 1992; Bading *et al*., 1993; reviewed in Ghosh and Greenberg, 1995; Bito *et al*., 1997; Hardingham and Bading, 1998), a key mechanism through which neurons retain information that they receive from the environment. Signalling pathways activated by calcium entry into excitable cells through either L-type voltage-gated calcium channels or *N*-methyl-D-aspartate (NMDA) receptors, can induce gene transcription by activating transcription factors which interact with the serum-response element (SRE) or cAMP-response element (CRE) (Sheng *et al*., 1988; Bading *et al*., 1993). CRE-dependent transcription, which has been particularly well studied, requires calcium transients in the cell nucleus and is mediated by the CRE-binding protein, CREB (Hardingham *et al*., 1997). Phosphorylation of CREB on Ser133, which can be catalyzed by several protein kinases (reviewed in Chawla and Bading, 1998), is important for calcium-activated transcription. However, recent experiments have demonstrated the existence of a second critical regulatory step for CRE/CREB-dependent gene expression: activation of the transcriptional coactivator, CREB-binding protein (CBP), that is recruited to the CRE via phospho(Ser133)-CREB, by nuclear calcium and calcium/calmodulin (CaM)-dependent protein kinase IV (Chawla *et al*., 1998). Moreover, CBP alone, when artificially tethered to a promoter, is sufficient to mediate transcriptional activation in response to nuclear calcium signals and CaM kinase IV (Chawla *et al*., 1998). These findings suggest that DNA-binding proteins other than CREB, that are capable of recruiting CBP to a promoter, may function as calcium-responsive transcriptional activators. In this study, the CBP-interacting transcription factor, c-Jun (Bannister *et al*., 1995), was chosen to test this hypothesis. c-Jun, which forms part of the activating protein-1 (AP-1) complex, is known to be a signal-regulated transcription factor. Its activity is induced by certain environmental stress signals such as UV irradiation, inflammatory cytokines such as tumour necrosis factor-α and treatment of cells with inhibitors of mRNA translation (Kyriakis *et al*., 1994; reviewed in Karin *et al*., 1997). Activation of c-Jun by stress is controlled by the classical stress-induced signalling cascade leading to c-Jun phosphorylation by the c-Jun N-terminal protein kinase (JNK/SAPK1) on two key regulatory sites, Ser63 and Ser73 (Radler-Pohl *et al*., 1993; Dérijard *et al*., 1994; Kyriakis *et al*., 1994). Here we report that increasing the intracellular calcium concentration by opening L-type voltage-gated calcium channels represents a second pathway for c-Jun activation. Stimulation of c-Jun-mediated transcription by calcium signals occurs in the absence of JNK/SAPK1 activation, is independent of Ser63 and Ser73, but requires CaM kinase activation and CBP function.

Results

c-Jun contains a calcium-regulated transactivation domain

The regulation of c-Jun function by calcium signalling pathways was tested with an altered specificity variant of

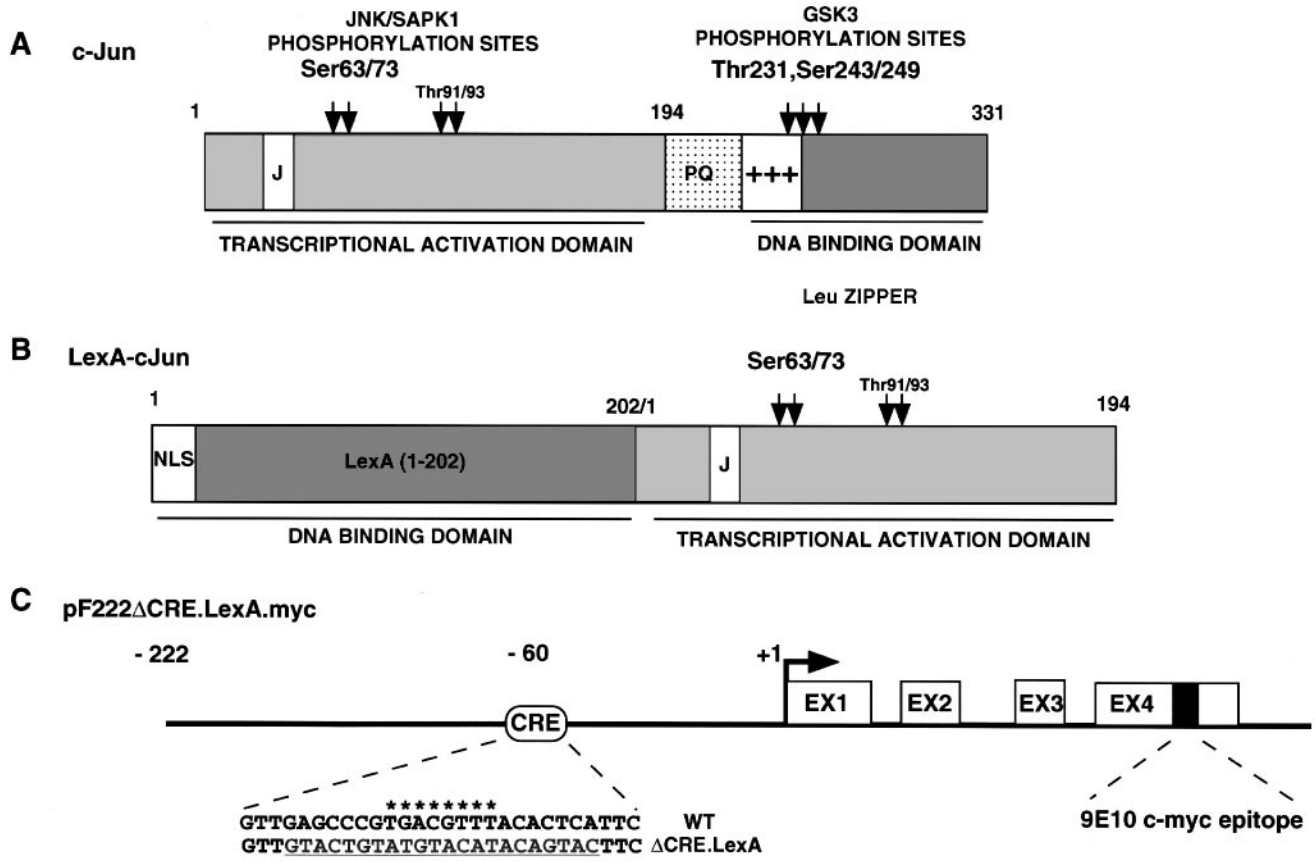


Fig. 1. Schematic representation of c-Jun, the LexA–c-Jun fusion protein, and the pF222ΔCRE.Lex.myc reporter construct. (**A**) The c-Jun N-terminal transactivation domain, docking site for the JNK/SAPK1 kinase (J) and phosphorylation sites for JNK/SAPK1 at amino acid positions 63, 73, 91 and 93 are indicated. The c-Jun C-terminal half is comprised of a proline/glutamine-rich domain (PQ), and a basic/leucine zipper which contains phosphorylation sites for glycogen synthase kinase 3 (GSK3). (**B**) The Lex–c-Jun fusion consists of an SV40 nuclear localization signal (NLS) followed by the bacterial repressor LexA (residues 1–202) and the 194 N-terminal amino acids of c-Jun containing the JNK/SAPK1 phosphorylation sites. (**C**) The reporter pF222ΔCRE.Lex.myc is based on the human *c-fos* gene containing 222 bp of the proximal promoter. The c-*fos* CRE at position –60 relative to the start site of transcription has been replaced with a binding site for the LexA dimer and a 9E10 myc epitope was inserted in the fourth exon of c-*fos*.

c-Jun (Figure 1). We used a fusion protein consisting of the DNA-binding domain of the bacterial repressor LexA (residues 1–202) and the N-terminal 194 amino acids of c-Jun which were previously shown to contain a signal-regulated transcription activation domain (Price *et al*., 1996). A plasmid encoding this LexA–c-Jun protein was transfected into the mouse pituitary cell line, AtT20, together with a reporter pF222ΔCRE.Lex.myc. This reporter gene contains the human c-*fos* gene including 222 base pairs of the proximal promoter. The *c-fos* CRE at position –60 relative to the start site of transcription was mutated to generate a binding site for the LexA dimer. The reporter also contains a 9E10 myc epitope in the fourth exon of c-*fos* which allows measurements of transcriptional responses at the single cell level by immunocytochemistry in addition to gene expression analysis in populations of cells by RNase protection assay. Two stimulation paradigms were compared: (i) activation of calcium flux through L-type voltage-gated calcium channels by KCl (50 mM)-induced membrane depolarization in the presence of the L-type calcium channel agonist, FPL 64176 (KCl–FPL stimulation; Hardingham *et al*., 1997); and (ii) treatment of the cells with the protein synthesis inhibitor anisomycin (500 ng/ml), a well-characterized activator of the JNK/SAPK1 signalling pathway and c-Jun-mediated transcription in many cells types (Kyriakis *et al*., 1994; Gille *et al*., 1995; Price *et al*., 1996). In AtT20 cells transfected with the control plasmid expressing the LexA DNA-binding domain only, transcription from pF222ΔCRE.Lex.myc is low in both unstimulated cells and in cells following KCl–FPL or anisomycin stimulation (Figure 2, lanes 1–3). However, in cells expressing LexA–c-Jun, transcription from pF222ΔCRE.Lex.myc is induced upon either KCl–FPL stimulation (K/F; Figure 2, lanes 4–7) or anisomycin treatment (Figure 2, lanes 8–11) demonstrating that the N-terminus of c-Jun contains a calcium and stress-regulated transcriptional activation domain. The calcium-induced LexA–c-Jun-mediated transcription is comparable in magnitude to that obtained using the prototypical calcium-regulated transcription factor CREB in the context of a similar reporter system (data not shown). However, due to the high basal activity of LexA–c-Jun, which correlates with a high basal level of JNK activity in AtT20 cells (see below), the fold induction of transcription following KCl–FPL treatment is ~2- to 3-fold. Similar results were obtained using the heterologous reporter gene construct Lex_2 thymidine kinase–chloramphenicol acetyltransferase (TK–CAT) (Price *et al*., 1996) (data not shown). While KCl–FPL and anisomycin treatment stimulated transcription from pF222ΔCRE.Lex.

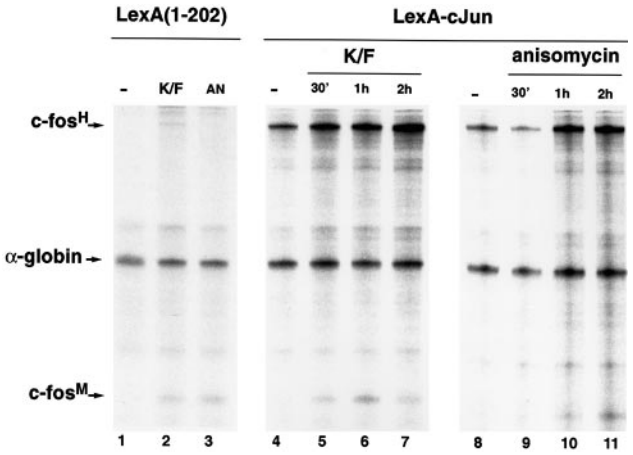


Fig. 2. c-Jun contains a calcium-regulated transcriptional activation domain. RNase protection analysis of LexA–c-Jun-mediated expression from the transfected human c-*fos*-based reporter gene, pF222ΔCRE.Lex.myc (c-fos^{H}), expression of the endogenous mouse c-*fos* gene (c-fos^{M}), and expression of the transfected human α-globin gene in unstimulated cells (–) and in cells stimulated for the indicated times with KCl–FPL (K/F) or anisomycin (500 ng/ml). AtT20 cells were transfected with pF222ΔCRE.Lex.myc, the LexA–c-Jun expression plasmid, and the human α-globin gene (plasmid pSVα1) to normalize for transfection efficiency.

myc to a similar extent, the kinetics of the responses were different (calcium-induced transcription precedes anisomycin-induced transcription; Figure 2, compare lane 5 with lane 9) suggesting that calcium and stress signals control c-Jun function via distinct mechanisms.

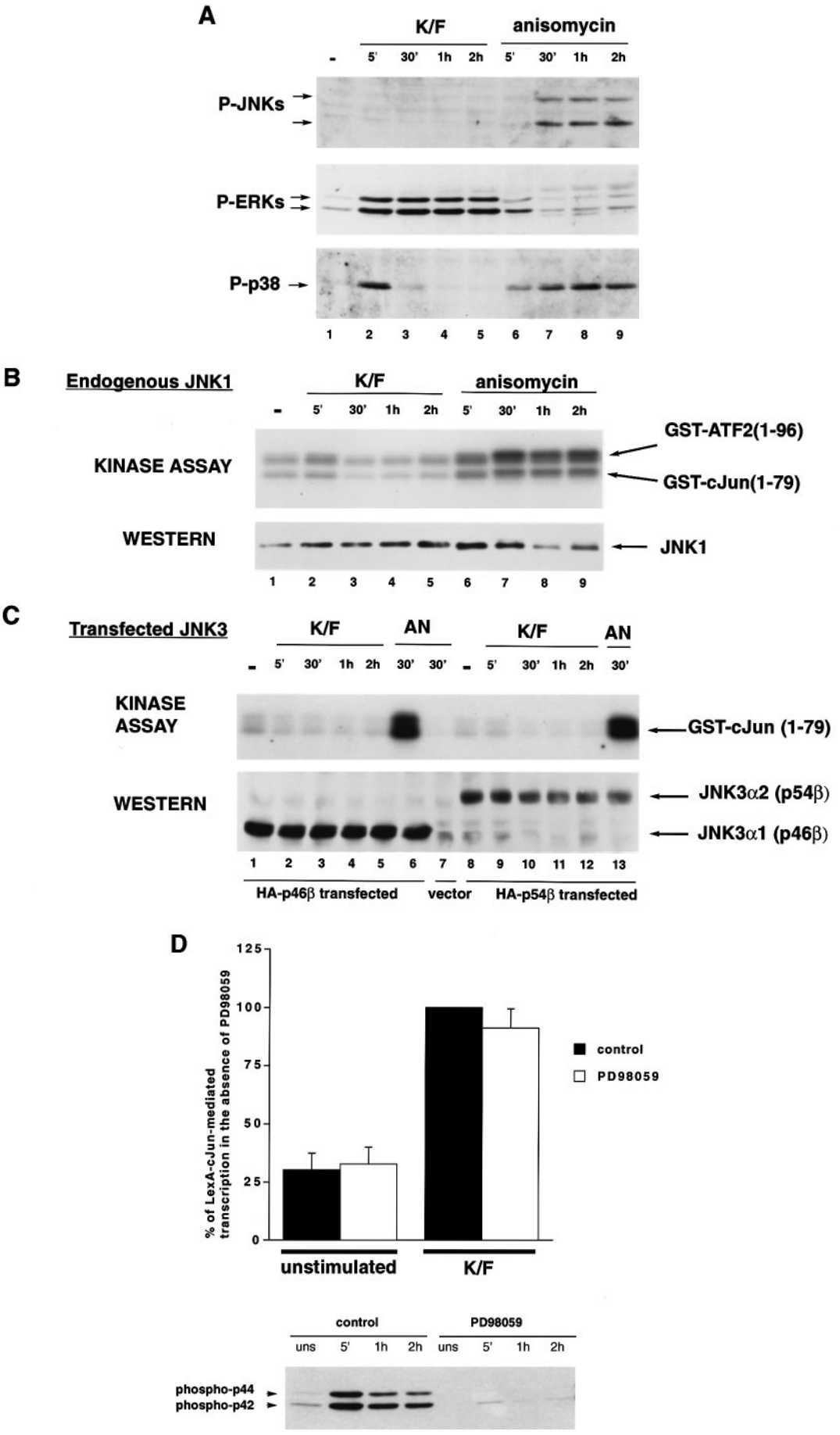
Calcium flux through L-type calcium channels fails to activate JNK/SAPK1 in AtT20 cells and does not induce c-Jun phosphorylation on Ser63/Ser73

Phosphorylation of c-Jun amino acid residues Ser63 and Ser73 by JNK/SAPK1 is the key regulatory event that controls c-Jun activation by stress signals. We therefore investigated JNK/SAPK1 activity and c-Jun phosphorylation on Ser63/Ser73 in cells following activation of L-type calcium channels. There are three JNK/SAPK1 genes that give rise to at least ten isoforms, they are all similarly regulated by stress signals (Gupta *et al*., 1996). All isoforms share a stretch of amino acids which contain phosphorylation sites for the JNK/SAPK1 upstream activator kinase, SAPK/ERK kinase (SEK) (Sánchez *et al*., 1994). We first assessed JNK/SAPK1 activation by Western blot analysis using antibodies specific for the SEK phosphorylation sites on JNK/SAPK1 which are indicative of stimulation of their enzymatic activity (Figure 3A). While in extracts from cells treated with anisomycin strong signals corresponding to the phosphorylated 46 and 54 kDa isoforms of JNK/SAPK1 were obtained (Figure 3A, lanes 6–9), phospho-JNK/SAPK1 immunoreactivity was undetectable upon KCl–FPL stimulation (Figure 3A, lanes 1–5), indicating that calcium transients do not induce phosphorylation or activation of JNK/SAPK1. Two other members of the mitogen-activated protein (MAP) kinase family, the extracellular signal-regulated kinases (ERKs) and the p38 MAP kinase, were assayed in parallel using antibodies specific for their phosphorylated (i.e. activated) forms. We found that calcium signalling pathways are potent activators of ERKs, which confirms previous reports (Bading and Greenberg, 1991; Rosen *et al*., 1994; Johnson *et al*., 1997), while treatment of the cells with anisomycin only moderately increases ERK activity (Figure 3A). Both KCl–FPL and anisomycin induced p38 MAP kinase activity, although with different kinetics: activation of p38 MAP kinase by KCl–FPL was transient, peaking at ~5 min after stimulation, while anisomycin induced a sustained increase in p38 MAP kinase activity. The absence of JNK/SAPK1 activation by KCl–FPL suggests a profound difference between the signalling cascades involved in activation of c-Jun-mediated transcription by calcium entry into cells and stress. The selective activation of p38 MAP kinase but not JNK/SAPK1 in AtT20 cells resembles the calcium regulation of these kinases in cerebellar granule cells: calcium entry via NMDA receptors efficiently activates p38 MAP kinase but only poorly stimulates JNK/SAPK1 (Kawasaki *et al*., 1997).

The failure of calcium transients in AtT20 cells to increase JNK/SAPK1 activity was confirmed in immune-complex kinase assays. Individual JNK/SAPK1 isoforms (either endogenously expressed or produced by an expression vector transiently transfected into AtT20 cells) were immunoprecipitated and assayed, in an immune complex, for kinase activity using bacterially expressed glutathione *S*-transferase (GST)–Jun(1–79) or GST–activating transcription factor 2 (ATF2)(1–96) as substrates. Similar to the results obtained with the phospho-JNK immunoblots, anisomycin stimulated the activity of endogenous JNK1 (Figure 3B, lanes 6–9) and JNK2 (data not shown). In contrast, KCl–FPL treatment decreased their activity (Figure 3B, lanes 1–5). Because an antibody suitable for JNK3 immunoprecipitation was not available, the regulation of this enzyme was analyzed after transfection of an expression vector encoding haemagglutinin (HA)-epitope tagged versions of the 46 and 54 kDa isoforms of JNK3 (Figure 3C). Again, immune-complex kinase assays using antibodies to the HA tag showed that these JNK/SAPK1 isoforms are not activated by calcium signals (Figure 3C, lanes 1–5 and 8–12) while anisomycin increased their activity (Figure 3C, lanes 6 and 13). To determine whether we could detect any Jun-N-terminus protein kinase activity that increases after L-type calcium channel activation in AtT20 cells, we prepared soluble cell extracts from cells before and after stimulation and assayed for *in vitro* kinase activity using GST–Jun(1–169) as a substrate. We found that under these condition extracts from anisomycin-treated cells but not from KCl–FPL-stimulated cells contain higher total GST–Jun(1–169) kinase activity than extracts prepared from untreated cells (data not shown).

Because of the observed strong stimulation of ERK activity caused by KCl–FPL treatment, we examined whether ERK activation is required for LexA–c-Jun-mediated, calcium-dependent transcription. Treatment of AtT20 cells with the MAP kinase/ERK kinase 1 (MEK1) inhibitor, PD98059 (50 μM), that effectively blocked the activation of ERKs by calcium flux through L-type calcium channels (Figure 3D, bottom panel), did not reduce calcium-activated, LexA–c-Jun-dependent transcription (Figure 3D, upper panel). These results indicate that c-Jun regulation by calcium signals functions independently of stimulation of the ERK-signalling pathway.

The lack of induction of JNK/SAPK1 activity upon calcium entry into AtT20 cells through L-type calcium


A
K/F
anisomycin
- 5' 30' 1h 2h 5' 30' 1h 2h
P-JNKs
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P-p38
1 2 3 4 5 6 7 8 9
B
Endogenous JNK1
K/F
anisomycin
- 5' 30' 1h 2h 5' 30' 1h 2h
KINASE ASSAY
GST-ATF2(1-96)
GST-cJun(1-79)
WESTERN
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KINASE ASSAY
GST-cJun (1-79)
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% of LexA-cJun-mediated transcription in the absence of PD98059
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75
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phospho-p44
phospho-p42

channels indicates that this stimulation does not lead to c-Jun phosphorylation on Ser63 and Ser73. Indeed, Western blot analysis using antibodies specific for either c-Jun phosphorylated on Ser63 (Figure 4, lanes 11–20) or for c-Jun phosphorylated on Ser73 (data not shown) showed that anisomycin but not KCl–FPL treatment leads to LexA–c-Jun phosphorylation of these residues (Figure 4, compare lane 11 with lanes 12–15 and lane 11 with lanes 16–19). Analysis of the same samples with an antibody to the LexA portion of the fusion protein (Figure 4, lanes 1–10) revealed that anisomycin treatment caused a characteristic shift in the electrophoretic mobility of LexA–c-Jun which is caused by phosphorylation of the N-terminus of c-Jun (Pulverer *et al*., 1991; Price *et al*., 1996) (Figure 4, lanes 6–9). In contrast, KCl–FPL stimulation did not yield any shift in LexA–c-Jun mobility (Figure 4, lanes 1–5), suggesting that the c-Jun N-terminus is not phosphorylated upon activation of calcium signalling pathways. The antibody specific to phospho(Ser63)-c-Jun failed to detect the phosphorylated form of the endogenous c-Jun protein in AtT20 cells. However, endogenous c-Jun protein is detectable using a c-Jun-specific antibody and undergoes the characteristic phosphorylation-induced shift in electrophoretic mobility upon anisomycin or UV treatment but not following calcium entry through L-type calcium channels (data not shown). Together, these data suggest that in AtT20 cells, calcium signals fail to activate JNK/SAPK1 and do not cause phosphorylation of c-Jun on Ser63 and Ser73.

Activation of c-Jun-mediated transcription by calcium signalling pathways is insensitive to mutation of Ser63/Ser73

To test directly the importance of Ser63 and Ser73 in calcium-stimulated c-Jun function we tested the ability of LexA–c-Jun(S63/73L) which contains a serine to leucine mutation at amino acid positions Ser63 and Ser73 in the c-Jun transactivation domain (Radler-Pohl *et al*., 1993; Dérijard *et al*., 1994; Kyriakis *et al*., 1994), to activate the reporter gene pF222ΔCRE.Lex.myc. Upon DNA transfection, this mutant protein was expressed at levels similar to that observed with LexA–c-Jun (data not shown). Consistent with previous reports, we found that mutation of c-Jun Ser63 and Ser73 reduced anisomycin-induced transcription (Figure 5). These mutations also lowered the levels of transcription in unstimulated cells, suggesting that the high basal activity of LexA–c-Jun is due to the high basal activity of JNK/SAPK1. In contrast to the stress response, calcium-induced transcription mediated by LexA–c-Jun(S63/73L) is similar to that obtained with wild-type LexA–c-Jun. These results demonstrate that calcium entry through L-type calcium channels controls c-Jun function by a signalling pathway which functions independently of JNK/SAPK1 activation and does not require c-Jun residues Ser63 and Ser73.

Calcium regulation of c-Jun activity requires CaM kinase activity and CBP function

CaM kinases, that are rapidly activated upon calcium entry through ligand- and/or voltage-gated ion channels (Nose *et al*., 1985; Fukunaga *et al*., 1992; Bading *et al*., 1993), are known regulators of gene expression (Morgan and Curran, 1986; Bading *et al*., 1993; Lerea and McNamara, 1993; Matthews *et al*., 1994; Sun *et al*., 1994). We therefore investigated the possibility that CaM kinases also control c-Jun regulation by calcium signalling pathways and assayed transcriptional activation by the LexA–c-Jun protein in the presence of the CaM kinase inhibitor, KN-62. This inhibitor was previously shown to specifically inhibit calcium-regulated transcription mediated by CREB and CBP (Hardingham *et al*., 1997; Chawla *et al*., 1998). Similar to the CREB/CBP-mediated responses, calcium-activated but not anisomycin-induced transcription mediated by LexA–c-Jun was reduced by KN-62 (Figure 6). KN-62 treatment also reduced calcium-induced activity of LexA–c-Jun(S63/73L) indicating that both wild-type and mutant c-Jun are regulated by a CaM kinase-dependent signalling pathway.

Because c-Jun can interact with CBP in a manner which does not depend on c-Jun phosphorylation on Ser63 and Ser73 (Bannister *et al*., 1995), and CBP is known to mediate transcriptional activation upon nuclear calcium transients and stimulation of CaM kinase IV (Chawla *et al*., 1998), we hypothesized that the CaM kinase signalling pathway that controls c-Jun function may target CBP. To test a possible involvement of CBP in calcium regulation of c-Jun function, we microinjected into the cells an expression vector for the adenovirus protein, E1A, which is known to disrupt CBP function (Arany *et al*., 1995; Bannister and Kouzarides, 1995). Expression of the reporter gene, pF222ΔCRE.Lex.myc was analyzed immunocytochemically using the 9E10 antibody. As shown in Figure 7, expression of E1A inhibited calcium-activated transcription by LexA–c-Jun. In contrast, expression of a mutant form of E1A, E1AΔCR1, that fails to interact with CBP (Bannister and Kouzarides, 1995), had little effect on LexA–c-Jun regulation by calcium signals (Figure 7; quantitation of the microinjection experiments is in the figure legend). These results suggest that CBP or a closely related molecule such as p300 (Arany *et al*., 1995; Lundblad *et al*., 1995) is critical for c-Jun to function as a calcium-regulated transcription factor.

Because calcium regulation of c-Jun appears to occur via the activation of its associated coactivator CBP, CBP-

Fig. 3. Regulation of JNK/SAPK1, ERK and p38 MAP kinase by calcium signals. (**A**) Western blot analysis of JNK/SAPK1, ERKs and p38 MAP kinase in unstimulated AtT20 cells and in cells treated for the indicated times with KCl–FPL or anisomycin using antibodies to the phosphorylated (i.e. activated) forms of these kinases. Equal amounts of protein from whole-cell extracts were separated by SDS–PAGE and blotted with anti-phospho-JNK/SAPK1, anti-phospho-ERK or anti-phospho-p38 MAP kinase antibodies. (**B** and **C**) Immune-complex kinase assays for various JNK/SAPK1 isoforms. Endogenous JNK1 and HA-tagged p46 and p54 isoforms of JNK3 transfected into AtT20 cells were immunoprecipitated from unstimulated AtT20 cells or cells after KCl–FPL or anisomycin treatment and assayed for kinase activity using GST–c-Jun(1–79) and GST–ATF2(1–96). (**D**) Calcium-stimulated, Lex–c-Jun-mediated transcription does not require activation of the ERK pathway. Upper panel: quantitative analysis of DNA transfection and RNase protection experiments (n = 3) using the pF222ΔCRE.Lex.myc/LexA–c-Jun reporter system as described in Figure 2. AtT20 cells were stimulated with KCl–FPL in either the absence or the presence of the MEK1 inhibitor PD98059 (50 μM). Bottom panel: immunoblot analysis of the activation of endogenous ERKs (p42 and p44) using phospho-ERK-specific antibodies. AtT20 cells were analyzed at the times indicated after KCl–FPL stimulation in the presence or absence of PD98059 (50 μM).

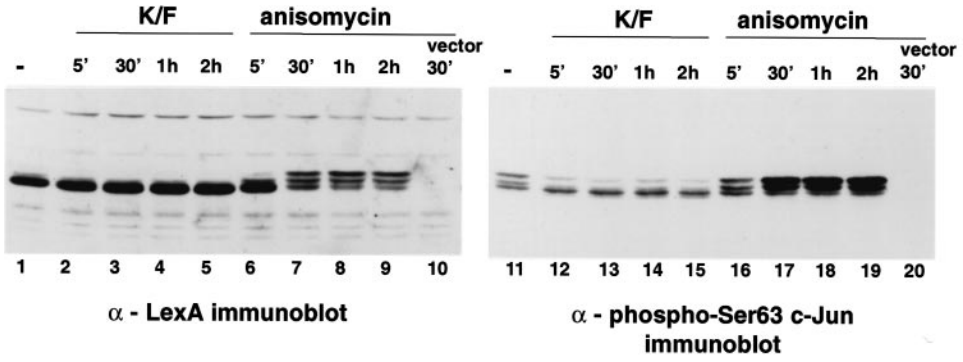

Fig. 4. Calcium influx does not induce phosphorylation of c-Jun on Ser63. AtT20 cells transfected with an expression vector encoding LexA–c-Jun(1–194) (lanes 1–9 and 11–19) or with vector control (lanes 10 and 20) were treated with KCl–FPL or anisomycin for the times indicated. Whole-cell extracts were prepared and proteins were analyzed by immunoblotting with antibodies against either LexA (left panel) or phospho-Ser63 (right panel).

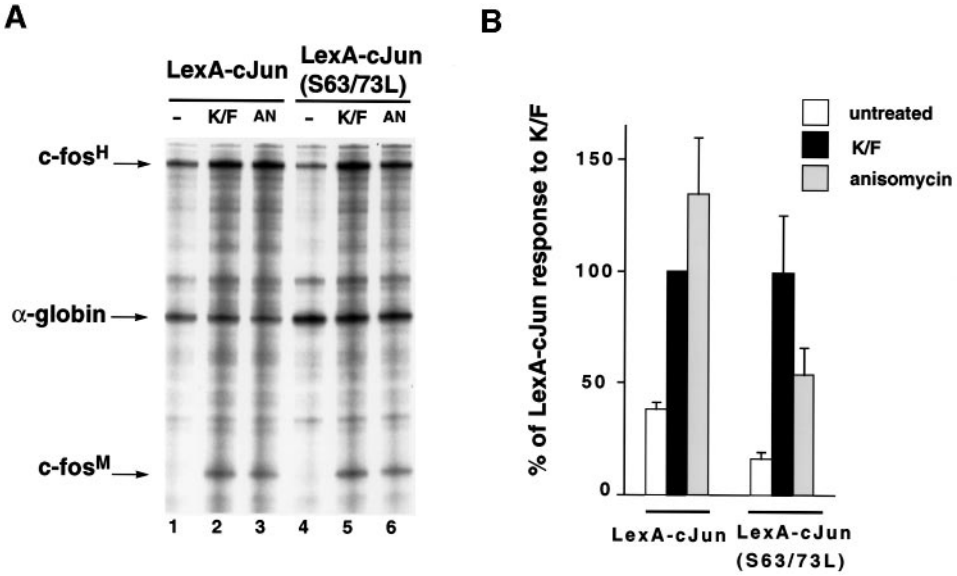

Fig. 5. c-Jun amino acid residues Ser63 and Ser73 are not required for calcium-induced c-Jun-mediated transcription. RNase protection analysis of LexA–c-Jun-mediated and LexA–c-Jun(S63/73L)-mediated expression from pF222ΔCRE.Lex.myc (c-fos^H), expression of the endogenous mouse c-*fos* gene (c-fos^M), and expression of the transfected human α-globin gene in unstimulated cells (–) and in cells stimulated 90 min with KCl–FPL (K/F) or anisomycin (AN). AtT20 cells were transfected with pF222ΔCRE.Lex.myc, the LexA–c-Jun or LexA–c-Jun(S63/73L) expression plasmid, and the human α-globin gene (plasmid pSVα1) to normalize for transfection efficiency. A representative experiment is shown in (**A**); quantitative analysis of four independent experiments is shown in (**B**).

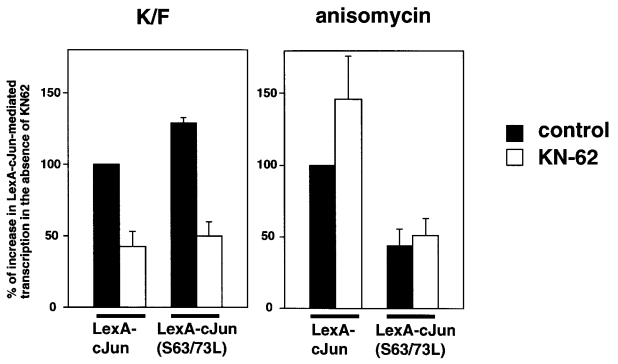

Fig. 6. CaM kinases are required for c-Jun activation by calcium signals. RNase protection analysis was used to measure LexA–c-Jun-mediated and LexA–c-Jun(S63/73L)-mediated expression from pF222ΔCRE.Lex.myc in unstimulated AtT20 cells and in cells 90 min after KCl–FPL or anisomycin stimulation in the absence (black bars) or presence (white bars) of KN-62 [n = 7, LexA–c-Jun; n = 3, LexA–c-Jun(S63/73L)]. Cells were treated with KN-62 (10 μM) 20 min before stimulation.

activating stimuli other than calcium entry into the cell should also induce c-Jun-dependent transcription. Since CREB, the prototypical CBP-interacting factor, and CBP itself when artificially tethered to the promoter, are potent mediators of cAMP-induced transcription (Chrivia *et al*., 1993; Chawla *et al*., 1998), we tested c-Jun regulation upon increasing intracellular concentrations of cAMP by treatment of the cells with forskolin, an activator of adenylate cyclase. We found that indeed under these conditions LexA–c-Jun-mediated transcription is activated (Figure 8). LexA–c-Jun activation by cAMP is insensitive to KN-62, indicating that cAMP-dependent protein kinase (PKA) rather than CaM kinases mediate this transcriptional response. Forskolin treatment did not induce any detectable increase in the phosphorylation of c-Jun at Ser63 and Ser73, failed to increase phosphorylation of JNK/SAPK1 on their activator sites, and did not increase JNK/SAPK1 activity (data not shown). Moreover, similar to the regulation by calcium signalling pathways, cAMP-induced c-Jun-dependent transcription did not require c-Jun amino acids

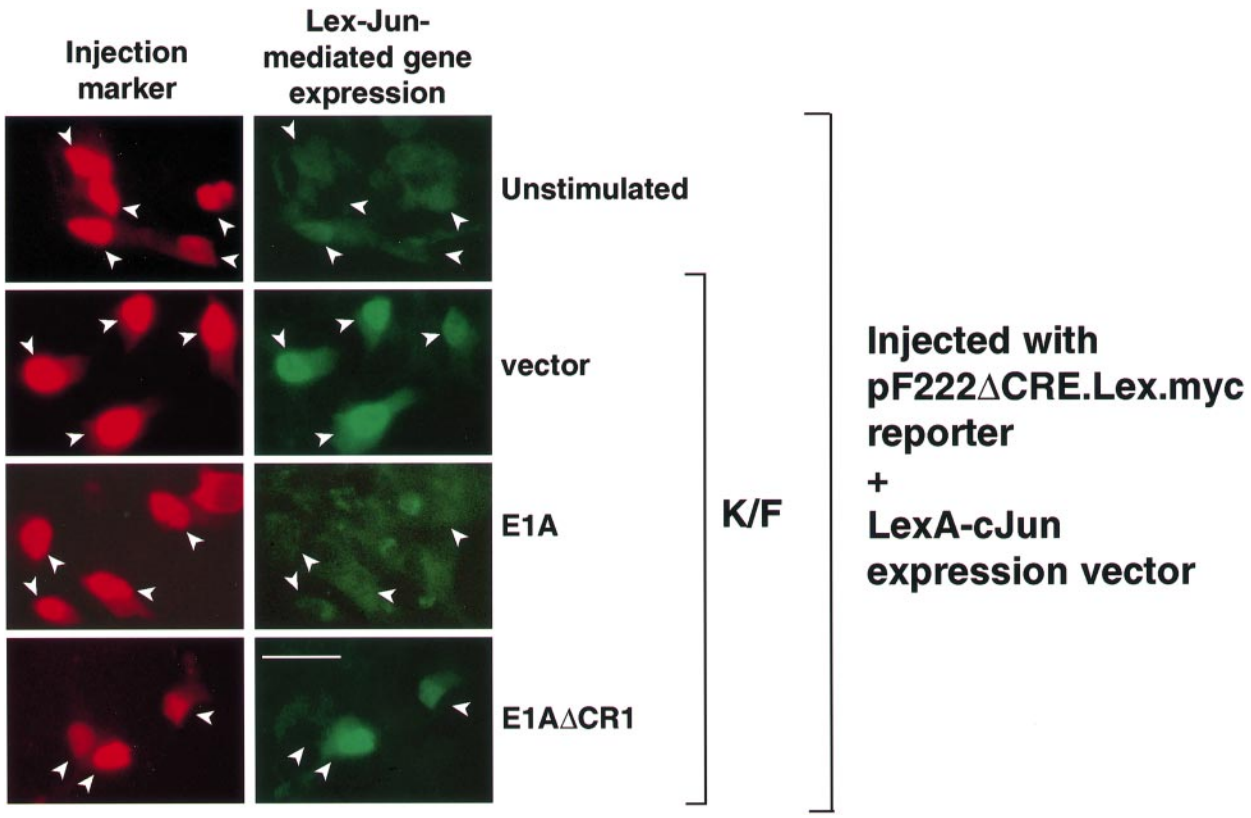

Fig. 7. Disruption of CBP function inhibits calcium-activated, LexA–c-Jun-mediated transcription. AtT20 cells were microinjected with the reporter gene pF222ΔCRE.Lex.myc plus the expression vector for LexA–c-Jun together with expression vectors for either E1A or E1AΔCR1, or a control plasmid (RSV-globin). The Texas Red fluorescence image identifies microinjected cells. In cells microinjected with the expression vector for E1A, reporter gene expression was (mean ± SEM of six independent experiments) 58 ± 5% (94 cells) of that obtained in control cells (307 cells analyzed); in cells microinjected with the expression vector for E1AΔCR1, reporter gene expression was (mean ± SEM of three independent experiments) 96 ± 9% (75 cells) of that obtained in control injected cells (290 cells analyzed). Scale bar is 60 μm.

Ser63 and Ser73 (Figure 8). These results suggest that calcium entry into cells or increases in cAMP induce c-Jun-mediated transcription via a JNK/SAPK1-independent signalling pathway, that targets c-Jun-bound CBP.

Discussion

While expression of the c-*jun* gene is induced by a number of extracellular stimuli including growth factors and neurotransmitters (Bartel *et al*., 1989; Szekeley *et al*., 1990; Angel and Karin, 1991; Bading *et al*., 1995), stress signals were thought to be the main activators of c-Jun function (Karin *et al*., 1997). Our experiments uncovered a novel c-Jun-regulating signalling pathway that is triggered by membrane depolarization and calcium influx through L-type voltage-gated calcium channels. This pathway does not require c-Jun residues Ser63 and Ser73, operates independently of JNK/SAPK1 activation and, thus, is mechanistically distinct from c-Jun regulation by stress.

The calcium-induced increase in c-Jun-mediated transcription, while dependent on CaM kinase activity, appears to take place without any detectable change in c-Jun phosphorylation: there is no calcium-induced increase in c-Jun phosphorylation on Ser63 and Ser73 and no detectable shift in the electrophoretic mobility of the LexA–c-Jun fusion, an indication of phosphorylation of the c-Jun N-terminus (Pulverer *et al*., 1991; Price *et al*., 1996). These data, together with our observation that E1A blocks c-Jun activation by calcium signals, suggest that the c-Jun-interacting protein, CBP (or a closely related protein such as p300), rather than c-Jun itself is the target for CaM kinase-mediated regulation. Indeed, CBP is a signal-regulated coactivator and control of CBP function by nuclear calcium and CaM kinase IV has recently been shown to be a key regulatory event in calcium-induced transcription by CREB (Chawla *et al*., 1998), the proto-

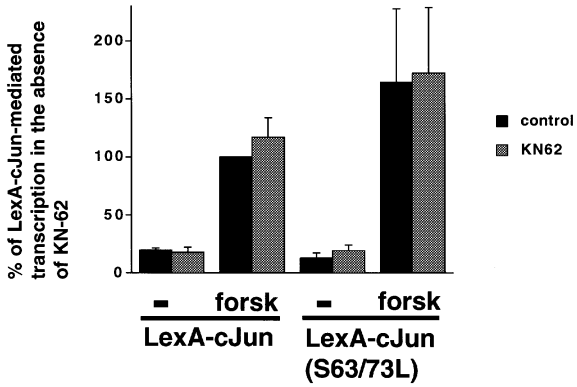

Fig. 8. c-Jun transcriptional activity is induced by increasing intracellular levels of cAMP. RNase protection analysis was used to measure LexA–c-Jun-mediated and LexA–c-Jun(S63/73L)-mediated expression from pF222ΔCRE.Lex.myc in unstimulated AtT20 cells and in cells 90 min after treatment with forskolin in the absence (black bars) or presence (grey bars) of KN-62 (n = 4, LexA–c-Jun; n = 4, LexA–c-Jun(S63/73L). Cells were treated with KN-62 (10 μM) for 20 min before stimulation.

typical CBP-interacting protein (reviewed in Janknecht and Hunter, 1996; Goldman *et al*., 1997). Thus, c-Jun and CREB form a new group of calcium-responsive transcription factors which function through recruitment of CBP and activate transcription upon stimulation of a calcium/CaM kinase signalling pathway. The exact nature of c-Jun and CREB-dependent gene expression responses, however, is distinct. CBP recruitment by CREB is tightly regulated and requires phosphorylation of CREB on Ser133 (Chrivia *et al*., 1993; Nakajima *et al*., 1997). While this phosphorylation event can be catalyzed by a number of protein kinases (Gonzalez and Montminy, 1989; Sheng *et al*., 1991; Tan *et al*., 1996; Xing *et al*., 1996; Chawla *et al*., 1998; reviewed in Chawla and Bading, 1998), their basal activity in unstimulated cells is generally very low. Consequently, CREB is not phosphorylated on Ser133 in unstimulated cells, CBP recruitment does not take place, and the basal level of CREB/CBP-dependent transcription is low. In stimulated cells, CREB is phosphorylated on Ser133, binds CBP which upon activation by nuclear calcium and CaM kinase IV causes a dramatic increase in transcription. In contrast to the two-step regulation of CREB-dependent gene expression, the interaction of c-Jun N-terminal transactivation domain with CBP appears to be independent of extracellular signals and, at least *in vitro*, does not require c-Jun phosphorylation on Ser63 and Ser73 (Bannister *et al*., 1995). Constitutive interaction of c-Jun and CBP may explain the high basal activity of the LexA–c-Jun protein and the moderate (2- to 4-fold) induction of transcription upon calcium entry into the cell which resembles the calcium regulation of CBP tethered to the promoter by fusion to the Gal4 DNA-binding domain (Chawla *et al*., 1998). The ability of LexA–c-Jun to activate gene expression upon increased intracellular levels of cAMP which, similarly to the calcium response is independent of c-Jun residues Ser63 and Ser73, is also likely to be due to the activation of CBP, a mediator of cAMP-induced transcription (Chrivia *et al*., 1993; Chawla *et al*., 1998). Thus, control of CBP function by calcium and cAMP signals may represent a general mechanism through which CBP-interacting transcription factors can mediate transcriptional activation. This model, termed the 'coactivator control model' predicts that transcription factors other than CREB and c-Jun can function as calcium/cAMP-regulated activators and may prove to be a mechanism through which calcium and cAMP signals can modulate the expression of many genes.

The regulation of c-Jun activity by calcium signals may be particularly relevant in the immune system and in neurons. c-Jun forms part of the nuclear factor of activated T cells (NFAT)/AP-1 transcription factor complex that, for example in activated T-cells, controls expression of the immune response-regulating cytokine, IL-2 (Serfling *et al*., 1995; Rao *et al*., 1997). While translocation of NFAT to the nucleus following antigen-induced calcium spikes is critical for induction of IL-2 gene expression, a possible regulation of the NFAT/AP1 complex by calcium signals could represent a second level of transcriptional control. In addition to their role in immunity and inflammation, calcium signals are associated with electrical activation of neurons and induce transcriptional responses which may be responsible for long-lasting adaptive changes in the mammalian central nervous system (Ghosh and Greenberg, 1995; Bading *et al*., 1997; Bito *et al*., 1997; Berridge, 1998; Hardingham and Bading, 1998). The c-*jun* gene is one of a number of genes that are induced following, for example, treatment of hippocampal neurons with the excitatory neurotransmitter glutamate (Szekeley *et al*., 1990; Bading *et al*., 1995). Regulation of c-Jun function by calcium signals may be an additional mechanism through which electrical activity modulates gene expression in the nervous system.

Materials and methods

Reporter genes and DNA plasmids

The human c-*fos* gene-based reporter, pF222ΔCRE.Lex.myc is identical to pF222myc (Hardingham *et al*., 1997) except that it contains a LexA-binding site instead of the c-*fos* CRE at position –60 relative to the transcription start site. An appropriate LexA-binding site-containing fragment was generated by PCR overlap extension using the oligonucleotides listed below. The final PCR product was digested with *Not*I and inserted between two *Not*I sites flanking the CRE. The *c-fos* transcriptional unit contains the 9E10 myc epitope inserted in the fourth exon as described previously (Hardingham *et al*., 1997).

Oligonucleotides used:

1a: 5′ CCCACGAGACCTCTGAGACAG3′

1b: 5′GTACTGTATGTACATACAGTACAACCACGGTGGCGCCAGAGGG3′

2a: 5′GTACTGTATGTACATACAGTACTTCATAAAACGCTTGTTATAA3′

2b: 5′TGCAGCGGGAGGATGACGCCT3′

The LexA–c-Jun protein is encoded by the plasmid murine leukaemia virus (MLV)–NLexA–c-Jun(1–194) (Price *et al*., 1996) and contains the entire bacterial repressor LexA, preceded by an SV40 nuclear localization signal, and fused to amino acids 1–193 of the human c-Jun. The plasmid encoding LexA–c-Jun(S63/73L), MLV–NLexA–c-Jun(1–194)Ser63/73Leu, was constructed by inserting a *Xho*I–*Sac*II fragment from plasmid RSV–Gal–c-Jun(1–166)Ser63/73Leu (Radler-Pohl *et al*., 1993) between the *Bam*HI and *Sac*II sites of MLV–NLexA–c-Jun(1–194). For immunoblot analysis and microinjection, LexA–c-Jun was expressed from the plasmid pEF-NLexA–c-Jun(1–194) (Price *et al*., 1996). JNK3 expression plasmids HA-SAPKp54β/MT2 and HA-SAPKp46β/MT2 (Sánchez *et al*., 1994) and expression plasmids for E1A and ΔE1A (Bannister and Kouzarides, 1995) have been described.

Stimulation of cells and preparation of cell extracts

AtT20 cells were stimulated two days after plating on 10 cm dishes by either addition of 500 ng/ml anisomycin, 10 μM forskolin, or 0.41 volumes of membrane depolarization solution containing 5 μM FPL 64176 (RBI Ltd) (Hardingham *et al*., 1997). Extracts for immunoprecipitations were prepared by washing the cells twice with ice-cold phosphate-buffered saline followed by lysis of the cells for 5 min on ice in 1 ml lysis buffer [HEPES 20 mM pH 7.4, 137 mM NaCl, 5 mM EDTA, 50 mM glycerophosphate, 10% glycerol, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 0.1 μg/ml okadaic acid, 1 mM benzamidine, 5 μg/ml pepstatin, 5 μg/ml aprotinin, 1 mM phenylmethyl sulfonylfluoride, 10 μg/ml leupeptin]. Lysates were transferred to a microcentrifuge tube and centrifuged in a chilled microcentrifuge at 14000 r.p.m. for 20 min. The soluble supernatant was transferred to a fresh tube and its protein concentration measured using the Bradford assay (Bio-Rad). Approximately 1.2 mg of total protein were used per immunoprecipitation reaction. For Western blotting, extracts were prepared from 60 mm dishes according to Marais *et al*. (1993).

Immunoprecipitation and kinase assays

Approximately 1.2 mg of soluble protein were incubated with 20 μl of agarose-conjugated antibodies against the C-terminus of JNK1 (C17) (Santa Cruz) with gentle agitation for 3 h at 4°C. After centrifugation at 2000 r.p.m. in an Eppendorf microcentrifuge the pellet was washed three times with lysis buffer, once with lithium wash (0.5 M LiCl, 20 mM HEPES pH 7.4, 0.1% Triton X-100, 1 mM DTT) and once with kinase buffer (20 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS) pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM DTT, 0.1 mM orthovanadate and 0.1 μg/ml okadaic acid) and resuspended in 27 μl kinase buffer. To this slurry 1 μg of GST–Jun (1–79) or

GST–ATF2(1–96) (Santa Cruz) and 8 µl of 250 µM ATP containing ~5 µCi of [γ-^{32}P]ATP were added. After incubation for 20 min at 30°C 15 µl of 4× Laemmli buffer was added, the reactions boiled for 2 min, centrifuged briefly and the supernatant was loaded onto a 10% SDS acrylamide gel. The portion of the gel containing the phosphorylated substrate was subsequently cut off, dried and exposed to X-ray film while the rest of the gel was immunoblotted for detection of the immunoprecipitated kinases.

RNase protection assays

Isolation of total RNA and RNase protection assay was performed as described previously (Bading *et al*., 1993; Johnson *et al*., 1997).

Antibodies and immunoblot analysis

Proteins separated on 10% SDS polyacrylamide gels were transferred overnight to PVDF membranes in transfer buffer containing 0.01% SDS and 20% methanol. After blocking in Tris-buffered saline (TBS) containing 5% dry milk, incubations with antibodies were done in blocking solution for at least 3 h or overnight. Signals were detected using chemiluminescence. Anti-phospho JNK, anti-phospho-p38 MAP kinase and anti-phospho-c-Jun(Ser63) were from New England Biolabs. Anti-phospho-ERK was from Promega. Goat anti-LexA, anti-JNK1(C-17) and anti-c-Jun (H-79) were from Santa Cruz. The monoclonal antibody to the HA epitope used in immunoprecipitation of JNK3 isoforms was from Babco.

Microinjection

Microinjection of plasmids and analysis of expression of myc-tagged reporter was carried out as described previously (Hardingham *et al*., 1997).

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References

Angel,P. and Karin,M. (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta*, **1072**, 129–157.

Arany,Z., Newsome,D., Oldread,E., Livingstone,D.M. and Eckner,R. (1995) A family of transcriptional adaptor proteins targeted by the E1a oncoprotein. *Nature*, **374**, 81–84.

Bading,H. and Greenberg,M.E. (1991) Stimulation of protein tyrosine phosphorylation by NMDA receptor activation. *Science*, **253**, 912–914.

Bading,H., Ginty,D.D. and Greenberg,M.E. (1993) Regulation of gene expression in hippocampal neurons by distinct calcium signaling pathways. *Science*, **260**, 181–186.

Bading,H., Segal,M.M., Sucher,N.J., Dudek,H., Lipton,S.A. and Greenberg,M.E. (1995) *N*-methyl-D-aspartate receptors are critical for mediating the effects of glutamate on intracellular calcium concentration and immediate early gene expression in cultured hippocampal neurons. *Neuroscience*, **64**, 653–664.

Bading,H., Hardingham,G.E., Johnson,C.M. and Chawla,S. (1997) Gene regulation by nuclear and cytoplasmic calcium signals. *Biochem. Biophys. Res. Commun*., **236**, 541–543.

Bannister,A.J. and Kouzarides,T. (1995) CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J*., **14**, 4758–4762.

Bannister,A.J., Oehler,T., Wilhelm,D., Angel,P. and Kouzarides,T. (1995) Stimulation of c-Jun activity by CBP: c-Jun residues Ser63/73 are required for CBP induced stimulation *in vivo* and CBP binding *in vitro*. *Oncogene*, **11**, 2509–2514.

Bartel,D.P., Sheng,M., Lau,L.F. and Greenberg,M.E. (1989) Growth factors and membrane depolarization activate distinct programs of early response gene expression: dissociation of *fos* and *jun* induction. *Genes Dev.*, **3**, 304–313.

Berridge,M.J. (1998) Neuronal calcium signaling. *Neuron*, **21**, 13–26.

Bito,H., Deisseroth,K. and Tsien,R. (1997) Ca^{2+}-dependent regulation in neuronal gene expression. *Curr. Opin. Neurobiol*., **7**, 419–429.

Chawla,S. and Bading,H. (1998) Function of nuclear and cytoplasmic calcium in the control of gene expression. In Verkhratsky,A and Toescu,E.C. (eds), *Integrative Aspects of Calcium Signaling*. Plenum Press, New York, NY, pp. 59–78.

Chawla,S., Hardingham,G.E., Quinn,D.R. and Bading,H. (1998) CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. *Science*, **281**, 1505–1509.

Chrivia,J.C., Kwok,R.P.S., Lamb,N., Hagiwara,M., Montminy,M.R. and Goodman,R.H. (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. *Nature*, **365**, 855–859.

Clapham,D.E. (1995) Calcium signalling. *Cell*, **80**, 259–268.

Dérijard,B., Hibi,M., Wu,I.-H., Barret,T., Su,B., Deng,T., Karin,M. and Davis,R.J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, **76**, 1025–1037.

Fukunaga,K., Soderling,T. and Miyamoto,E. (1992) Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C activation by glutamate in cultured rat hippocampal neurons. *J. Biol. Chem*., **267**, 22527–22533.

Ghosh,A. and Greenberg,M.E. (1995) Calcium signalling in neurons: molecular mechanisms and cellular consequences. *Science*, **268**, 239–247.

Gille,H., Strahl,T. and Shaw,P. (1995) Activation of ternary complex factor Elk-1 by stress-activated protein kinases. *Curr. Biol*., **5**, 1191–1200.

Goldman,P.S., Tran,V.K. and Goodman,R.H. (1997) The multifunctional role of the co-activator CBP in transcriptional regulation. *Recent Prog. Horm. Res*., **52**, 103–119.

Gonzalez,G.A. and Montminy,M.R. (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*, **59**, 675–680.

Gupta,S., Barrett,T., Whitmarsh,A.J., Cavanagh,J., Sluss,H.K., Dérijard,B. and Davis,R.J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. *EMBO J*., **15**, 2760–2770.

Hardingham,G.E. and Bading,H. (1998) Nuclear calcium: a key regulator of gene expression. *Biometals*, **11**, 345–358.

Hardingham,G.E., Chawla,S., Johnson,C.M. and Bading,H. (1997) Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature*, **385**, 260–265.

Janknecht,R. and Hunter,T. (1996) Transcriptional control: versatile molecular glue. *Curr. Biol*., **6**, 951–954.

Johnson,C.M., Hill,C.S., Chawla,S., Treisman,R. and Bading,H. (1997) Calcium controls gene expression via three distinct pathways that can function independently of the Ras/microtubule associated protein kinases (ERKs) signaling cascade. *J. Neurosci*., **17**, 6189–6202.

Karin,M., Liu,Z.-g. and Zandi,E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol*., **9**, 240–246.

Kawasaki,H., Morooka,T., Shimohama,S., Kimura,J., Hirano,T., Gotoh,Y. and Nishida,E. (1997) Activation and involvement of p38 mitogen-activated protein kinase in glutamate-induced apoptosis in rat cerebellar granule cells. *J. Biol. Chem*., **272**, 18518–18521.

Kyriakis,J.M., Banerjee,P., Nikolakaki,E., Dai,T., Rubie,E.A., Ahmad,M.F., Avruch,J. and Woodgett,J.R. (1994) The stress-activated protein kinase subfamily of c-jun kinases. *Nature*, **369**, 156–160.

Lerea,L.S. and McNamara,J.O. (1993) Ionotropic glutamate receptor subtypes activate c-*fos* transcription by distinct calcium-requiring intracellular signaling pathways. *Neuron*, **10**, 31–41.

Lerea,L.S., Butler,L.S. and McNamara,J.O. (1992) NMDA and non-NMDA receptor-mediated increase of *c-fos* mRNA in dentate gyrus neurons involved calcium influx via different routes. *J. Neurosci*., **12**, 2973–2981.

Lundblad,J.R., Kwok,R.P., Lawrence,M.E., Harter,M.L. and Goodman,R.H. (1995) Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional coactivator CBP. *Nature*, **374**, 85–88.

Marais,R., Wynne,J. and Treisman,R. (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell*, **73**, 381–393.

Matthews,R.P., Guthrie,C.R., Wailes,L.M., Zhao,X., Means,A.R. and McKnight,G.S. (1994) Calcium/calmodulin-dependent protein kinase types II and IV differentially regulate CREB-dependent gene expression. *Mol. Cell. Biol*., **14**, 6107–6116.

Means,A.R. (1988) Molecular mechanisms of action of calmodulin. *Recent Prog. Horm. Res*., **44**, 223–262.

Means,A.R., Vanberkum,M.F.A., Bagchi,I., Lu,K.P. and Rasmussen,C.D. (1991) Regulatory functions of calmodulin. *Pharmacol. Therap*., **50**, 255–270.

Morgan,J.I. and Curran,T. (1986) Role of ion influx in the control of c-*fos* expression. *Nature*, **322**, 552–555.

Nakajima,T., Uchida,C., Anderson,S.E., Parvin,J.D. and Montminy,M. (1997) Analysis of a cAMP-responsive activator reveals a two component mechanism for transcriptional induction via signal-dependent factors. *Genes Dev.*, **11**, 738–747.

Nose,P.S., Griffith,L.C. and Schulman,H. (1985) Ca^{2+}-dependent phosphorylation of tyrosine hydroxylase in PC12 cells. *J. Cell Biol.*, **101**, 1182–1190.

Price,M.A., Cruzalegui,F.H. and Treisman,R. (1996) The p38 and ERK MAP kinase pathways cooperate to activate ternary complex factors and c-*fos* transcription in response to UV light. *EMBO J.*, **15**, 6552–6563.

Pulverer,B., Kyriakis,J.M., Avruch,J., Nikolakaki,E. and Woodgett,J.R. (1991) Phosphorylation of c-jun mediated by MAP kinases. *Nature*, **353**, 670–674.

Radler-Pohl,A., Sachsenmaier,C., Gebel,S., Auer,H.-P., Bruder,J.T., Rapp,U., Angel,P., Rahmsdorf,H.J. and Angel,P. (1993) UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. *EMBO J.*, **12**, 1005–1012.

Rao,A., Luo,C. and Hogan,P.G. (1997) Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.*, **15**, 707–747.

Rosen,L.B., Ginty,D.D., Weber,M.J. and Greenberg,M.E. (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. *Neuron*, **12**, 1207–1221.

Sánchez,I., Hughes,R.T., Mayer,B.J., Yee,K., Woodgett,J.R., Avruch,J., Kyriakis,J.M. and Zon,L.I. (1994) Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-jun. *Nature*, **372**, 794–798.

Serfling,E., Avots,A. and Neumann,M. (1995) The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochim. Biophys. Acta*, **1263**, 181–200.

Sheng,M., Dougan,S.T., McFadden,G. and Greenberg,M.E. (1988). Calcium and growth factor pathways of *c-fos* transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.*, **8**, 2787–2796.

Sheng,M., Thompson,M.A. and Greenberg,M.E. (1991) CREB: A Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science*, **252**, 1427–1430.

Sun,P., Enslen,H., Myung,P.S. and Maurer,R.A. (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes Dev.*, **8**, 2527–2539.

Szekeley,A.M., Costa,E. and Grayson,D.R. (1990) Transcriptional program coordination by *N*-methyl-D-aspartate-sensitive glutamate receptor stimulation in primary cultures of cerebellar neurons. *Mol. Pharmacol.*, **38**, 624–633.

Tan,Y., Rouse,J., Zhang,A., Cariati,S., Cohen,P. and Comb,M.J. (1996) FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.*, **15**, 4629–4642.

Xing,J., Ginty,D.D. and Greenberg,M.E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science*, **273**, 959–963.

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