

The translation inhibitor anisomycin induces Elk-1-mediated transcriptional activation of *egr-1* through multiple mitogen-activated protein kinase pathways

Soon Young Shin¹, Joon Ho Lee¹,
Byung Wook Min² and Young Han Lee^{1,3}

¹Division of Molecular and Life Sciences
College of Science and Technology
Hanyang University
Ansan 426-791, Korea

²Department of Surgery
Korea University College of Medicine
Ansan 425-707, Korea

³Corresponding author: Tel, 82-31-400-5517;
Fax, 82-31-416-9781; E-mail, younghan@hanyang.ac.kr

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Abbreviations: ANX, anisomycin; CHX, cycloheximide; P_{egr-1} , *egr-1* gene promoter

Abstract

The early growth response-1 gene (*egr-1*) encodes a zinc-finger transcription factor Egr-1 and is rapidly inducible by a variety of extracellular stimuli. Anisomycin (ANX), a protein synthesis inhibitor, stimulates mitogen-activated protein kinase (MAPK) pathways and thereby causes a rapid induction of immediate-early response genes. We found that anisomycin treatment of U87MG glioma cells resulted in a marked, time-dependent increase in levels of Egr-1 protein. The results of Northern blot analysis and reporter gene assay of *egr-1* gene promoter (P_{egr-1}) activity indicate that the ANX-induced increase in Egr-1 occurs at the transcriptional level. Deletion of the serum response element (SRE) in the 5'-flanking region of *egr-1* gene abolished ANX-induced P_{egr-1} activity. ANX induced the phosphorylation of the ERK1/2, JNK, and p38 MAPKs in a time-dependent manner and also induced transactivation of Gal4-Elk-1, suggesting that Elk-1 is involved in SRE-mediated *egr-1* transcription. Transient transfection of dominant-negative constructs of MAPK pathways blocked ANX-induced P_{egr-1} activity. Furthermore, pretreatment with specific MAPK pathway inhibitors, including the MEK inhibitor U0126, the JNK inhibitor SP600125, and the p38 kinase inhibitor SB202190, completely

inhibited ANX-inducible expression of Egr-1. Taken together, these results suggest that all three MAPK pathways play a crucial role in ANX-induced transcriptional activation of P_{egr-1} through SRE-mediated transactivation of Elk-1.

Keywords: anisomycin; cycloheximide; early growth response protein 1; ets-domain protein Elk-1; mitogen-activated protein kinases; serum response element

Introduction

The *egr-1* gene, which is also known as *G0S30*, *NGFI-A*, *zif268*, *krox24*, and *Tis8*, encodes a zinc-finger-containing transcription factor and is an immediate-early response gene that is rapidly induced by stress, injury, mitogens, and differentiation factors in diverse cell types (Forsdyke, 1985; Sukhatme *et al.*, 1988; Edwards *et al.*, 1991; Liu *et al.*, 1998). Egr-1 regulates the expression of genes that are involved in growth control and apoptosis by transactivating p21, p53, PTEN, TGF- β 1, fibronectin, and Gadd45 (Liu *et al.*, 1998; Virolle *et al.*, 2001; Krones-Herzig *et al.*, 2003; Ragione *et al.*, 2003; Thyss *et al.*, 2005; Baron *et al.*, 2006). Expression of Egr-1 in several tumor cells is low (Huang *et al.*, 1997), and the ectopic expression of *egr-1* in tumor cells attenuates cell proliferation and tumorigenicity and increases cell attachment (de Belle *et al.*, 1999; Liu *et al.*, 1999; 2000). These results suggest that Egr-1 plays a key role in the regulation of cell growth and death in response to mitogens and stress signals.

Extracellular growth factors such as EGF, FGF, and PDGF cause the rapid and transient transcriptional induction of *egr-1*. This induction is mediated in part by the serum response element (SRE) in the 5'-flanking region of the gene (Christy and Nathans, 1989; Clarkson *et al.*, 1999; Tsai *et al.*, 2000). The ternary complex factor (TCF) forms a ternary complex on the *egr-1* SRE with the MADS (MCM1-agamous-ARG80-deficiens-SRF)-box protein serum response factor (SRF) (Treisman, 1994; Sharrocks, 2002). The TCFs represent a subfamily of Ets-domain transcription factors that contains at least three members, Elk-1 (Rao *et al.*, 1989), Sap-1 (Dalton and Treisman, 1992), and Sap-2/Net/Erp (Dalton and Treisman, 1992; Lopez *et al.*, 1994).

Mitogen-activated protein kinase (MAPK) pathways play major roles in the transduction of mitogenic and stress stimuli into nuclear responses. The TCFs are known targets of the three major MAPKs, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase, and TCF phosphorylation by MAPKs causes an increase in SRE-mediated transcriptional activation (Price *et al.*, 1995; Sharrocks, 2002). ERK-mediated phosphorylation of Elk-1 has been well established as correlating with increased transcriptional activation of *egr-1* (Hipskind *et al.*, 1994; Watson *et al.*, 1997; Hodge *et al.*, 1998; Guha *et al.*, 2001; Schrott *et al.*, 2001). In general, the ERK cascade is activated by growth factors and mitogens, whereas the JNK and p38 kinase cascades respond to cytokines and stress stimuli.

Inhibition of protein synthesis in Rat1 fibroblasts has been reported to induce transcriptional activation of *egr-1* (Shin *et al.*, 2002), and we have also observed that treatment of 3Y1 fibroblasts with cycloheximide (CHX), a protein synthesis inhibitor, is sufficient to increase the transcriptional rate of *egr-1* (Shin *et al.*, 2002). The translation inhibitor anisomycin (ANX) induces *c-fos* gene expression by stimulating MAPK signaling (Zinck *et al.*, 1995; Barros *et al.*, 1997), suggesting that ANX is able to modulate intracellular signaling pathways, independently of a block to translation. However, the signaling process whereby *de novo* inhibition of translation stimulates *egr-1* transcription has not been fully elucidated.

In the present study, we investigated the effects of the translation inhibitors ANX and CHX on the induction of *egr-1* transcription in U87MG human glioma cells. We show that the SRE of *egr-1* mediates the induction of *egr-1* transcription by ANX. Moreover, we present evidence that all three major MAPK cascades, ERK1/2, JNK1/2, and p38 kinase, play important roles in ANX-induced Elk-1 transactivation, which in turn stimulates transcription of the *egr-1* gene.

Materials and Methods

Reagents

DMEM and LipofectAMINE 2000 were purchased from Life Technologies, Inc. (Gaithersburg, MD), and fetal bovine serum was from Hyclone (Logan, Utah). Anisomycin, cycloheximide, U0126, SP600125, and SB202190 were obtained from Calbiochem (San Diego, CA). Antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK1/2 (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), and p38 MAPK were purchased from New England BioLabs (Beverly, MA). Antibodies against Egr-1, ERK2, JNK1 and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

U87MG human glioma cells were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum as described previously (Ahn *et al.*, 2006). To investigate the effects of protein synthesis inhibitors, U87MG cells were starved in DMEM containing 0.5% FBS for 24 h, and then stimulated with ANX or CHX for various lengths of time, as indicated.

Plasmids

The serial deletion mutant constructs of the *egr-1* promoter expressing the firefly luciferase were provided by Dr. H. Eibel (Department of Orthopedic Surgery, Research Laboratory University of Tübingen Medical Center, Germany) and described elsewhere (Aicher *et al.*, 1999). pCMV/ β -gal plasmid for normalization of transfection efficiency was obtained from Clontech. Dominant-negative MEK1 (pCGN1/DNMEK1), dominant negative ERK2 (pHA-ERK2 K52R), dominant negative JNK1 (pSR α /HA-JNK T183A/Y185F), and kinase-dead p38 kinase (pcDNA3/flag-p38 T180A/Y182F) plasmids were kindly gifted by Dr. Min, DS (Pusan National University). *Trans*- and *cis*-acting reporter plasmids, pFR-Luc, pFA2-Elk1 and pSRF-Luc, were obtained from Stratagen (La Jolla, CA).

Western blot analysis

Cells were lysed as described previously (Kim *et al.*, 2006), and 20- μ g aliquots of lysate were subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose filters that were incubated with primary antibodies and developed with the ECL detection system (Amersham Pharmacia Biotech., Piscataway, NJ). In some experiments, blots were re-probed with anti-GAPDH antibody for use as an internal control.

egr-1 promoter assay

U87MG glioma cells were seeded into 35-mm dishes (6×10^5 cells/dish) and co-transfected with 0.5 μ g of 5'-deletion constructs of the *egr-1* promoter (P_{egr-1}) (Aicher *et al.*, 1999) and 0.2 μ g of the plasmid pCMV/ β -gal using LipofectAMINE 2000 reagents (Life Technologies, Inc.) according to the manufacturer's instructions. Where indicated, 0.5 μ g of a mammalian expression vector encoding dominant-negative (dn)-MEK1, dn-ERK2, dn-JNK1, or kinase-dead (kd)-p38 kinase was included. Twenty-four hours post-transfection, cells were treated with ANX (10 ng/ml). Cells were harvested after 6-12 h, protein extracts were prepared using Reporter lysis buffer

(Promega), and 1–5 µg of protein was assayed for luciferase activity. Luminescence was measured using a luminometer (model TD 2020; Berthold, Tübingen, Germany).

Transactivation assay

pFA2-Elk1 encodes a fusion protein consisting of the yeast Gal4 DNA-binding domain (aa 1–147) and the activation domain of Elk-1 (aa 307–427). pFR-Luc contains five repeats of the Gal4-binding element upstream of a luciferase reporter gene. The *trans*-acting activity of Elk-1 was measured in U87MG cells using a luciferase reporter assay after the cells were co-transfected with 0.5 µg of the *trans*-activator plasmid pFA2-Elk1 and 50 ng of the luciferase reporter plasmid pFR-Luc. To assay SRF-dependent transcriptional activation, we used plasmid pSRF-Luc, which contains five tandem repeats of the SRF-binding sequence. After transfection, cells were treated with or without ANX, as indicated, and assayed for luciferase activity.

Results

Translation inhibitors induce expression of Egr-1 protein

CHX inhibits protein synthesis by blocking the peptidyl synthase activity of eukaryotic ribosomes (Dieci *et al.*, 1995; Christner *et al.*, 1999), whereas ANX inhibits translation by binding to the 60S ribosomal subunit and blocking formation of peptide bonds (Barbacid and Vazquez, 1974). We have previously shown that CHX at low concentrations (0.1–1 µg/ml), but not at high concentrations (>10 µg/ml), causes accumulation of *egr-1* mRNA in 3Y1 fibroblasts by increasing the rate of *egr-1* transcription rather than by stabilizing the *egr-1* mRNA (Shin *et al.*, 2002). To determine whether other protein synthesis inhibitor ANX activates *egr-1* trans-

cription, we examined the efficacy of CHX and ANX on Egr-1 expression in human U87MG glioma cells. When serum-starved U87MG cells were treated with 0.1 µg/ml CHX, the amount of Egr-1 protein increased within 1 h and reached a peak at 2 h, after which the Egr-1 level dropped considerably (Figure 1A). ANX treatment at 10 ng/ml also markedly increased the amount of Egr-1 protein, but at >50 ng/ml, the amount of Egr-1 protein decreased dramatically, so that the Egr-1 level was only slightly higher than it was in untreated cells (Figure 1B). This result suggests that only a narrow concentration range of ANX is effective at increasing Egr-1 expression. A time-course study showed that ANX increased the level of Egr-1 protein within 2 h; the level peaked at 4 h and then substantially decreased by 6 h (Figure 1C). In contrast, ANX did not affect the amount of GAPDH protein in the cells.

SRE regions are essential for anisomycin-induced activation of *egr-1* transcription

To examine whether ANX causes accumulation of Egr-1 protein by increasing the *egr-1* transcription rate, U87MG cells were transfected with P_{egr-1} plasmid containing an *egr-1* promoter-controlled luciferase reporter gene. As shown in Figure 2A, treatment with ANX significantly increased *egr-1* promoter activity to 1.7 ± 0.3 -fold ($n = 3$), 1.9 ± 0.3 -fold ($n = 3$), and 1.4 ± 0.2 -fold ($n = 3$) at 5, 10, and 20 ng/ml, respectively, as compared to untreated cells, demonstrating that ANX activates the transcription of the *egr-1* gene. To further dissect the response element responsible for the ANX-induced activation of P_{egr-1} , internal deletion mutants of P_{egr-1} (Aicher *et al.*, 1999) were transfected into U87MG cells. Deletion of the proximal *SRE5* (δ *SRE5*, -89 to -108), a cluster of three SREs (δ *SRE2-4*, -326 to -376), or the distal *SRE1* (δ *SRE1*, -394 to -410) resulted in complete loss of ANX-induced activation of the mutant P_{egr-1} , compared with the wild-type

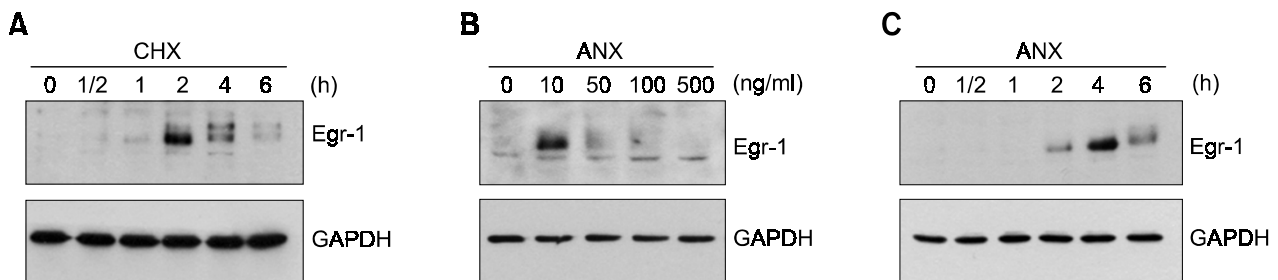


Figure 1. Effect of translation inhibitors on Egr-1 expression in U87MG cells. Serum-starved U87MG cells were exposed to 1 µg/ml CHX (A) or 10 ng/ml ANX (C) for various lengths of time, or were exposed to various concentrations of ANX for 2 h (B). Protein lysates were prepared and were then analyzed by Western blotting using an anti-Egr-1 antibody. GAPDH was used as an internal control. Similar results were obtained in two other independent experiments.

(WT) P_{egr-1} . However, at present, we cannot explain the reason why either deletion of SRE regions completely abrogates the ANX response. Further studies will be required whether SRE binding proteins including co-activator/co-repressor cooperate among them. In contrast, deletion of the proximal cAMP-response element (CRE) 2 ($\delta CRE2$, -127 to -134), the distal CRE1 ($\delta CRE1$, -623 to -630), or the Egr-1 binding sites ($\delta EBS1$, -685 to -695; $\delta EBS2$, -589 to -597)

resulted in both a high basal level of reporter gene activity and in strong ANX-induced reporter gene activity, suggesting that the CRE and EBS regions are negative regulators of Egr-1 expression. These results demonstrate that SRE regions are essential for ANX-induced activation of P_{egr-1} .

Anisomycin stimulates Elk-1 transactivation

Elk-1 is a ternary complex factor (TCF) that forms a complex with the serum response factor (SRF) on the SRE of P_{egr-1} and plays a crucial role in growth factor induction of *egr-1* transcription (Chen *et al.*, 2004). To assess whether Elk-1 is transactivated in response to ANX treatment, we utilized a *trans*-acting reporter assay system. U87MG cells were co-transfected with Gal4-Elk-1 DNA and plasmid pFR-Luc. Gal4-Elk-1 encodes a protein consisting of the yeast Gal4 DNA-binding domain fused to the activation domain of Elk-1 (aa 307-428), and pFR-Luc contains five repeats of the Gal4-binding element upstream of a luciferase reporter gene. Treatment of cells with 10 ng/ml ANX resulted in an increase of Gal4-Elk-1 transactivation approximately 2.5-fold compared with that of untreated control cells (Figure 3A). To determine whether ANX stimulates SRF-dependent transcriptional activation, pSRF-Luc containing five tandem repeats of the SRF-binding sequence was used instead of pFR-Luc. In this case, ANX had no effect on SRF transactivation (Figure

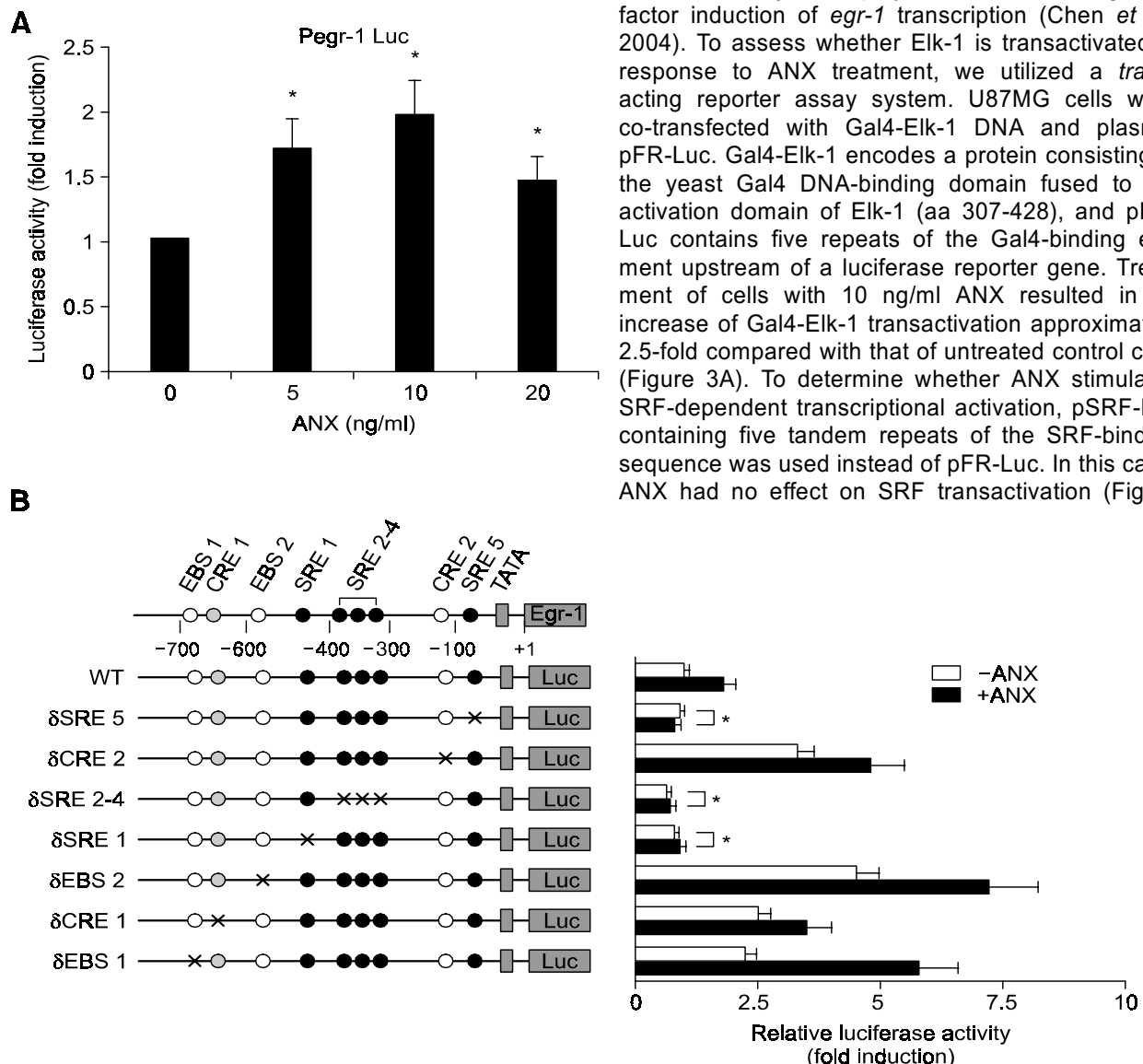


Figure 2. Effect of ANX on transcription of *egr-1*. U87MG cells were transfected with a wild-type P_{egr-1} -luciferase reporter gene construct. (A) After 24 h of transfection, cells were treated with 0, 5, 10, or 20 ng/ml ANX for 6 h. The cells were then lysed, and the lysates were analyzed for luciferase assay. (B) U87MG cells were transfected with a P_{egr-1} -luciferase reporter gene construct containing deletion mutations in P_{egr-1} . After 24 h, transfected cells were treated with 0 or 10 ng/ml ANX for 6 h, and luciferase assays were conducted. In both (A) and (B), the results are expressed as fold increase \pm S.D. of three independent experiments performed in triplicate, and are normalized for transfection efficiency using the pCMV/ β -gal reporter gene as a control. The statistical significance of the assays was evaluated using the Student's *t*-test (*, $P < 0.01$ compared with untreated control cells).

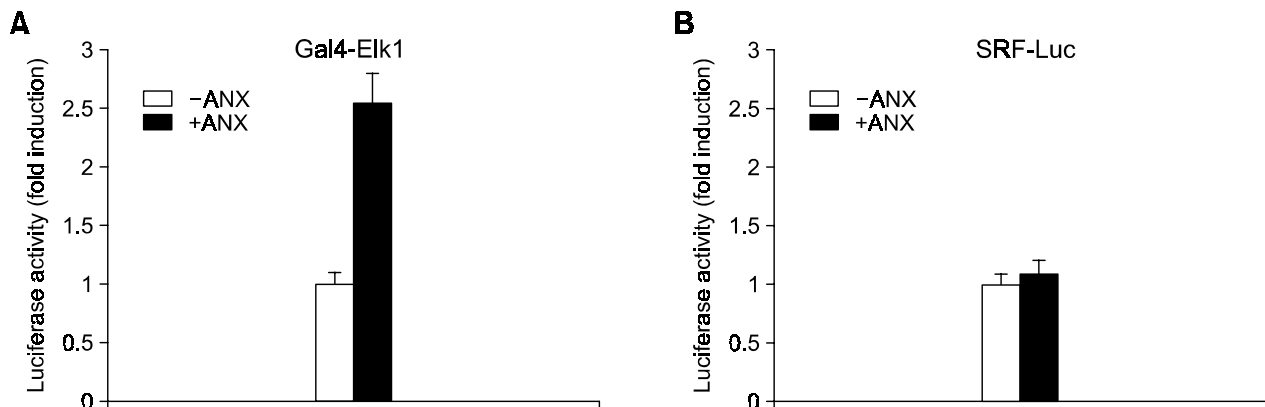


Figure 3. Effect of ANX on the *trans*-acting activity of Elk-1. U87MG cells were transfected with (A) plasmids pFA2/Gal4-Elk1 (50 ng) and pFR-Luc (0.5 μ g), or (B) plasmid pSRF-Luc (0.5 μ g), which contains five tandem repeats of the SRF-binding sequence. After 24 h of transfection, the cells were treated with 0 or 10 ng/ml ANX for 12 h, and then assayed for luciferase activity. The results are expressed as fold increase \pm S.D. of three independent experiments performed in triplicate, and are normalized for transfection efficiency using the pCMV/ β -gal reporter plasmid as a control.

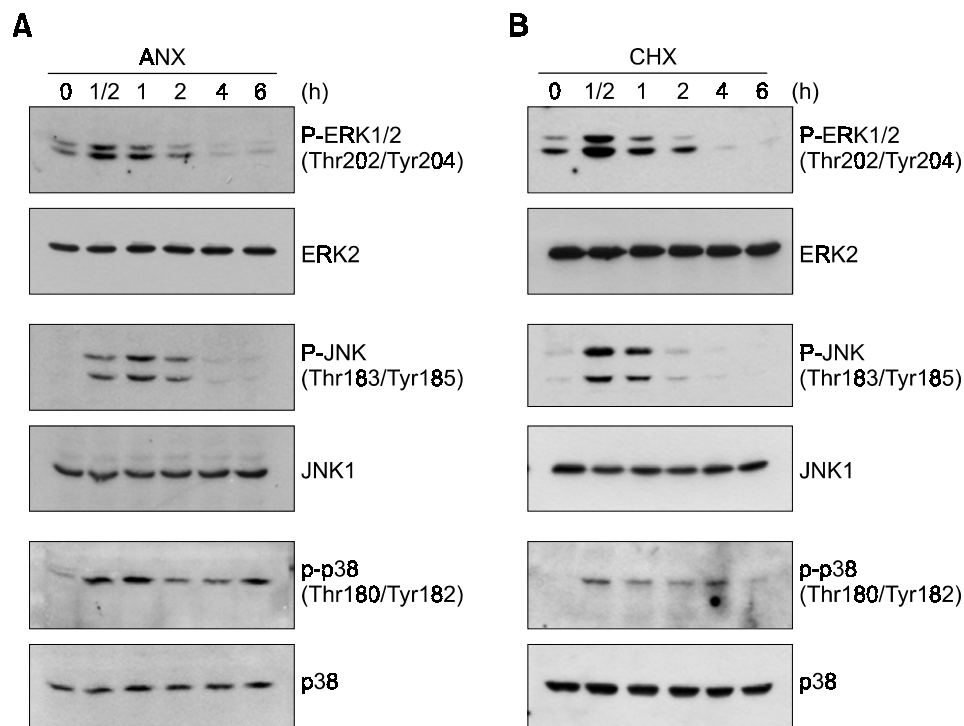


Figure 4. Activation of multiple MAPKs by ANX and CHX. Serum-starved U87MG cells were exposed to 10 ng/ml ANX (A) or 1 μ g/ml CHX (B) for the indicated lengths of time, and Western-blot analysis was performed with antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK (Thr183/ Tyr185), or phospho-p38 (Thr180/ Tyr182). The ERK2, JNK, or p38 kinase was used as an internal control. Similar results were obtained in two other independent experiments.

3B), suggesting that activation of the transcription factor Elk-1, but not of SRF, is responsible for SRE-mediated activation of *egr-1* transcription by ANX.

Anisomycin modulates multiple MAPK pathways

Elk-1 is phosphorylated and activated by multiple MAPK pathways in response to a variety of extracellular stimuli (Janknecht *et al.*, 1993). ANX has been shown to efficiently induce intracellular MAPK

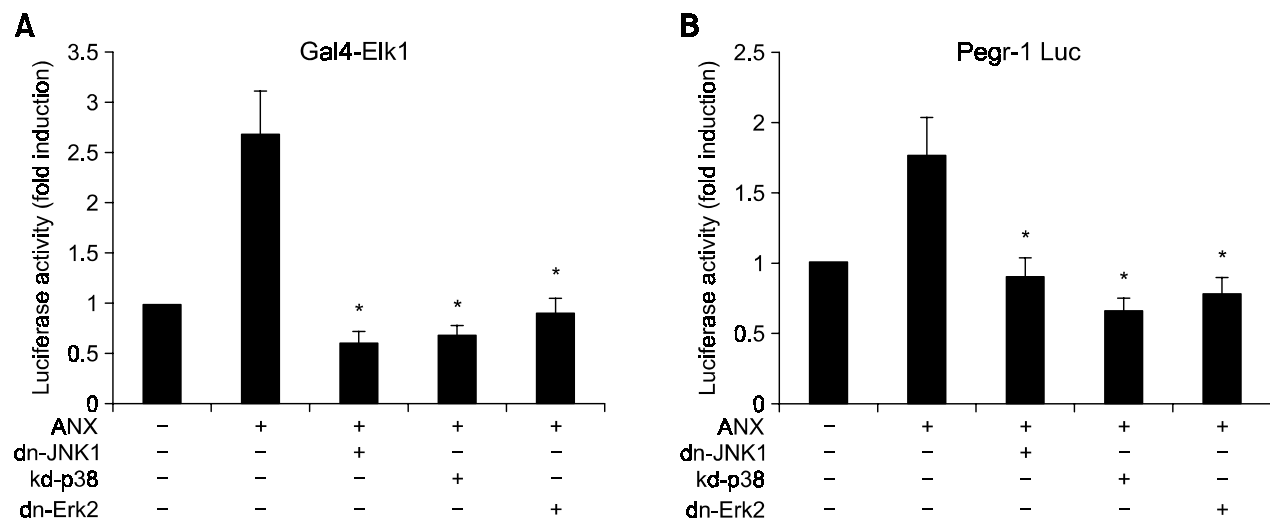


Figure 5. Multiple MAPKs are involved in ANX-induced transactivation of Elk-1 and activation of P_{egr-1} . U87MG cells were transfected with (A) plasmids pFA2/Gal4-Elk1 and pFR-Luc, or (B) a wild-type P_{egr-1} -luciferase reporter gene construct and a DNA construct encoding dn-JNK1, kd-p38 kinase, or dn-ERK2, as indicated. After 24 h of transfection, the cells were treated with ANX for 6 h and then assayed for luciferase activity. The results are expressed as fold increase \pm S.D. of three independent experiments performed in triplicate, and are normalized for transfection efficiency using the pCMV/ β -gal reporter plasmid as a control. The statistical significance of the assay was evaluated using the Student's *t*-test (*, $P < 0.01$ compared with untreated control cells).

signaling pathways that converge on Elk-1 activation, apparently independently of the ability of ANX to inhibit translation (Zinck *et al.*, 1995). To confirm the possible regulatory relationship between MAPK pathways and Elk-1 transactivation by ANX, we measured the phosphorylation status of MAPK pathway proteins using phospho-specific antibodies. When serum-starved U87MG cells were treated with ANX, increases in the level of phosphorylation of ERK1/2, JNK1/2, and p38 MAPKs were detected within 0.5 h after stimulation, while total amounts of these MAPKs did not change (Figure 4A), suggesting that these MAPKs are activated by ANX. The effect of CHX on the phosphorylation of these MAPKs was comparable to that of ANX (Figure 4B).

ERK, JNK, and p38 MAPKs are involved in anisomycin-induced transactivation of Elk-1 and activation of the *egr-1* promoter

To examine the possible involvement of different MAPK pathways in ANX-induced Elk-1 transactivation, Gal4-Elk-1/pFR-Luc *trans*-acting reporter constructs were transfected into U87MG cells along with plasmids encoding dn MAPKs. As shown in Figure 5A, ANX induction of Elk-1-dependent reporter gene expression was blocked by expression of dn-JNK1, kd-p38, or dn-ERK2 (Figure 5A). When we assessed the contribution of the different MAPKs to the ANX-induced transcriptional activity of P_{egr-1} , we observed that expression of dn-JNK1, kd-p38, or

dn-ERK2 abolished the ability of ANX to enhance P_{egr-1} activity, as shown in Figure 5B.

The ERK, JNK, and p38 MAPK inhibitors prevent anisomycin-induced accumulation of Egr-1 protein

To confirm that ERK1/2, JNK1/2, and p38 MAPKs contribute to ANX-induced Egr-1 expression, we examined the effect of pharmacological MAPK inhibitors on the accumulation of Egr-1. Western-blot analysis demonstrated that pretreatment with the MEK inhibitor U0126, the JNK inhibitor SP600125, and the p38 inhibitor SB202190 completely blocked the ANX-inducible accumulation of Egr-1 protein (Figure 6A). All of the MAPK inhibitors tested had a similar effect on CHX-induced Egr-1 expression (Figure 6B), suggesting that multiple MAPK signaling pathways may play a role in ANX-induced activation of the *egr-1* gene. Collectively, our results suggest that the protein synthesis inhibitors ANX and CHX stimulate Elk-1-mediated transcription of P_{egr-1} through multiple MAPK signaling pathways in U87 glioma cells.

Discussion

In this study, we examined the effects of the translation inhibitors ANX and CHX on the activation of Egr-1 expression. Our data indicate that ANX induces Elk-1 transactivation through all three major

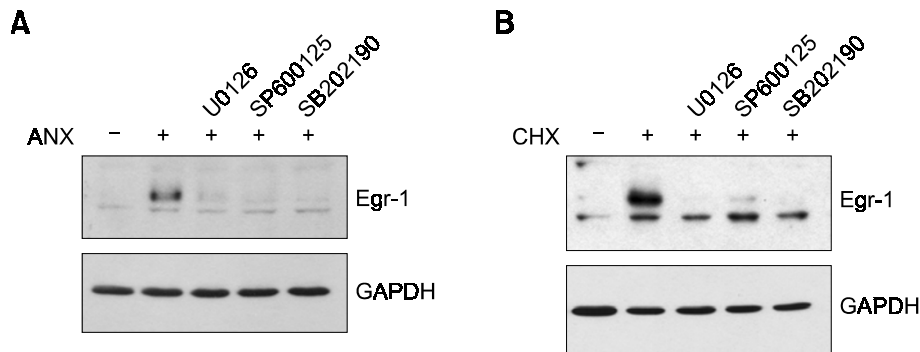


Figure 6. Effect of MAPK inhibitors on the ANX- and CHX-induced expression of Egr-1. Serum-starved U87MG cells were pretreated with U0126 (50 μ M), SP600125 (20 μ M), SB202190 (10 μ M), or no inhibitor for 1 h, and were then treated with 10 ng/ml ANX (A) or 10 μ g/ml CHX (B) for 2 h. Cells were then harvested, and Egr-1 expression was analyzed by Western blotting. GAPDH served as an internal control. Similar results were obtained in two other independent experiments.

MAPK pathways, including ERK, JNK, and p38 kinase, which in turn may play an important role in SRE-mediated *egr-1* transcription.

Previous studies have demonstrated that immediate-early response genes like *c-fos* and *egr-1* are hyper-induced when the cells are stimulated with growth factors in the presence of protein synthesis inhibitors (Cochran *et al.*, 1984; Kruijer *et al.*, 1984; Muller *et al.*, 1984). We also have reported the results of nuclear run-on transcription experiments indicating that pretreatment with the protein synthesis inhibitor CHX causes a hyper-induction of growth factor-stimulated *egr-1* transcription in rat 3Y1 fibroblasts (Shin *et al.*, 2002). Thus, the existence of a labile transcription repressor that maintains immediate-early response genes in an inactive state has been proposed. According to this model, protein synthesis inhibitors block this short-lived repressor, causing hyper-induction of immediate-early response genes (Greenberg *et al.*, 1986; Morello *et al.*, 1990). Given that ANX stimulates *c-fos* gene expression through activation of MAPK signaling pathways independently of its ability to inhibit protein synthesis (Zinck *et al.*, 1995; Barros *et al.*, 1997), we tested whether these pathways are involved in the transcriptional activation of *egr-1* by protein synthesis inhibitors in U87MG glioma cells.

In our experiments, we found that Egr-1 protein accumulated in U87MG cells when the cells were treated with CHX or ANX, another translation inhibitor. Using serial mutations in P_{egr-1} , we found that ANX reproducibly stimulated SRE-mediated transcription of *egr-1* (Figure 2). The *egr-1* SRE includes both a CA₂G box that binds the SRF, and an Ets motif that binds a TCF family member (Christy and Nathans, 1989; Watson *et al.*, 1997). The ERK, JNK, and p38 MAPKs are responsible for Elk-1 phos-

phorylation, which in turn correlates with increased transcription of *egr-1* (Lim *et al.*, 1998; Guha *et al.*, 2001; Chen *et al.*, 2004). Our data demonstrate that ANX rapidly stimulates phosphorylation of ERK, JNK, and p38 (Figure 4) and that Gal4-Elk1 transactivating activity is stimulated by ANX (Figure 3), strengthening the hypothesis that Elk-1 plays a role in coupling P_{egr-1} to multiple MAPK signaling cascades. Therefore, our data indicate that the accumulation of Egr-1 protein induced by translation inhibitors occurs *via* positive regulation at the transcriptional level, mediated, at least in part, by activation of Elk-1. At present, an unresolved question is how transfection with either one of dominant negative mutants of MAPKs, each of which blocks a specific MAPK signaling, is sufficient to cause complete shut-off of Elk-1 transactivation and *egr-1* expression by ANX (Figure 5). One possible explanation is ERK, JNK and p38 pathways cross-talked each other and this cooperation might be critical for ANX-induced Elk-1 transactivation. This view is supported by the notion that MAPK pathways often share upstream and downstream kinases and transcription factors that interact and integrate among different MAPK cascades (Cuschieri and Maier, 2005). Further study will be required to determine the detailed mechanisms underlying the regulation of ERK, JNK and p38 MAPK pathways by ANX.

Our data conflict with previous reports that ANX activates JNK and p38 MAPK, but does not activate ERK cascades (Hazzalin *et al.*, 1998). In other studies, however, ANX-induced activation of the ERK1/2 signaling pathway was observed in MCAS cells (Konishi *et al.*, 2004). These discrepancies may be attributable to the fact that the regulation of MAPK signaling pathways may differ in different cell

lines.

The current data are not sufficient to evaluate the possible mechanisms by which ANX or CHX regulate MAPK activities. One possible explanation is that *de novo* expression of MAPK phosphatases such as MKP-1, which is required for down-regulation of MAPK, is sensitive to translation inhibitors. If the putative labile repressor responsible for the hyper-induction of immediate-early response genes by translation inhibitors does indeed exist, then it must be associated with the inactivation of MAPK signaling pathways. Alternatively, ANX or CHX may directly stimulate an upstream kinase of MAPKs, independently of inhibition of protein synthesis. Further experiments are required to fully understand the regulatory mechanism underlying the increased phosphorylation of MAPKs induced by ANX and CHX.

In summary, this study demonstrate that ANX stimulates the *trans*-acting activity of Elk-1 in U87MG glioma cells *via* activation of the ERK1/2, JNK1/2, and p38 MAPKs, which leads to transcriptional activation of *egr-1*.

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