Stress-induced Stimulation of Early Growth Response Gene-1 by p38/Stress-activated Protein Kinase 2 Is Mediated by a cAMP-responsive Promoter Element in a MAPKAP Kinase 2-independent Manner*

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Malvyne Rolli, Alexey Kotlyarov, Kathleen M. Sakamoto‡, Matthias Gaestel§, and Armin Neininger

From the Martin-Luther-University Halle-Wittenberg, Innovationskolleg Zellspezialisierung, Hoher Weg 8, D-06120 Halle, Germany and the §University of California, Los Angeles, School of Medicine, Los Angeles, California 90095-1722

The p38/stress-activated protein kinase (p38/SAPK2) is activated by cellular stress and proinflammatory cytokines. Several transcription factors have been reported to be regulated by p38/SAPK2, and this kinase is involved in the control of expression of various genes. In human Jurkat T-cells, induction of the early growth response gene-1 (egr-1) by anisomycin is completely inhibited by SB203580, a specific inhibitor of p38/SAPK2a and -b. Northern blot and reporter gene experiments indicate that this block is at the level of mRNA biosynthesis. Using mutants of the egr-1 promoter, we demonstrate that a distal CAMP-responsive element (CRE; nucleotides -134 to -126) is necessary to control egr-1 induction by p38/SAPK2. Pull-down assays indicate that phospho-CRE binding protein (CREB) and phospho-activating transcription factor-1 (ATF1) bind to this element in a p38/SAPK2-dependent manner. In response to anisomycin, two known CREB kinases downstream to p38/SAPK2, MAPKAP kinase 2 (MK2) and mitogen- and stress-activated kinase 1 (MSK1), show increased activity. However, in MK2 --/ -- fibroblasts derived from mice carrying a disruption of the MK2 gene, the phosphorylation of CREB and ATF1 and the expression of egr-1 reach levels comparable with wild type cells. This finding excludes MK2 as an involved enzyme. We conclude that egr-1 induction by anisomycin is mediated by p38/SAPK2 and probably by MSK1. Phosphorylated CREB and ATF1 then bind to the CRE of the egr-1 promoter and cause a stress-dependent transcriptional activation of this gene.

Signal transduction via mitogen-activated protein (MAP) kinases plays a key role in a variety of cellular responses, including growth factor-induced proliferation, differentiation, and cell death (1–5). Several parallel MAP kinase signal transduction pathways have been defined in mammalian cells. These pathways include the extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK, also known as SAPK1), and p38 MAP kinases (SAPK2).

p38/SAPK2 is activated by bacterial lipopolysaccharide (6), physico-chemical changes in the extracellular milieu (heat, hyper- and hypo-osmolarity, UV irradiation, sodium arsenite, and anisomycin) (7–9), and proinflammatory cytokines (e.g. IL-1β and tumor necrosis factor-α) (5, 10, 11).

The activation of several transcription factors is regulated by the p38/SAPK2 pathway, and hence this pathway is involved in the control of expression of various genes including interferon-γ (12), tumor necrosis factor-α, IL-1, IL-8 (13, 14), IL-6 (10), inducible nitric-oxide synthase (15), and as shown more recently, E-selectin (16) and vascular cell adhesion molecule-1 (17). In vitro studies demonstrate that the transcription factor ATF2 is phosphorylated and activated by p38/SAPK2 (18, 19).

In addition, p38/SAPK2 activates the Elk-1 (19), CHOP (20), MEF2C (21), and SAP-1 (22) transcription factors. In addition to these factors, p38/SAPK2 also phosphorylates and thereby activates numerous downstream kinases (i.e. MNK1/2 (23, 24), MAPKAP kinase 2 (MK2) (25, 26), PRAK (27), and MSK1 (28)), implicated in the regulation of eIF-4E, the phosphorylation of CREB, and the induction of several immediate-early (IE) genes (i.e. c-fos, fosB, c-jun, junB, and junD) (29). The downstream kinase MK2 activated by p38/SAPK2 may contribute to c-fos induction via phosphorylation of CREB (30) and serum response factor (31). More recently, p38/SAPK2 has been shown to be essential for c-fos induction under conditions in which ERKs are not activated, such as upon anisomycin stimulation (32), possibly through the ability of this kinase to phosphorylate ternary complex factor directly (33).

A group of pyridinyl imidazole compounds have been identified as highly specific inhibitors of p38α/β/SAPK2a,b (34–36). The inhibitory effect of these compounds toward p38/SAPK2 is attributed to binding of the drug to the ATP binding pocket of the kinase (37). The compound SB203580 inhibits p38/SAPK2 with an IC50 of 0.6 μM and exhibits no effect even at 100 μM on the activities of 12 other protein kinases tested, including ERK2 and JNK/SAPK1 (35). In the past 2 years, SB203580 has been employed extensively to explore the specific roles of p38/SAPK2 in cellular responses in a variety of experimental systems (5, 10, 35, 38–40).

The immediate early gene egr-1 (also known as NGFI-A, zif268, TTS8, and krox24) encodes a transcription factor containing a DNA binding domain formed by three zinc finger motifs. This protein (Egr-1) binds to a specific GC-rich se-
Mechanism of Stress Induction of egr-1

**RESULTS**

**Anisomycin Stimulates a Transient p38/SAPK2-dependent Induction of egr-1**—To examine the regulation of egr-1 induction by the p38/SAPK2 pathway, Jurkat T-cells were treated with anisomycin for different times in the presence or absence of 5 mM anisomycin for different times in the presence or absence of 5 mM

**Mechanism of Stress Induction of egr-1**

The expression of egr-1 in Jurkat T-cells is regulated by stress-inducing factors such as anisomycin. Anisomycin stimulates a transient p38/SAPK2-dependent induction of egr-1, which is rapid and transient. This induction is mediated by the p38/MAPK pathway, which is activated in response to anisomycin treatment. The p38/SAPK2 pathway is a critical regulator of egr-1 expression, and its activation is essential for the induction of egr-1 under stress conditions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—Human Jurkat T-cells were cultured in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were grown in 75-cm² culture flasks. Mouse embryonic fibroblasts were grown in 75-cm² culture flasks. Mouse embryonic fibroblasts were transfected by liposome-mediated transfer using Lipo-2000 (Invitrogen) to mediate gene transfer. The correct mutated sequence was confirmed by DNA sequencing.

**Protein Kinase Assays**—Cells were washed three times with ice-cold saline and harvested in lysis buffer (20 mM Tris acetate, pH 7.4, 10 mM magnesium acetate, 0.1 mM EDTA, 50 μM ATP) containing 1.5 μg (γ-32P)ATP, and 4 μg of purified His-tagged MK2 (p38/SAPK2 assay), 3 μg of GST-c-jun(1-79) (JNK/ SAPK1 assay) (Alexis Corp., San Diego, CA), or 3 μg of Hsp27 (MK2 assay) as substrate. Proteins were resolved by electrophoresis in 7.5–20% SDS-polyacrylamide gel. Gels were dried, and kinase activity was determined by bio-imaging using the Cyclone imaging system (Packard Instrument Co.). Equal loading of RNA was confirmed by phosphorimaging.

**Pull-down Assay**—A DNA fragment (nt 232 to 109) spanning the direct CRE (nt 136 to 124) of the egr-1 promoter was amplified from Jurkat T-cells by PCR and cloned into pCMV-Myc vector (Promega). The promoter fragment was ligated into the pCMV-Myc vector to generate a plasmid containing the egr-1 promoter and Myc epitope tag. The plasmid was transfected into HEK293 cells using LipofectAMINE (Life Technologies, Inc.). The cells were lysed in RIPA buffer and subjected to immunoprecipitation with an antibody against Myc (clone 9E10). The immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

**Western Blot**—The expression of egr-1 was evaluated by Western blotting. Cells were lysed in lysis buffer containing 200 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 10 mM EDTA, and 0.5 μg/ml aprotinin. The lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with antibodies against egr-1 and secondary antibodies conjugated to horseradish peroxidase. The blots were developed using an enhanced chemiluminescent substrate and visualized byautoradiography.

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**Fig. 1.** Anisomycin-induction of egr-1 depends on activation of p38/SAPK2. Kinetics of p38/SAPK2 (A) and JNK/SAPK1 (B) activation by anisomycin. JUKAT T-cells were treated with 10 μg/ml anisomycin (Ani) for the different times indicated in the presence (+) and absence (−) of 10 μM SB203580 (SB). p38/SAPK and JNK/SAPK1 activity was determined in cell lysates by a kinase assay using His-tagged MK2 (His-MK2) and GST-c-jun-(1–79) as substrate, respectively. C, Analysis of egr-1 mRNA expression in Jurkat T-cells treated as in A. 10 μg of total RNA was analyzed by Northern blotting using egr-1 and glyceraldehyde-3-phosphate dehydrogenase (gapdh) DNA probes.

The specific p38α, β/SAPK2α, b inhibitor SB203580. p38/SAPK2 activity was determined in cells lysates by kinase assays using His-tagged MK2 as a substrate. p38/SAPK2 activation was already detectable 30 min after stimulation and a high kinase activity was maintained for more than 3 h (Fig. 1A). p38/SAPK2 activation by anisomycin was almost completely blocked in the presence of SB203580 (Fig. 1A, + SB). We also monitored JNK/SAPK1 activity in the cell lysates by using GST-c-jun-(1–79) as a substrate. JNK/SAPK1 shows a kinetics of anisomycin-stimulation similar to p38/SAPK2 (Fig. 1B). However, SB203580 treatment does not inhibit JNK/SAPK1 stimulation in Jurkat T-cells. Total RNA was isolated in parallel and subject to Northern blot analysis (Fig. 1C). egr-1 mRNA was not detectable in cells grown under control conditions. A low level of egr-1 mRNA becomes detectable after 30 min of anisomycin treatment, increases, and reaches its maximum at about 2 h. SB203580 completely blocked anisomycin-induced egr-1 mRNA expression. The similar kinetics of p38/SAPK2 activation and egr-1 expression and, especially, the similar effects of SB203580 indicate that the p38/SAPK2 pathway is directly involved in the regulation of egr-1 expression at the level of mRNA induction.

**A Putative cAMP-responsive Element in the egr-1 Promoter Is Essential for p38/SAPK2-dependent Induction.** To analyze the mechanism of egr-1 induction by the p38/SAPK2 pathway, human egr-1 promoter-CAT reporter gene constructs were transiently transfected into human 293 cells. The transfected cells were treated with anisomycin in the presence or absence of SB203580, and reporter gene activity was measured (Fig. 2). The full-length promoter (−600) revealed a high reporter gene activity after anisomycin treatment, which was reduced to approximately 50% in the presence of SB203580 (Fig. 2, −600). 5′-Deletion mutants of the egr-1 promoter, lacking potential binding sites for Egr-1, SP1 (−480), and serum response factor (−180), presented a lower reporter gene activity compared with the full-length promoter, but CAT activity showed a similar inhibition by SB203580 treatment (Fig. 2, −480, −235, −180). Further 5′-deletion of the egr-1 promoter (−116) resulted in a loss of the reduction of reporter gene activity by SB203580. A putative cAMP-responsive element (CRE) with the sequence 5′-TCACGTCA-3′ was identified within position −134 to −126 in the human egr-1 promoter (55). Site-directed mutagenesis of this potential CRE to a CREB/ATF1 nonbinding sequence 5′-TCTCATCA-3′ (−600m) (56) within the full-length promoter (−600) results in a complete loss of SB203580 sensitivity of the CAT reporter gene activity (Fig. 2, −600m). To make sure that the transcriptional effects observed are not specific for anisomycin or for the JNK/SAPK1 pathway but for the p38/SAPK pathway, we transfected human 293 cells with a constitutive active mutant of the p38/SAPK2 activator kinase MKK6, MKK6EX (19). As shown in Fig. 2C, MKK6EX also activates the egr-1 wild type promoter (−600) but not the mutated one (−600m). Together with the data from the deletion experiments, these findings demonstrate that p38/SAPK2-dependent egr-1 induction is mediated by the CRE within position −134 to −126. These results further indicate that transcription factors binding to this element are targets of the p38/SAPK2 pathway.

**CREB and ATF1 Are Phosphorylated and Bound to the CRE as a Result of p38/SAPK2 Activation.** To determine whether the transcription factors CREB and ATF1 were involved in egr-1 induction, we performed pull-down assays using a biotinylated DNA fragment spanning the distal CRE (nt −134 to −126) of the egr-1 promoter and nuclear extracts of Jurkat T-cells stimulated with anisomycin in the presence or absence of SB203580. Western blot analysis performed with phospho-CREB/ATF1 antibodies revealed that CREB and ATF1 are already phosphorylated to a low extent in nonstimulated Jurkat T-cells (Fig. 3A, nuclear extract, C). After anisomycin treatment CREB and ATF1 are highly phosphorylated (Fig. 3A, nuclear extract, Ani). This phosphorylation is completely blocked by SB203580 treatment (Fig. 3A, nuclear extract, SB). Pull-down assays revealed that phospho-CREB and phospho-ATF1 bind to the wild type CRE of the egr-1 promoter (−226 to −109) in anisomycin-treated cells (Fig. 3, A and B, wtCRE, Ani). In the presence of SB203580, no binding of CREB, phospho-CREB, or phospho-ATF1 could be detected (Fig. 3, A and B, wtCRE, SB). In cells grown under control conditions, phospho-ATF1 binds also to the wild type CRE (Fig. 3A, wtCRE, C). Binding of CREB, phospho-CREB, or phospho-ATF1 was not detected using the mutant CRE (−226 to −109, A-132T, G-130A) (Fig. 3, A and B, mCRE). These data indicate a phosphorylation-specific binding of CREB and ATF1 to the CRE. Because it is clear that p38/SAPK2 itself is not able to directly phosphorylate CREB and ATF1, it becomes highly probable that protein serine/threonine kinases downstream to p38/SAPK2 are involved.

**Analysis of the Involvement of Protein Kinases Downstream to p38/SAPK2.** The only CREB-kinases downstream to p38/SAPK2 known so far are MK2 and MSK1. To determine whether MK2 or MSK1 or both kinases are responsible for the anisomycin-induced SB 203580-dependent egr-1 induction, we decided to analyze stress-dependent egr-1 expression in cells lacking MK2 activity. For that reason, MEFs derived from mice with a targeted disruption of MK2 (−/− MEF) (68) and from wild type mice (+/+ MEF) were treated with anisomycin for 120 min in the presence or absence of SB 203580. p38/SAPK2 and MK2 activation by anisomycin was verified in these cells by kinase assays using cell lysates and His-tagged MK2 or Hsp25 as a substrate. MSK1 activity were determined in parallel by immunocomplex kinase assays using CREBtide as sub-
FIG. 2. The distal CRE (nt −134 to −126) is necessary for activation of the egr-1 promoter by p38/SAPK2. A, schematic representation of the egr-1 promoter fragments fused to the CAT gene. The putative regulatory elements of the egr-1 promoter are indicated: EBS, Egr-1 binding site; Sp-1, Sp1-binding G/C-rich element; SRE, serum response element; CRE, cAMP-responsive element. Successive truncations (−480, −235, −180, and −116) of the full-length (−600) promoter were examined. In addition, a loss of function mutation of the distal CRE (nt −134 to −126) in the full-length egr-1 promoter (−600m) was used. B and C, CAT reporter gene assay. B, 293 cells were transfected with the constructs shown in A using the LipofectAMINE reagent. 5 h after transfection, cells were stimulated by anisomycin (Ani) and absence (Ani) of SB203580 for 30 min. C, 293 cells were transfected with the full-length promoter construct (−600) and the mutated construct (−600m) and co-transfected with pcDNA3-FLAG-MKK6EE (19) and pcDNA3 as a control. After 24 h, cells were harvested and CAT activity was measured. The data shown (means ± S.D.) were obtained from two different experiments performed in triplicate.

FIG. 3. CREB and ATF1 bind to the distal CRE in a phosphor-ylation-dependent manner. Nuclear extracts from control (C), anisomycin (Ani)-treated, or anisomycin- and SB203580 (SB)-treated 293 cells were incubated with a biotinylated DNA fragment spanning the wild type CRE (wtCRE, nt −226 to −109) or the mutated CRE (mutCRE, −226 to −109, 125T→A, 136C→A). Bound proteins were identified by Western blotting using antibodies against phosphorylase-CREB/ATF1 (A) and CREB (B). C, histone acetyltransferase, and CREB in vivo. The immediate-early gene egr-1 is rapidly and transiently induced in response to multiple mitogenic signals (41, 49, 56), by the serine/threonine protein phosphatase inhibitor okadaic acid (44, 45), by diverse types of DNA damaging agents (46–48), and by several stress treatments (42, 50, 57). Recently, it has also been shown that the muscarinic acetylcholine receptors activate the Egr family of transcription factors (58). In contrast to the numerous studies concerning egr-1 induction, the understanding of the signaling pathways contributing to egr-1 activation in response to stress and other stimuli is rather incomplete. In B-lymphocytes, egr-1 induction by antigen receptor stimulation is dependent on the p21ras pathway (59). The insulin-induced egr-1 expression in 32D cells requires MEK1 activation but not phosphatidylinositol 3-kinase activity (43). In NIH-3T3 cells, stress treatment such as heat shock, sodium arsenite, ultraviolet radiation, and anisomycin induces egr-1 promoter activity involving p38/SAPK2 and/or JNK/SAPK1 (50). In contrast, in rat mesangial cells, lysophosphatic acid-mediated egr-1 gene expression does not depend on p38/SAPK2 but on MEK1 activation (49). In our studies we have used anisomycin, a stress stimulus that does not activate ERKs (29, 32) but does activate JNK/SAPK1 and p38/SAPK2. Similar to the results obtained for NIH-3T3 cells (50), we found that anisomycin induction of egr-1 in human Jurkat T-cells is completely inhibited by SB203580, whereas JNK/SAPK1 activity remains unchanged. We suggest that in these cells p38α, p38β, and p38αδ are the main kinases responsible for egr-1 gene expression after anisomycin stimulation.

The egr-1 promoter contains several putative regulatory elements, including two Sp1-binding sites, five putative SREs, two putative CREs, an Egr-1 binding site, and a tetra-decanoyl phorbol acetate-responsive element (55). In BA/F3 cells, activation of the egr-1 promoter by granulocyte/macrophage-colony stimulating factor is controlled by SREs (nt −56 to −116 and −235 to −480) through pathways that involve Janus kinase 2 and Ras (60). In TF-1 cells it has been demonstrated that, in addition to binding of serum response factor to the SRE, CREB is also bound to proximal CRE (nt−57 to −76) of the egr-1 promoter to induce its activity after granulocyte/macrophage-colony stimulating factor or IL-3 stimulation (56). The transcriptional activation of egr-1 by granulocyte/macrophage-colony stimulating factor necessitates the phosphorylation of
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May indicate a further contribution by JNK/SAPK1 through phosphorylation of Elk-1 as described (50).

ATF1 and CREB are members of the CREB/ATF family implicated in cAMP- and calcium-induced transcriptional activation. Although CREB can bind to DNA as a homodimer (63), two other bZIP proteins, ATF1 and CREM, which are highly related to CREB, are known to form hetero-dimers with CREB (64–66). We have shown that the phosphorylation of CREB and ATF1 after p38/SAPK2 activation allows their binding to the egr-1 promoter and causes a transient induction of egr-1.

Two downstream kinases of p38/SAPK2, MK2 and MSK1, were implicated in the phosphorylation of CREB after activation of the p38/SAPK2 pathway. Whereas CREB has been shown to be a poor substrate for MK2 in vitro (30), MK2 has been demonstrated to be involved in stress- and fibroblast growth factor-dependent activation of CREB and ATF1 (67). Using cells that lack MK2 activity, we demonstrate that this enzyme is not necessary for the phosphorylation of CREB and ATF1 in vivo. Because the CREB-kinase MSK1 (28) is highly activated in a SB203580-dependent manner in MK2−/− cells after anisomycin treatment, this enzyme is a good candidate for direct phosphorylation of these transcription factors. However, very recently a third CREB-kinase controlled by p38/SAPK2, RSK-B (52), was identified, which at this time can not be excluded as a contributor to anisomycin-induced egr-1 expression.

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Malvyne Rolli, Alexey Kotlyarov, Kathleen M. Sakamoto, Matthias Gaestel and Armin Neininger

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