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The p38 SAPK Is Recruited to Chromatin via Its Interaction with Transcription Factors*[S]

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In mammals, the stress-activated protein kinase (SAPK) p38 coordinates a rapid and complex transcriptional program to adapt to sudden changes in the extracellular environment. Although a number of genes have been reported to be under the control of p38, the basic mechanisms of transcriptional regulation by this SAPK remain uncharacterized. Here we show that in response to osmotic shock, anisomycin- or TNFα-activated p38 SAPK is recruited to stress-induced genes. The MAPKK MKK6 is also found at stress-responsive promoters. The recruitment of RNA polymerase II complex to the target promoters requires p38 activity. Moreover, when tethered to DNA as a LexA fusion protein, p38 activates transcription in a stress-regulated manner. Thus, p38 activity allows for recruitment of RNA polymerase and transcription initiation. p38 directly phosphorylates and interacts with the transcription factor Elk1. p38 activity is necessary for the recruitment of Elk1 to the c-Fos promoter, and knocking down Elk1 by siRNAs compromises both p38 recruitment to the c-Fos promoter and c-Fos transcriptional up-regulation upon osmostress. In addition, p38 recruitment to the osmoinducible gene Cox2 and the TNFα target gene IL8 is mediated by the transcription factors AP1 and NFκB, respectively. Therefore, anchoring of active SAPK to target genes is mediated by transcription factors. The presence of active p38 at open reading frames also suggests the involvement of the SAPK in elongation. Taken together, SAPK recruitment to target genes appears to be a broad mechanism to regulate transcription that has been preserved from yeast to mammals.

Exposure to stress requires rapid and efficient adaptive responses to maximize cell survival. Cells have signal transduction systems designed to produce rapid outcomes in response to extracellular stimuli. Eukaryotic organisms contain multiple MAPK families organized in discrete cascades that respond to mitogens and stress. A prototype member of the SAPK family is the p38 stress-activated protein kinase (SAPK), which plays an essential role in proper cell adaptation to extracellular stimuli (1, 2). Both mitogenic (MAPKs) and stress activated protein kinases (SAPKs) have been widely reported to regulate gene expression through the direct phosphorylation and activation of transcription factors, which are responsible for the activation of target genes. However, this canonical molecular mechanism of gene expression regulation has been challenged by numerous studies showing that activated MAPKs regulate gene expression by using a broad range of molecular mechanisms (3). In yeast, activation upon osmostress of the SAPK Hog1, the homolog of the mammalian p38 SAPKs, results in major reprogramming of the gene expression capacity of cells by regulation of several steps of the transcription process. Active Hog1 not only directly phosphorylates several transcription factors to alter their activities (4, 5), but also associates to chromatin at stress-responsive promoters (6). Once at the promoters, Hog1 serves as a platform to recruit general transcription factors, chromatin-modifying activities, and RNA Pol II (7, 8). In addition, Hog1 plays a role during elongation when recruited at open reading frames (ORFs) (9, 10).

To date, the knowledge of how higher eukaryote MAPKs regulate gene expression is more limited, in part due to the higher complexity imposed by the presence of at least three parallel MAPKs cascades: the JNK, p38 SAPK, and ERK cascades, capable of responding to a broad range of environmental stresses and physiological stimuli (11). However, it is known that there is a strong structural and functional preservation of MAP kinases and adaptive responses from yeast to mammals (12). Of note, recruitment to chromatin of ERK MAPK together and other signaling kinases have been reported (13–15). Also, there is evidence that p38 is recruited to muscle-specific gene promoters during muscle development suggesting that indeed SAPKs might play an important role at the chromatin (16).

A recent wide-genome study has shown that osmostress-activated p38 SAPK induces a complex time-dependent gene expression pattern (17). Although it has been well established that p38 SAPK contributes to the stabilization of 3′ AU-rich mRNAs (18), the fact that stress-induced p38 SAPK quickly promotes the accumulation of certain mRNAs suggests that p38 SAPK activation directly up-regulates stress-induced gene transcription. The evidence that yeast Hog1 directly binds to stress-induced gene promoters to drive transcription prompted

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us to ask whether similar mechanisms of gene expression control could be working in mammalian cells. In contrast to yeast Hog1, activation of mammalian p38 occurs in response to several stimuli. Osmostress signaling is mediated by the MAP3K MEKK4/MTK1 (19). A more recent study has shown that osmostress promotes the recruitment of the malcavernin/OSM protein to membrane ruffles, where it serves as a scaffold that brings together the MAP3K MEKK3 with the upstream activator Rac (20). Upon TNFα binding to the TNF receptor, a cascade of events leads to the activation of a plethora of MAP3Ks such as NIK, MEKK1, and ASK1(21). On the other hand, anisomycin binding to ribosomes induces a ribotoxic stress signaling pathway involving the MAP3K MLK7/ZAK(22, 23). These MAP3Ks will then activate the MAP2Ks MKK3/6, which will phosphorylate and activate p38. Here, we show that regardless the signaling pathway used to activate p38, this SAPK is recruited to stress-responsive genes mediating the recruitment of RNA polymerase II complex. In response to osmostress, p38 is recruited and regulates gene expression of genes such as c-Fos by binding to Elk1 transcription factor, which serve as anchor to locate p38 SAPK activity to stress-responsive promoters. We furthered extended our observations to AP1 and NFκB as anchors for p38. Moreover, stress-activated p38 SAPK was also found to be recruited to ORFs. Altogether, p38 SAPK recruitment to stress-induced promoters through the interaction with a specific transcription factor and to the ORFs appears to be a rather common molecular mechanism used to regulate gene expression that has been preserved throughout evolution from yeast to mammals.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells and immortalized wild-type and p38α knockout mouse embryonic fibroblasts (MEFs)[4] (24) were maintained in Dulbecco's modified Eagle's medium (Biological Industries) containing 10% fetal calf serum (Sigma) and supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GIBCO) and cultured in a 5% CO_2 humidified incubator at 37 °C. Cells were treated either with 100 mM NaCl or with 0.1 μg/ml TNFα (Preprotech) for the appropriate times. When indicated, cells were incubated with 10 μM SB203580 (Calbiochem) for 30 min, 5 μM BIRB 0796 (Axon Medchem) for 2 h, or with 50 μM Bay 11-7082 for 1 h (Sigma) prior to the treatments. HeLa cells were transiently transfected with the indicated plasmids or small interference RNAs (siRNAs) using the FuGENE 6 transfection reagent (Roche Applied Science) or DharmaFECT Duo Transfection Reagent (Dharmacon) respectively according to the manufacturer's directions for at least 48 h before treatments and harvesting.

Plasmids, siRNAs, and Antibodies—The following plasmids were used: pCDNA3 (Invitrogen), pEFmlinkMKK6^{DD} was obtained from Dr. A. R. Nebreda (CNIO, Madrid), pCDNA3-3HA-p38α was provided by Dr. D. Engelberg (The Hebrew University of Israel, Jerusalem), pELK1-Flag was gifted by Dr. A. Fellicielo (Università Federico II, Naples), pL8G5luc was donated by Dr. T. C. Südhof (Stanford University School of Medicine, Standford, CA), pCDNA3-LexA-DBD (expressing the LexA DNA binding domain) was generated from pCDNA3-LexA-Vp16 by removing the VP16 fragment by double digestion with KspI and BamHI followed by klenow dNTP fill-in and T4 ligase re-ligation; pCDNA3-LexA-DBD-p38α was generated by cloning the mouse p38α SAPK in-frame with the LexA-DBD. Briefly the p38α SAPK was amplified by PCR with oligonucleotides containing flanking KspI and BamHI restriction sites for directional cloning into the KspI- and BamHI-digested pCDNA3-LexA-Vp16. p38α-DsRed and p38α-GFP were obtained by cloning mouse p38α in-frame with the fluorescent protein markers DsRed and GFP, respectively. All cloned constructs were checked by sequencing. The following siRNAs were used: Elk1 (sc-35290) and GFP (sc-45924) siRNAs were from Santa Cruz Biotechnology; the c-Jun siRNA (6203) was from Cell Signaling. The antibodies used were as follows: mouse monoclonal anti-HA, mouse monoclonal anti-Myc, mouse monoclonal anti-Flag (Sigma, S2220), mouse monoclonal anti-RNA Pol II (Abcam, clone 8WG16), rabbit polyclonal anti-IκB-α (Santa Cruz Biotechnology, sc-371), rabbit polyclonal anti-p38α SAPK (Santa Cruz Biotechnology, sc-535), rabbit monoclonal anti-pp38 SAPK (Cell Signaling, clone 3D7), rabbit polyclonal anti-JNK (Cell Signaling, 9252), rabbit polyclonal anti-ppJNK (Cell Signaling, clone 81E11), mouse monoclonal anti-ppERK (Cell Signaling, clone E10), rabbit monoclonal anti-ERK (Cell Signaling, clone 137F5), rabbit polyclonal anti-c-Jun (Cell Signaling, clone 60A8) and rabbit polyclonal anti-HSP27 (Stressgen, SPA-523).

Western Blot and Coimmunoprecipitation Analysis—Cells were washed with ice-cold PBS and scraped into 500 μl of IP/lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 2 mM EDTA, 50 mM NaF, 50 mM β-glycerophosphate, 1 mM sodium vanadate, supplemented with the protease inhibitors 1 mM PMSF, 1 mM benzamidine, 200 μg/ml leupeptin, and 200 μg/ml pepstatin). The lysates were then cleared by microcentrifugation. A tenth of the lysate was retained as a whole cell extract input, and the remaining 90% was subjected to immunoprecipitation. Briefly, protein A-Sepharose beads (GE Healthcare) were washed twice with cold IP/lysis buffer and incubated with the appropriate antibody at 4 °C for at least 1 h. The protein A-Sepharose beads conjugated to the antibodies were then added to cleared cell lysates and incubated overnight at 4 °C with orbital agitation. Immunoprecipitates were then washed four times with cold IP/lysis buffer before the addition of SDS-loading buffer. Both whole cell extracts and immunoprecipitates were then subjected to SDS-PAGE electrophoresis, transferred to Immobilon P membranes (Millipore), and further analyzed by Western blotting. Proteins were visualized with HRP-conjugated anti-rabbit or an anti-mouse antibodies and the Enhanced Chemiluminiscence kit (GE Healthcare).

RT-PCR mRNA Analysis—Total RNA was purified from HeLa cells using the RNeasy kit (Qiagen) following the manufacturer's instructions. 100 ng of total RNA was then converted to cDNA using the reverse transcriptase Superscript kit (Invitrogen) according to the manufacturer's protocol. The mRNA levels of the c-Fos, Elk1, interleukin 8 (IL8), and Cox2 were analyzed by PCR or real-time PCR using a DNA Biosystems 7700 sequence detector and the SYBR Green kit (Applied

[4] The abbreviation used is: MEF, mouse embryonic fibroblast.

Biosystems). Real-time PCRs were performed in triplicates and referenced to the GAPDH mRNA levels. The following oligonucleotides were designed with Primer3 (25): *c-Fos* ORF For: AAAAGGAGAATCCGAAGGGA and Rev: GCAACCCACAGAGTACCTAC; Elk1 ORF For: TTTAATGGGTTGGGAGTCTT and Rev: AGACAAAGGAATGGCTTCTC; IL8 ORF) For: TGCCTGACTTAAGGAATCAT, and Rev: CAAAAACTTCTCCACAACCC; Cox2 ORF For: AACATTTTTTTGAAAATTTCAG Rev: ATCTCTAATGGATTCTTCTTACTCAC.

Luciferase Reporter Assay—Treated HeLa cells were washed twice with cold PBS and lysed with 1× cell culture lysis reagent (Promega). Cell lysates were cleared by microcentrifugation. Luciferase reporter activity was measured from cell supernatants using a Luciferase Reporter Assay kit (Promega) and a Microlumat LB 960 luminometer (Berthold Technologies). The total amount of protein found in the cell extracts was measured with the Bradford reagent (Bio-Rad). Protein-corrected luciferase reporter activities were performed in triplicates and represented as a fold-induction over the pCDNA3-LexA-DBD signal, which was considered as the basal.

Chromatin Immunoprecipitation (ChIP) Assays—Protein-DNA interactions were cross-linked in cell cultures by the direct addition of 1% (v/v) formaldehyde (Sigma) for 20 min at room temperature. Cross-linking was stopped by the addition of 0.125 M glycine for 5 min at room temperature. Cells were washed and harvested in PBS containing 4 μg/ml Complete Protease Inhibitor (Roche Applied Science). Pelleted cells were then lysed on ice for 10 min in 50 mM Tris-HCl, pH 8.1, 1% (w/v) SDS, 10 mM EDTA containing 4 μg/ml of Complete Protease Inhibitor Mixture. Lysates were sonicated in a Bioruptor water bath (Diagenode) set at full power with 0.5 min sonication/0.5 min resting intervals at 4 °C for 12 min. Next samples were centrifuged, and the chromatin was quantified from the supernatants with a nanodrop apparatus. Under these sonication conditions, DNA was fragmented in a range of 200–700 bp. 10% of the volume was retained as an input, and ~40 μg of chromatin was used per IP and diluted in ChIP buffer (6.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl supplemented with 4 μg/ml Complete Protease Inhibitor Mixture). Dynabeads (Invitrogen) were conjugated by orbital mixing overnight at 4 °C with the appropriate antibody before being added to the diluted cell extracts. After a further overnight incubation at 4 °C, dynabeads were serially washed with the following buffers: low ionic strength (120 mM Tris-HCl, pH 8.1, 0. 1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), high ionic strength (120 mM Tris-HCl, pH 8.1, 0. 1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), LiCl buffer (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P40, 1% deoxycholate, 1 mM EDTA) and TE (10 mM Tris-HCl, pH 8.1, 1 mM EDTA). Protein-DNA complexes were eluted from the dynabeads by incubation at room temperature with elution buffer (0.1 M $NaHCO_3$, 1% SDS). Protein-DNA cross-linking was reversed by adding 200 mM NaCl and incubated for 4 h at 65 °C in 200 mM NaCl followed by incubation for 1 h at 45 °C in 40 mM Tris-HCl, pH 6.5, 10 mM EDTA, and 20 μg of proteinase K solution (Novagen). DNA was then extracted with phenol/chloroform and precipitated. Immunoprecipitated DNA fragments were analyzed by PCR or real-time PCR as described above Real-time PCRs were performed in triplicates, referenced to the inputs, and represented as fold induction over the mock transfected cells, which was considered as the basal binding. Negative controls were also made in the absence of DNA to rule out nonspecific DNA amplifications. In addition, in all experiments that we performed ChIPs on tagged proteins (either HA or Myc), we always carried out a mock ChIP where the corresponding empty vector plasmid had been transfected. The mock sample is precipitated using the same antibody as the rest of the ChIPs. In that case, any amplification coming from the mock IP is regarded as background. In the case that an endogenous protein is ChIPed, then we carried out either a no-antibody control or an anti-tubulin antibody. In ChIP experiments carried out in this study to detect binding of the endogenous p38 SAPK in wild-type MEFs as well as in MEFs cells lacking p38α, we used the same antibody (anti-p38) in both types of cells. PCR primers for individual genes were designed with Primer3 (25) to generate DNA fragments between 100 and 200 base pairs. The sequences used are the followings: c-Fos promoter For: GAGCAGTTCCCGTCAATCC and Rev: GCATTTCGCAGTTCCTGTCT; Cox2 promoter For: CCCCTCTGCTCCCAAATT and Rev: CGCTCACTGCAAGTCGTAT; IL8 promoter, For: GGGCCATCAGTTGCAAATC and Rev: TTCCTTCCGGTGGTTTCTTC; GAPDH promoter For: TACTAGCGGTTTTACGGGCG, and Rev: TCGAACAGGAGGAGCAGAGAGCGA.

Statistical Analysis—Data analysis was carried out with Prism software (GraphPad). Responses among different treatments were analyzed with one-way analysis of variance followed by Bonferroni's multiple comparison test. All experiments were carried out in triplicate. The results are presented as means ± S.D.

RESULTS

The p38 SAPK Is Recruited to Osmostress-induced Genes—To understand how p38 SAPK promotes gene transcription, we focused our studies on c-Fos transcription regulation upon osmotress. The treatment of HeLa cells with 100 mM NaCl provoked a rapid increase of phosphorylated p38 SAPK with a peak between 45′ and 60′ which steadily decreased over time (Fig. 1*A*). p38 SAPK phosphorylation correlated with its accumulation into the nucleus (supplemental Fig. S1). Importantly, osmostress specifically induced p38 phosphorylation and activation, as assessed by monitoring the p38 SAPK downstream target HSP27, with neither affecting the phosphorylation of JNK nor ERK under the experimental conditions tested (Fig. 1*B*). Notably, osmostress-induced p38 activation correlated with c-Fos mRNA accumulation. c-Fos induction was fully prevented by pretreating HeLa cells with the specific p38 inhibitor SB203580. Osmostress did not have any effect on the mRNA levels of the housekeeping gene GAPDH which was used as a control (Fig. 1*C*). These results clearly show that osmostress up-regulates c-Fos transcription in a p38 SAPK-dependent manner.

To assess whether binding of p38 was occurring at stress-regulated genes, we analyzed the recruitment of p38 at c-Fos. We transfected HeLa cells with a HA-tagged p38 and performed a ChIP analysis using an anti-HA monoclonal antibody to specifically pull down p38. Osmostress specifically enhanced

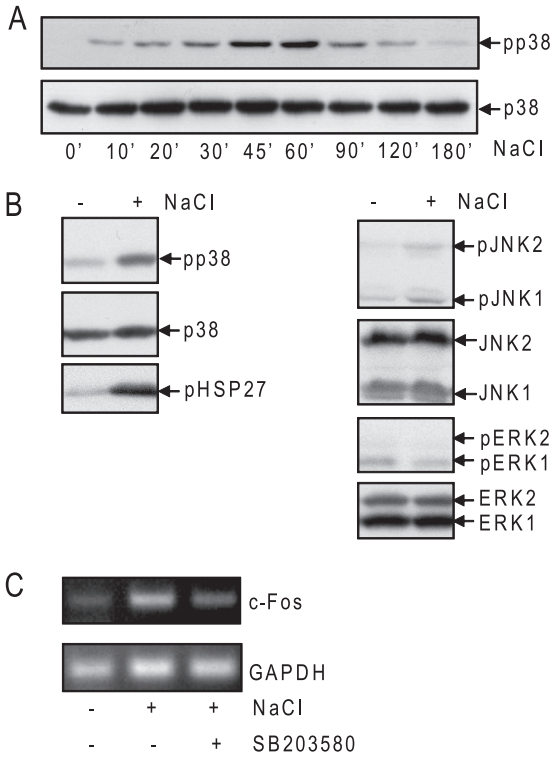

FIGURE 1. **Osmostress specifically phosphorylates p38 SAPK and induces c-Fos transcription.** *A*, HeLa cells were stimulated with 100 mM NaCl for the indicated times and analyzed by Western blotting with the indicated antibodies. *B*, HeLa cells were stimulated with 100 mM NaCl for 45 min and then analyzed by Western blotting with the indicated antibodies. Representative Western blots are shown in *A* and *B*. *C*, HeLa cells were stimulated with 100 mM NaCl for 2 h either in the absence or presence of SB203580. The mRNA levels of c-Fos and GAPDH were analyzed by PCR.

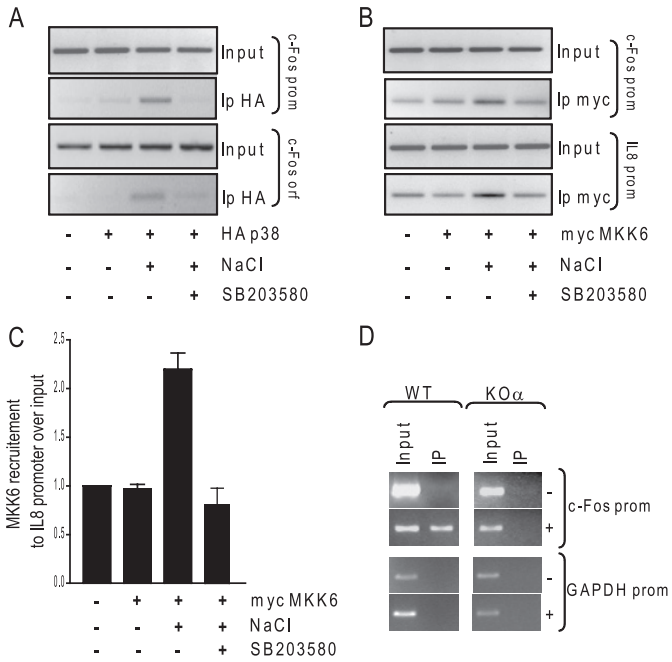

FIGURE 2. **Osmostress induces p38 SAPK and MKK6 recruitment to the c-Fos gene.** *A*, HeLa cells were transfected with pCDNA3–3HA-p38α and treated with 100 mM NaCl for 45 min either in the absence or presence of SB203580. Samples were subjected to ChIP analysis using an anti-HA antibody. *B*, HeLa cells were transfected with pEFmlinkMKK6 or treated with 100 mM NaCl for 45 min either in the absence or presence of SB203580. ChIP analysis was performed using an anti-Myc antibody. *C*, relative MKK6DD recruitment to the IL8 promoter. The values represent the mean $\pm$ S.D. of three independent experiments performed in triplicate. *, statistically significant ($p \leq 0.05$). *D*, wild type and p38α SAPK knockout MEFs were treated with 100 mM NaCl for 45 min and subjected to ChIP assay using an anti-p38α antibody. Immunoprecipitated DNA fragments were analyzed by PCR.

the recruitment of p38 at the c-Fos promoter (Fig. 2*A*). Importantly, the activity of p38 was essential for this process because the addition of the p38 inhibitor SB203580 fully prevented osmostress-induced p38 recruitment at the c-Fos promoter (Fig. 2*A*). In yeast, it has also been shown that the p38 SAPK ortholog Hog1 is recruited at osmostress-induced gene ORFs in addition to promoters (8, 9). Then, we monitored the presence of p38 SAPK at the coding regions of c-Fos and found that p38 was also present at ORFs. Notably, the presence of p38 at the ORF was also dependent on its activity because it was abolished by the p38 inhibitor SB203580 (Fig. 2*A*). Therefore, p38 is recruited to both promoter and ORF regions of stress-responsive genes such as c-Fos.

Previous reports have shown that MEK1 is recruited to ERK dependent genes (26). We then assessed whether MKK6, a MAPKK for p38, is able to bind to stress-responsive genes. We performed ChIP assays monitoring Myc-tagged MKK6 and found that the MAP2K is able to bind to c-Fos and IL8 also in response to high osmolarity. Importantly, binding of MKK6 is also abolished in the presence of SB203580 (Fig. 2*B*). Although this binding was weak, it is consistently observed in several independent experiments (Fig. 2*C*).

To further characterize the binding of p38 at c-Fos in response to osmostress, we treated wild-type and p38α knockout MEFs with osmostress and ChIP p38 using a specific anti-p38α antibody. NaCl cell treatment specifically induced the recruitment of the endogenous p38 SAPK to the mouse c-Fos promoter after 45′ of salt addition on wild-type MEFs but not in p38α knockout MEFs (Fig. 2*D*). p38 was not recruited to the GAPDH housekeeping gene promoter (Fig. 2*D*). Therefore, p38 binds at the c-Fos osmostress-responsive promoter in response to high osmolarity.

Recruitment of RNA PolII at Stress-responsive Promoters Requires p38 Activity—Transcription initiation requires the loading of the RNA Pol II and the basal transcription machinery to promoters. In addition, we have previously shown that p38 and RNA Pol II interact *in vivo* (7), and therefore it might be a possibility that recruiting activated p38 SAPK to stress-induced gene promoters, such as the c-Fos promoter, is a step necessary to induce gene transcription. To confirm that osmostress specifically induced the RNA Pol II recruitment at c-Fos promoter, we performed a ChIP analysis on HeLa cells stressed with NaCl for 15′ and 30′ using a specific RNA Pol II monoclonal antibody. RNA Pol II was not present at the c-Fos promoter under non-stress conditions. Meanwhile, the addition of salt induced the recruitment of the RNA Pol II to the c-Fos promoter after 30′ of osmostress. In contrast to c-Fos, RNA pol II was always present at the promoter of the housekeeping gene GAPDH (Fig. 3*A*). We then assessed whether the presence of RNA Pol II required p38 activity. HeLa cells were pretreated with the p38 inhibitor SB203580 prior to salt addition, and then RNA Pol II recruitment to the c-Fos promoter was followed by ChIP. Binding of RNA Pol II was completely abolished in cells with inhibited p38 (Fig. 3*B*). Altogether, RNA Pol II recruitment at the

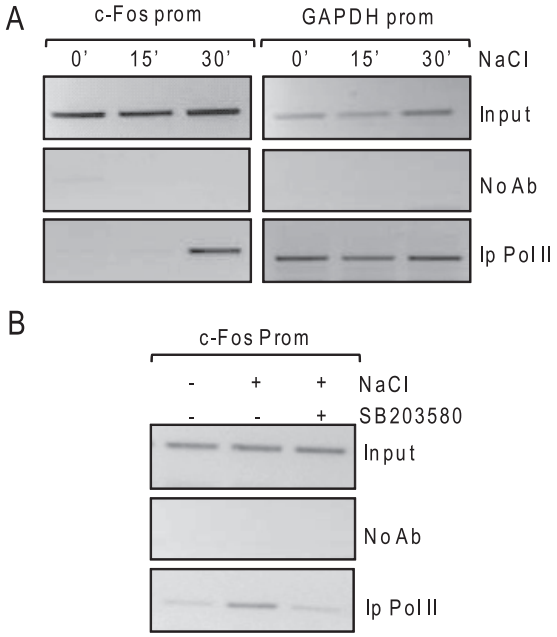

FIGURE 3. **Osmostress induces RNA Pol II recruitment at the c-Fos promoter.** *A*, HeLa cells were treated with 100 mM NaCl for the indicated times. Binding of RNA Pol II to the c-Fos promoter was determined by ChIP. *B*, cells were treated with 100 mM NaCl for 30 min and subjected to ChIP analysis using anti-RNA Pol II antibody in the absence or presence of 10 μM SB203580. Immunoprecipitated DNA fragments were analyzed by PCR and ethidium bromide DNA-agarose staining.

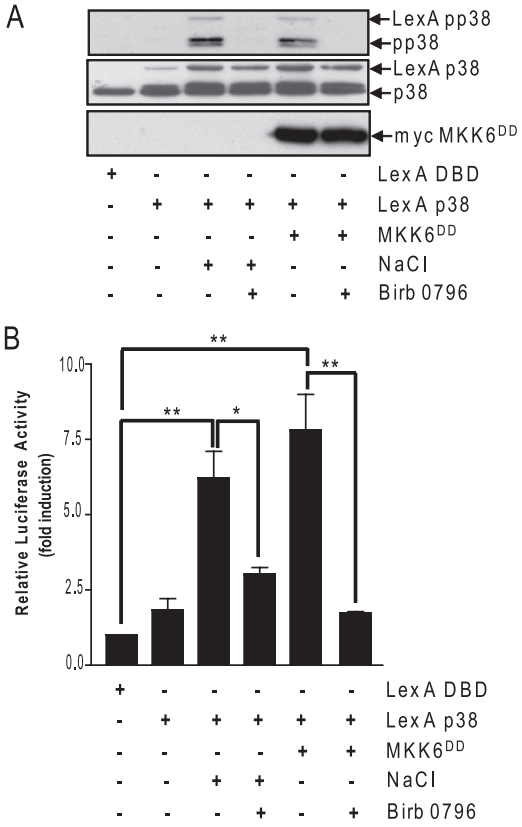

FIGURE 4. **Promoter-bound p38 SAPK drives gene transcription.** *A*, HeLa cells were transfected with the shown plasmids. Cells were treated with 100 mM NaCl for 45 min in the absence or presence of BIRB 0796. The inhibitor was added 24 h before harvesting. Lysates were analyzed by Western blotting with the indicated antibodies. Representative Western blots are shown. *B*, HeLa cells were transfected with the shown plasmids. Cells were treated with 100 mM NaCl in the absence or presence of BIRB 0796 for 24 h before harvesting. The luciferase reporter activity was determined and corrected by the total amount of protein and represented as fold induction over the pCDNA3-LexA-DBD signal, which was considered as 1. The values represent the mean ± S.D. of three independent experiments performed in triplicate. **, statistically significant ($p \leq 0.01$) and *, statistically significant ($p \leq 0.05$).

osmostress-induced c-Fos promoter correlates with transcription activation and accumulation of the c-Fos mRNA in a p38-dependent manner.

Promoter-bound p38 SAPK Drives Gene Transcription—We next addressed whether tethering p38 to a promoter was sufficient to stimulate gene expression. We fused full-length p38 to the LexA DNA binding domain. The LexA-p38 fusion protein was then co-transfected in HeLa cells in the presence of an artificial gene promoter carrying 6× LexA DNA binding sites upstream of the Luciferase reporter. LexA-p38 activation was induced either by co-transfecting the active p38 MAPKK $MKK6^{DD}$ or by treating cells with 100 mM NaCl. Ectopically expression of $MKK6^{DD}$ and osmostress specifically induced the phosphorylation of both the endogenous p38 SAPK and the LexA-p38 SAPK similarly (Fig, 4*A*) without either affecting the phosphorylation of JNK or ERK (data not shown). Tethering LexA-p38 at the reporter gene did not produce a significant increase in the luciferase reporter activity, which indicates that binding of p38 to a gene promoter is not sufficient to activate gene transcription. However, both $MKK6^{DD}$ and osmostress induced the transcriptional activity of the LexA-p38 fusion protein (Fig. 4). Therefore, once recruited at the promoter, active p38 is able to induce gene expression. Correspondingly, impairing p38 SAPK activation by addition of the p38 inhibitor (Birb 796) prevented the LexA-Luc gene promoter activation (Fig. 4). Thus, active promoter-bound SAPK kinase is sufficient to drive gene transcription.

Recruitment of p38 at the c-Fos Promoter Requires the Transcription Factor Elk1—Because p38 does not have a defined DNA binding domain, its binding to gene promoters might not be direct. This prompted us to ask how p38 was recruited to the c-Fos promoter. It has been shown that the c-Fos promoter can be regulated by several transcription factors which respond to a broad range of cell stimuli (27). Among those transcription factors, the Elk1 transcription factor had previously been shown to be targeted by MAPKs (28, 29). Thus we first asked whether p38 SAPK and Elk1 could interact with each other. We co-transfected HeLa cells with HA-tagged p38 and Flag-tagged Elk1 and performed two immunoprecipitations either with anti-HA or anti-Flag specific monoclonal antibodies. The transcription factor Elk1 specifically co-immunoprecipitated with p38 MAPK and the p38 MAPK co-immunoprecipitated with Elk1 indicating that both proteins can interact with each other *in vivo* (Fig. 5*A*). Such an interaction suggested that Elk1 might be a substrate for p38. To address this question we co-transfected HeLa cells with HA-tagged p38 and Flag-tagged Elk1 in the absence or presence of the constitutive active p38 MAPKK $MKK6^{DD}$. Elk1 phosphorylation status was analyzed by following the change in the electrophoretic mobility of the Elk1 protein on a SDS/PAGE gel by Western blot using the anti-Flag antibody. Indeed p38 SAPK activation produced a slower migrating band corresponding to phosphorylated Elk1 protein. Notably, the phosphorylation of Elk1 was fully blocked by treat-

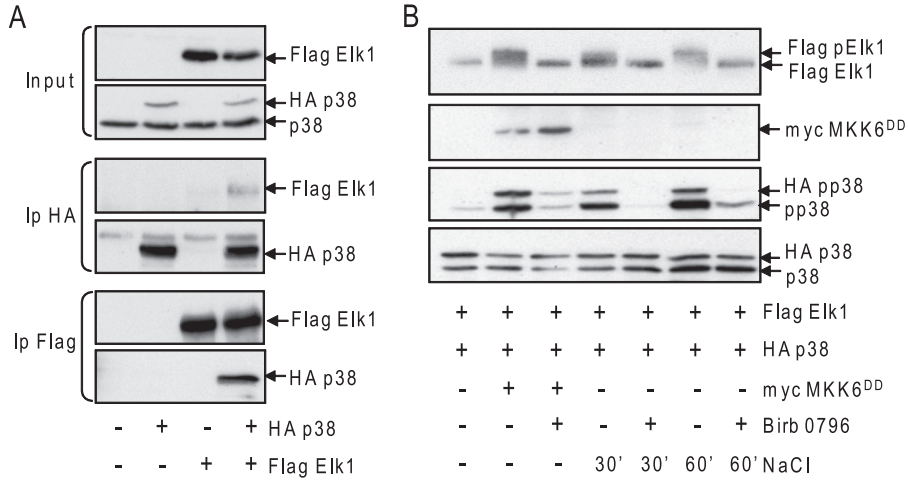

FIGURE 5. **p38 SAPK targets and interacts with the transcription factor Elk1.** *A*, HeLa cells were co-transfected with pELK1-Flag and pCDNA3–3HA-p38α. Cell lysates were immunoprecipitated either with anti-HA or anti-Flag antibodies and analyzed by Western blotting with the indicated antibodies. *B*, HeLa cells were transfected with pELK1-Flag, pCDNA3–3HA-p38α, and pEFmlinkMKK6^{DD}. When indicated, cells were treated with 100 mM NaCl either in the presence or the absence of BIRB 0796. Cleared cell lysates were subsequently analyzed by Western blotting with the indicated antibodies. Representative Western blots are shown in *A* and *B*.

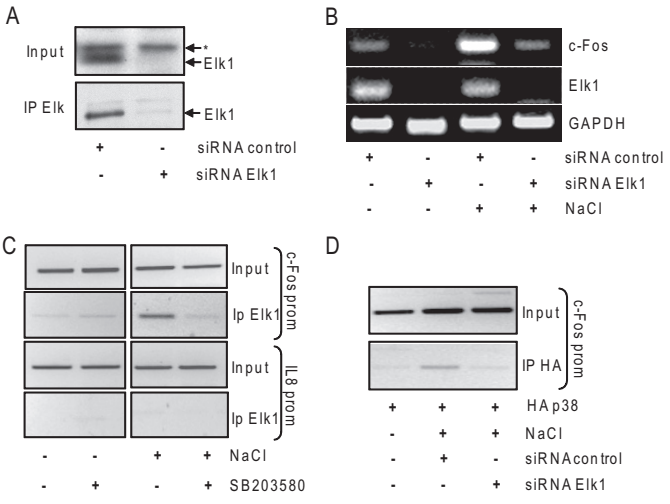

FIGURE 6. **p38 SAPK recruitment at the c-Fos promoter requires the transcription factor Elk1.** *A*, HeLa cells were transfected with either control or Elk1 siRNAs and the cell lysates were inmunoprecipitated with anti-Elk1 antibody. Both inputs and immunoprecipitates were analyzed by Western blotting with anti-Elk1 antibody. * marks a nonspecific band. *B*, HeLa cells were transfected with the indicated siRNAs and treated with 100 mM NaCl for 2 h. The mRNA levels of c-Fos, Elk1, and GAPDH were analyzed by PCR. *C*, HeLa cells were treated with 100 mM NaCl for 60 min either in the presence or absence SB203580 and subjected to ChIP assay using an anti-Elk1 antibody. *D*, HeLa cells were transfected with pCDNA3–3HA-p38α along with control or Elk1 siRNAs. Cells were treated with 100 mM NaCl for 60 min and subjected to ChIP assay with anti-HA antibody. Immunoprecipitated DNA fragments were subjected to PCR analysis.

ing the cells with a specific p38 inhibitor (Birb 0796). We then assessed whether osmostress also induced Elk1 phosphorylation. Cells were treated for 30 or 60 min with 100 mM NaCl in the presence or absence of the p38 inhibitor (Birb 0796), and Elk1 was monitored as before. As shown in Fig. 5*B*, an osmotic shock was also able to induce the phosphorylation of Elk1 protein in a p38-dependent manner.

The fact that p38 and the transcription factor Elk interact with each other and that Elk1 is a p38 substrate *in vivo* prompted us to ask whether this transcription factor might be responsible for p38 recruitment to the c-Fos promoter in response to osmostress. To address this issue we knocked down endogenous Elk1 protein levels with specifically designed siRNAs in HeLa cells. Elk1 protein levels were then analyzed by Western blot and immunoprecipitation. Elk1 protein levels were dramatically reduced by the specific siRNA whereas a nonspecific siRNA targeting the GFP protein used as a control did not have any effect on Elk1 protein levels (Fig. 6*A*). To assess the relevance of Elk1 on osmostress induction of c-Fos levels, HeLa cells were transfected with control GFP siRNAs and specific Elk1 siRNAs before being stressed with 100 mM NaCl. After treatments, total RNA was extracted, converted to cDNA, and analyzed by PCR. As expected, Elk1 mRNA levels were strongly diminished by the specific Elk1 siRNA and were not affected by the GFP control siRNA. Notably knocking down Elk1 clearly affected both c-Fos basal transcription and the induction of c-Fos mRNA upon osmostress (Fig. 6*B*). The fact that c-Fos expression was not totally suppressed by knocking down Elk1 suggests that an Elk1-independent mechanism may also be taking place. However, Elk1 plays a crucial role on c-Fos transcription activation upon osmostress. Of note, neither the Elk siRNA nor the GFP siRNA used as a control had any effect on the expression of the GAPDH housekeeping gene (Fig. 6*B*).

Taking into account that up-regulation of c-Fos upon osmostress required p38 activity and that Elk1 was targeted by the SAPK, we assessed the possibility that Elk1 recruitment to the c-Fos promoter was regulated by p38. Thus, we analyzed the recruitment by ChIP of the endogenous Elk1 transcription factor to the c-Fos promoter in osmostress-treated HeLa cells in the absence or the presence of a p38 inhibitor. The recruitment of the transcription factor Elk1 to the c-Fos promoter was enhanced upon osmostress in a p38-dependent manner (Fig. 6*C*). In contrast, Elk1 was not recruited to the p38-dependent gene IL8, which is not regulated by Elk1 (Fig. 6*C*). These observations suggested that Elk1 regulation by p38 is critical for c-Fos control and also posed the question whether regulated binding of Elk1 could be fundamental for p38 recruitment to the c-Fos promoter upon osmostress. To assess this possibility, we co-transfected HeLa cells with HA-tagged p38 in the presence of either Elk1 siRNAs or the control GFP siRNA and performed a ChIP analysis using a specific anti-HA monoclonal antibody. ChIP analyses showed that knocking down Elk1 protein levels strongly impaired HA-p38 recruitment to the c-Fos promoter (Fig. 6*D*). Correspondingly, the control GFP siRNA had no effect on stress-induced p38 recruitment to the c-Fos promoter (Fig. 6*D*). Taken together, we conclude that there is interdependent binding of the transcription factor and the

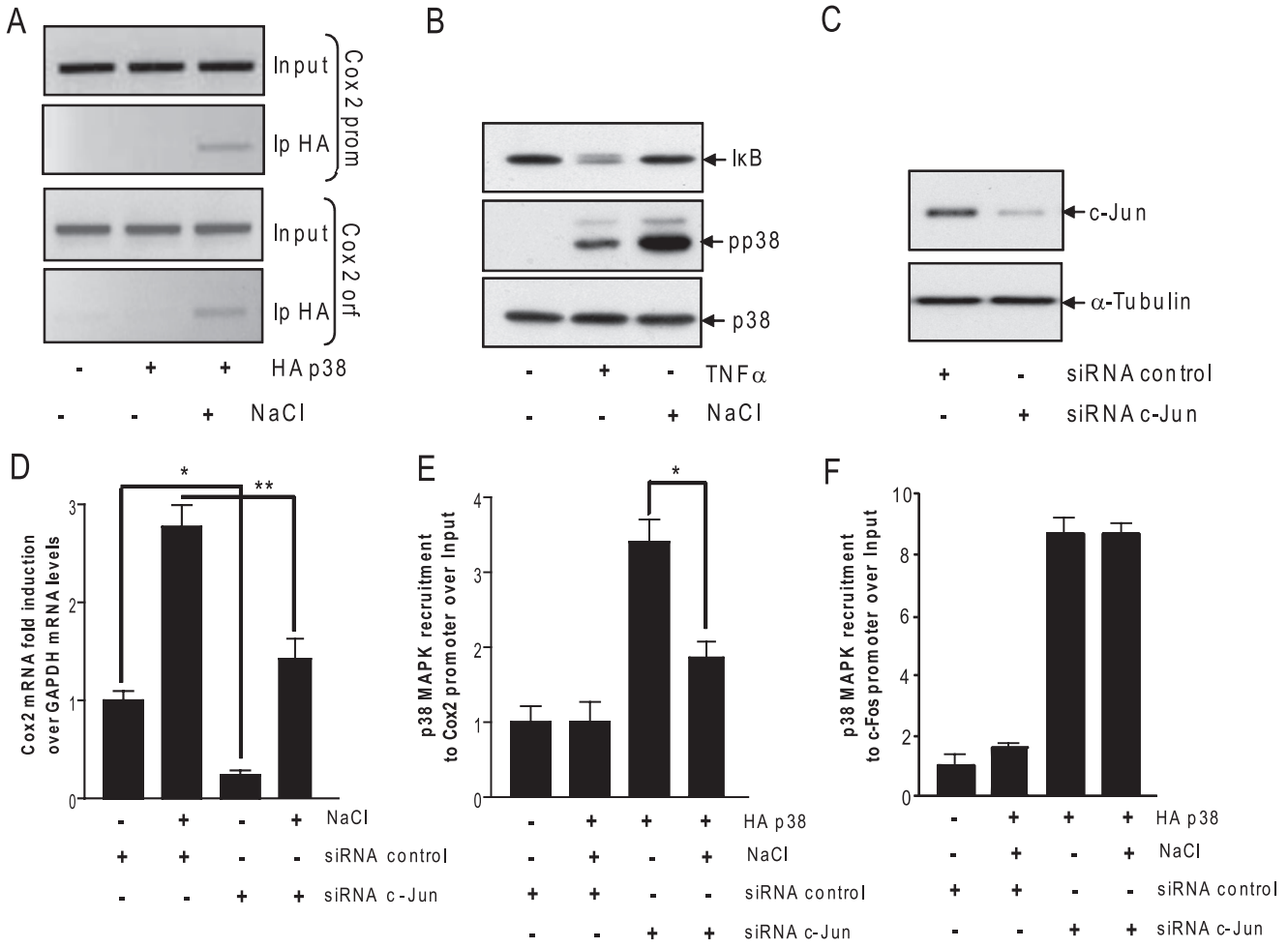

FIGURE 7. **p38 SAPK recruitment to the Cox2 genes requires AP-1 function.** *A*, HeLa cells were transfected with pCDNA3–3HA-p38α, stimulated with 100 mM NaCl for 60 min, and subjected to ChIP assay using an anti-HA antibody. Immunoprecipitated DNA fragments were subjected to PCR. *B*, HeLa cells were stimulated with 0.1 μg/ml TNFα for 5 min or 100 mM NaCl for 30 min. Cell lysates were subsequently analyzed by Western blotting with the indicated antibodies. *C*, HeLa cells were transfected with either control or c-Jun siRNAs. Cell lysates were subsequently analyzed by Western blotting with the indicated antibodies. Representative Western blot are shown in *B* and *C*. *D*, HeLa cells were transfected as in *C* and treated with 100 mM NaCl for 2 h. The mRNA levels of Cox2 were analyzed by real-time PCR, corrected by the GAPDH mRNA levels, and represented as fold induction. *E*, HeLa cells were co-transfected with pCDNA3–3HA-p38α along with control or c-Jun siRNAs. After the addition of 100 mM NaCl for 60 min, cells were subjected to ChIP analysis with an anti-HA antibody. Immunoprecipitated DNA fragments were analyzed by real-time PCR. Fold induction of p38α SAPK relative enrichment over the input to the Cox2 promoter is shown. Mock transfected cells were taken as 1. *F*, Hela cells were treated and processed as in *E*. Fold induction of p38α SAPK relative enrichment over the input to the c-Fos promoter is shown. Means and standard errors of a representative experiment performed in triplicate are shown in *D*, *E*, and *F*. **, statistically significant ($p \leq 0.01$) and *, statistically significant ($p \leq 0.05$).

SAPK at the c-Fos promoter in response to stress and that the transcription factor Elk1 is acting as an anchor for p38 SAPK recruitment to chromatin in response to osmostress.

Recruitment of p38 at Cox2 Promoter Requires the Transcription Factor c-Jun—The network of genes induced by p38 in response to osmostress is not limited to genes regulated by Elk1. For instance, in response to osmostress, Cox2 is strongly induced by p38 (17), and this induction is independent of the presence of Elk1 (data not shown). Expression of the Cox2 gene has been reported to be under the control of the transcription factors NFκB and AP1 (30). We first tested by ChIP whether p38 was recruited at Cox2 in response to osmostress and found that as in c-Fos, osmostress induced p38 recruitment at the Cox2 promoter and ORF (Fig. 7*A*). We then studied which transcription factor was relevant to induce Cox2 upon osmostress. We found that osmostress was not able to induce IκB degradation, and therefore NFκB remained inactive for the conditions used in this study (Fig. 7*B*). Having ruled out the involvement of NFκB on osmostress-induced Cox2 gene regulation, we decided to block AP1 function by knocking down c-Jun protein levels. Specifically designed siRNAs targeting c-Jun effectively diminished the endogenous levels of c-Jun protein (Fig. 7*C*), which led to a reduced Cox2 mRNA accumulation after salt addition (Fig. 7*D*). We then assessed whether knocking down c-Jun impaired recruitment of p38 in response to osmostress. ChIP analysis showed that indeed knocking down c-Jun protein levels strongly affected the recruitment of p38 at Cox2 gene (Fig. 7*E*). Notably, p38 SAPK recruitment to the c-Fos promoter was not affected in the absence of c-Jun (Fig. 7*F*). Therefore, in response to osmostress, different transcription factors depending on the promoter context target the p38 SAPK to chromatin.

Recruitment of Active p38 to Chromatin Is a General Mechanism—p38 SAPK is activated upon osmostress and in response to a great variety of extracellular stimuli (31). There-

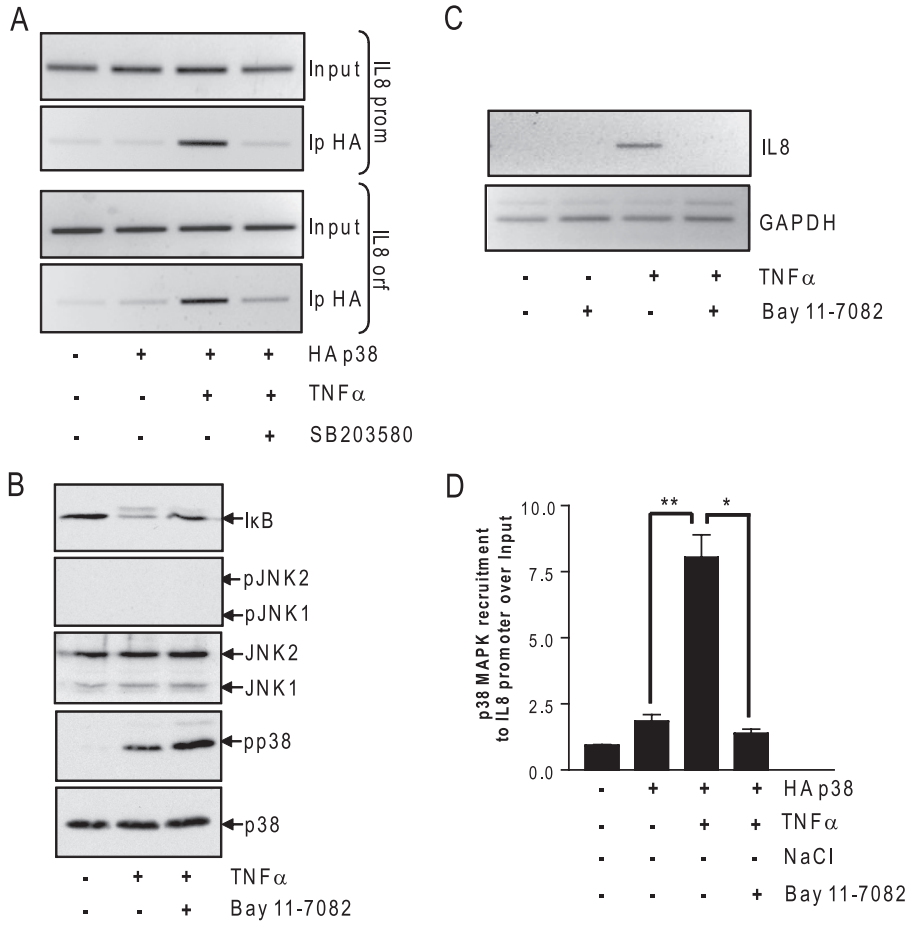

FIGURE 8. **p38 SAPK recruitment to the IL8 gene requires NF-κB function.** *A*, HeLa cells were transfected with pCDNA3–3HA-p38α, stimulated with 100 ng/ml TNFα for 60 min in the presence or absence of SB203580 and subjected to ChIP assay using an anti-HA antibody. Immunoprecipitated DNA fragments were subjected to PCR analysis. *B*, HeLa cells were stimulated with 100 ng/ml TNFα for 5 min. NFκB signaling was blocked by pretreating cells with 50 μM Bay 11-7082 1 h prior to TNFα addition. Cleared cell lysates were subsequently analyzed by Western blotting with the indicated antibodies. Representative Western blots are shown. *C*, HeLa cells were treated with 100 ng/ml TNFα for 2 h in the absence or presence of 50 μM Bay 11-7082. The mRNA levels of IL8 and GAPDH were analyzed by PCR. *D*, HeLa cells were transfected with pCDNA3–3HA-p38α and treated with 100 ng/ml TNFα for 45 min or in the absence or presence of 50 μM Bay 11-7082. Cell lysates were subjected to ChIP analysis with an anti-HA antibody. Immunoprecipitated DNA fragments were analyzed by real-time PCR. Fold induction of p38α SAPK relative enrichment over the input is shown. Mock transfected cells were taken as 1. The values represent the mean ± S.D. of two independent experiments performed in triplicate. **, statistically significant ($p \leq 0.01$) and *, statistically significant ($p \leq 0.05$).

fore, we decided to test whether stimuli other than osmostress were inducing recruitment of the SAPK to p38-responsive genes. Initially, we tested whether anisomycin was able to induce recruitment of p38. Anisomycin induces both p38 phosphorylation and nuclear accumulation with kinetics similar to osmostress (supplemental Fig. S2, *A* and *B*). c-Fos and Cox2 genes are known to be up-regulated in response to anisomycin in a p38 manner (17). We then assessed binding of p38 by ChIP at those genes in response to anisomycin. As shown in supplemental Fig. S2*C*, p38 was recruited to c-Fos and Cox2 genes, both promoter and ORFs, in response to anisomycin. Of note, inhibition of p38 activity prevented binding of p38 at those genes (supplemental Fig. S2*C*). In addition, anisomycin specifically induced the recruitment of the endogenous p38 SAPK to the mouse c-Fos promoter on wild-type MEFs but not in p38α knockout MEFs. No p38 recruitment was observed on the GAPDH housekeeping gene promoter (supplemental Fig. S2*D*). Therefore, activation of p38 by different stimuli leads to recruitment of p38 to chromatin.

It is known that gene regulation by p38 in response to different stimuli is controlled by selective transcription factors. For instance, the pro-inflammatory cytokine TNFα is a strong activator of p38 and NFκB signaling (32). Thus, we decided to assess whether activation of pro-inflammatory genes controlled by p38 required the NFκB-mediated recruitment of p38. First, we analyzed binding of p38 at IL8 in response to TNFα. ChIP analyses have shown that indeed in response to the cytokine, active p38 is recruited at the IL8 promoter and ORF at the concentrations of 10 and 100 ng/ml of TNFα (supplemental Fig. S3 and Fig. 8). Notably, the observed p38 recruitment is specific because an anti-tubulin antibody did not produce a significant enrichment of the signal (supplemental Fig. S3). NFκB function can be specifically blocked by the addition of the IKK chemical inhibitor Bay 11-7082 (33). The addition of this compound prior to the addition of TNFα prevented IκB phosphorylation and degradation without affecting p38 SAPK activation (Fig. 8*B*). The presence of the inhibitor prevented the NFκB nuclear translocation, and transcriptional up-regulation of NFκB target genes such as IL8 was impaired (Fig. 8*C*). Next, we addressed whether NFκB activation was needed for p38 recruitment to the IL8 gene promoter. A ChIP analysis showed that TNFα-induced binding of p38 was prevented by the chemical compound Bay 11-7082 (Fig. 8*D*). Altogether, these observations indicate that recruitment of p38 to stress-induced gene promoters is mediated by its association to different transcription factors, and this represents a broad molecular mechanism employed by this SAPK to regulate gene expression.

DISCUSSION

In yeast, activation of the SAPK Hog1, the homolog of the mammalian p38 SAPKs, is critical to modulate gene expression in response to osmostress. Interestingly, Hog1 associates to chromatin, which serves to regulate gene expression at initiation and during elongation (8, 34). Studies on Hog1 have served to link signaling kinases to chromatin. There is a strong structural and functional preservation of SAPKs and adaptive

responses from yeast to mammals (12) and thus, common gene regulatory mechanisms might be conserved between yeast and mammals.

Here, we have analyzed the basis for chromatin association of p38. We have found that in response to different stimuli that induces p38 activation and nuclear accumulation (osmostress, anisomycin, and TNFα) p38 is recruited to chromatin. Of note, inhibition of the kinase activity of p38 prevents its association to chromatin albeit do not prevent its nuclear accumulation (data not shown). Association of members of the signaling cascade is not restricted to p38, because MKK6 is also present when the SAPK is present there. Therefore, it could be that an active module of signaling that comprises the MAPK and the MAPKK is required to mediate gene expression regulation. A similar scenario has been described for ERK1 and its association to chromatin together with MEKK1 (26). The relevance of the association of the p38 SAPK to chromatin is confirmed by the ability of p38 to induce gene expression of a heterologous gene promoter when tethered to a LexA DNA binding domain. Of note, induction of gene expression only occurred when the SAPK was active either in response to stress or in the presence of a constitutive active MKK6 ($MKK6^{DD}$). Therefore, as previously shown in *Saccharomyces cerevisae*, recruitment of activated p38 SAPK to gene promoters contributes to osmostress-induced gene transcription initiation by facilitating the recruitment of the RNA pol II holoenzyme.

We have also observed the presence of the p38 SAPK at the coding regions of the c-Fos, IL8, and Cox2 genes, clearly suggesting that p38 SAPK might be traveling along with the RNA pol II with the nascent mRNA elongating machinery in a similar way as described in the yeast (9). Therefore, the results reported here show that p38 SAPK activity is not just necessary for gene promoter activation but might also be necessary for the nascent mRNA elongation of p38-dependent genes.

The lack of a defined DNA binding domain in p38 and the resemblance to the yeast SAPK suggested that the association of the SAPK to chromatin might be mediated by its association to transcription factors. The c-Fos promoter gene is regulated by a broad range of basal and inducible transcription factors in response to different stimuli (27). Of note, we have found that in response to osmostress, Elk1 mediates gene induction. Elk1 has been shown to be directly phosphorylated at multiple sites by mitogenic-induced ERKs as well as stress-activated JNKs and p38 SAPKs (28, 29). This evidence prompted us to study the relationship between p38 SAPK and Elk1 and how these proteins contributed to c-Fos gene regulation. Both proteins were able to interact with each other when expressed in HeLa cells, and Elk1 was phosphorylated *in vivo* by p38. Elk1 protein is essential to induce c-Fos and notably, p38 recruitment to the c-Fos promoter is impaired in the absence of Elk1 protein. Taken together, these observations suggest that osmostress-induced p38 phosphorylates the transcription factor Elk1, which can then bind to the c-Fos serum response element (SRF) along with activated p38. Binding of Elk1 and p38 seem to be interdependent because Elk1 cannot bind c-Fos when p38 is inactive and p38 cannot bind c-Fos in the absence of Elk1. This observation resembles the interdependency observed in yeast between Hog1 SAPK and the transcription factor Hot1 (7).

To address whether the recruitment of p38 via a transcription factor was a general mechanism, we extended our study to other genes up-regulated upon osmostress in a p38-dependent manner such as Cox2 (35). The Cox2 gene promoter can be activated by both NFκB and AP1 transcription factors (30). However osmostress does not activate the NFkB signaling, whereas p38 activation has been shown to directly up-regulate AP1 transcription activity (36, 37). We have found that specifically targeting the c-Jun protein with siRNAs compromised p38 recruitment to the Cox2 promoter.

Activation of p38 occurs in response to several stimuli. To determine whether recruitment of the SAPK to responsive genes via transcription factors was not limited to osmostress we analyzed binding of p38 at the IL8 gene promoter in response to TNFα (38). TNFα strongly mediates NFκB and p38 MAPK signaling (32) and induces the p38 MAPK recruitment to the IL8 promoter. Retaining NFκB inactive in the cytoplasm by blocking IKK-mediated IkB phosphorylation and proteosome degradation prevented the recruitment of the p38 SAPK to the IL8 promoter and completely impaired transcription activation of the IL8 gene. Taken together, anchoring activated p38 SAPK to specific stress-induced gene promoters by transcription factors appears to be a broad mechanism used by this SAPK to regulate gene expression.

Our results point out that transcription factor-mediated recruitment of p38 is critical for RNA Pol II recruitment and transcription initiation. Other MAPKs have been found to associate to promoters. For instance, progesterone-induced transcription initiation from the mouse mammary tumor virus (MMTV) requires the recruitment of a complex comprising the progesterone transcription factor/receptor, the activated Erk1 and the MAPK downstream kinase Msk1. These events correlated with H3 Ser-10 phosphorylation, eviction of the transcriptional repressor HP1γ, and subsequent chromatin remodeling involving H2A and H2B displacement, PCAF-mediated H3 Lys-14 acetylation and BAF complex anchoring to chromatin (13, 39). Similarly, p38 is targeted to specific muscle promoters Myog and Ckm during skeletal muscle differentiation. The association of p38 at these promoters seems to be important for recruiting the SWI-SNIF complex (16). A recent study from Lawrence *et al.* (15) has shown that active MAPKs are quickly loaded to the insulin promoter where their kinase activity modulates the acetylation state of H3 and H4 core histones. Thus, chromatin association of signaling kinases through transcription factors might be important for association of basal transcription machinery, chromatin modifying activities or remodeling factors. Taken together SAPK recruitment to target genes appears to be a broad mechanism to regulate transcription that has been preserved from yeast to mammals.

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