Transcriptional Regulation of EGR-1 by the Interleukin-1-JNK-MKK7-c-Jun Pathway*

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The proinflammatory cytokine interleukin (IL)-1 activates several hundred genes within the same cell. This occurs in part by activation of the MKK7-JNK-c-Jun signaling pathway whose precise role in the regulation of individual inflammatory genes is still incompletely understood. To identify the genes that are under specific control of activated JNK, we used a JNK-MKK7 fusion protein. Genome-wide microarray analysis revealed EGR-1 as the transcript that was most strongly induced by JNK-MKK7. IL-1-stimulated EGR-1 mRNA and protein expression were impaired in cells lacking JNK or c-Jun. Transcriptional activation of the EGR-1 promoter by JNK-MKK7 or by IL-1 required a single upstream AP-1 site and three distal serum-response elements (SRE). Reconstitution experiments in c-Jun-deficient cells revealed that c-Jun is required for EGR-1 transcription through both the AP-1 site and the distal SREs. By chromatin immunoprecipitation analysis, we found IL-1-inducible recruitment of c-Jun to the AP-1 site and to the region containing the three distal SREs. These experiments suggest that c-Jun plays a dual role in EGR-1 transcription. It directly binds to the AP-1 element, and at the same time it is essential for promoter activation through the three distal SREs by an indirect unknown mechanism. As predicted by TRANSFAC analysis and verified by ChIP experiments, IL-1-induced EGR-1 protein binds to the promoter regions of inflammatory mediators such as IL-6, IL-8, and CCL2. Furthermore, short interfering RNA-mediated suppression of EGR-1 partially suppresses IL-1-inducible transcription of IL-8, IL-6, and CCL2. In summary, we provide novel evidence for a complex c-Jun-mediated mechanism that is essential for inducible EGR-1 expression. We identify this pathway as a previously unrecognized part of a multistep gene regulatory network that controls cytokine and chemokine expression via the IL-1-MKK7-JNK-c-Jun-EGR-1 pathway.

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The inflammatory response is characterized by the coordinated induction or repression of a large number of inflammatory genes. These genes control leukocyte infiltration, innate or adaptive immune responses, and tissue remodeling. Prototypic proinflammatory cytokines such as interleukin (IL)3-1 or tumor necrosis factor (TNF) can activate or repress several hundred genes within the same cell (1). They mediate their gene-regulatory effects in part by activation of MAPK signaling pathways such as JNK, p38, and ERK. These pathways in turn activate a large number of transcriptional regulators that fall into different classes, i.e. AP-1, etc., C/EBP, and others. Although it is well known that JNKs phosphorylate and activate AP-1 components such as c-Jun or ATF-2, the precise role of individual transcription factors in regulation of individual inflammatory genes is comparably less understood. Moreover, most inflammatory genes bind several transcription factors simultaneously, the mRNA expression of which is themselves regulated at the transcriptional level. For example, the c-Jun promoter is regulated by a c-Jun-ATF-2 dimer in an autoregulated fashion (2, 3). But it is also regulated by lipopolysaccharide-induced p38-mediated activation of the transcription factor Me2c (4). Hence, an emerging picture is that the initial inflammatory gene expression program is a result of complex mutual control of transcription factor networks and their target genes.

A comprehensive understanding of these networks requires the identification of all target genes in a given pathway, a task that is still far from complete. This is exemplified by studies on the JNK-c-Jun pathway. Although JNKs are activated by almost every proinflammatory or stressful condition, their downstream target genes are poorly defined. This also applies to the downstream effector of JNK, c-Jun. One way of studying the role of JNK in gene regulation is selective activation of the pathway by ectopically expressed gain-of-function mutants. We have previously used an active mutant of MKK7, the only specific upstream activator of JNK (5). However, this mutant is a relatively weak activator of JNK and may thus not mimic the strong increase of activity of endogenous JNK that is found in response to proinflammatory cytokines such as IL-1. Hence, we

3 The abbreviations used are: IL, interleukin; AP-1, activating protein-1; SRE, serum-response element; MAP, mitogen-activated protein; TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; RNAI, RNA interference; siRNA, short interfering RNA; RT, reverse transcription; PBS, phosphate-buffered saline; TCF, ternary complex factor; TBS, Tris-buffered saline; Mef, murine embryonic fibroblast; kd, kinase dead.
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extended these studies using ectopically expressed fusion proteins of MKK7 and JNK. As shown previously, these fusion proteins display strong catalytic activity of JNK (6–9).

In this study we used this approach to identify novel downstream effectors of the JNK pathway. We found that JNK and c-Jun are required for induction of the stress-induced transcription factor encoded by the early growth response (EGR) gene-1. EGR-1 is a prototypic member of the family of zinc finger transactivators that consist of immediate early genes induced by a number of stimuli, including growth factors, mitogens, synaptic activity, nerve and tissue injury, ischemia, and apoptotic signals, in distinct cell types (10–17). EGR-1 was originally identified as an immediate-early gene rapidly activated by serum in fibroblasts (11) and by nerve growth factor in PC12 cells (10). Subsequently, EGR-1 has been shown to play a crucial role in cell growth, differentiation, transformation, cell survival, and cell death (18). More recently, its role in inflammatory processes, in particular in vascular injury, has been closely investigated. It is induced in response to a variety of proinflammatory conditions (16, 17, 19–21). In turn, EGR-1 expression induces the sustained expression of other inflammatory mediators, such as IL-6, MCP-1/CCL2 (22), and TNF-α (23), possibly allowing it to act as a “master switch” (reviewed in Ref. 12). Correspondingly, functionally significant EGR-1 DNA-binding motifs have been described within promoters of genes activated in inflammatory and proliferative cascades, including TNF-α (23), ICAM-1 (24), and IL-2 (25, 26). The EGR-1 promoter has been characterized in detail (27, 28), and its regulation is considered to occur mainly through a cluster of serum-response elements (SRE) (19) and in addition through a proximal cyclic AMP-response element (21, 28). A number of studies have shown that EGR-1 is regulated by ERK-dependent phosphorylation of Elk-1 (19, 27, 29, 30).

Here, we show that EGR-1 expression is also regulated by an additional signaling pathway. We identify c-Jun as an essential effector in EGR-1 transcriptional regulation and thus establish a novel link between the stress-activated JNK-c-Jun signaling pathway and EGR-1. We also show that c-Jun activates EGR-1 by its regulation is considered to occur mainly through a cluster of serum-response elements (SRE) (19) and in addition through a proximal cyclic AMP-response element (21, 28). A number of studies have shown that EGR-1 is regulated by ERK-dependent phosphorylation of Elk-1 (19, 27, 29, 30).

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EXPERIMENTAL PROCEDURES

Cells and Materials—HEK293IL-1R cells and human epidermal carcinoma KB cells have been described (31, 32). The following immortalized murine embryonic fibroblast lines were used as described previously (33, 34): wild type Mefs, Mefs deficient for JNK1/2 or for c-Jun, or Mefs isolated from c-Jun (S63A/S73A) mutant mice. Unless stated otherwise, cells were cultured in Dulbecco’s modified Eagle’s medium, complemented with 10% fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin. Antibodies against the following proteins or peptides were used in this study: EGR-1 (sc-110), c-Jun (sc-1694), HDAC3 (sc-11417), and Elk-1 (sc-355) from Santa Cruz Biotechnology; phospho-Ser-63 c-Jun and phospho-JNK antibodies from Cell Signaling; anti Myc (9E10) from Roche Applied Science; rabbit IgG from Rockland. Chicken antibody 681 against JNK was described in Ref. 35. PD98059 was from Alexis; SP600125 was from Tocris. Horseradish peroxidase-coupled secondary antibodies and protein A/G-Sepharose beads were from GE Healthcare. Human recombinant IL-1α was produced as described previously (36). Other reagents were from Sigma or Fisher and were of analytical grade or better.

Plasmids and Transfections—The expression plasmids pcDNA3-Myc-SAPKβ-MKK7 and the kinase dead (kd) mutant pcDNA3-Myc-SAPKβ-MKK7 kd have been described (7). SAPKβ is also known as JNK3. pEgr-1 (1.2 kb) luc (2860), pEgr-1 (0.5 kb) luc (2862), pEgr-1 (0.2 kb) luc (2864), pEgr-1 (0.15 kb) luc (2865) have been described (19). pUCH13.3 IL-6 promoter luc (37) and CCL2 promoter luc (ccl2 2.8/2 luc) (1) have been described. A 315-nucleotide fragment of the human IL-6 promoter (nucleotides −303 to +12, Ensembl ENSG00000136244) was amplified from genomic DNA by PCR to generate the IL-6 promoter-driven luciferase reporter plasmid pUHC13.3 IL-6 promoter luc. The fragment was cloned into the Xhol/Sall sites of plasmid pUHC13.3 using the primers (sense, 5'-agctcgagtcgcaagcatgcaagtctgc-3'; antisense, 5'--gcgcgtcgaccgagggca-gaag-3' as described in Ref. 37). SV40 β-galactosidase was from Promega.

pRSV-c-Jun and p-c-JunTAM67 were gifts from Peter Angel and Stephan Ludwig, respectively. HEK293IL-1R (plated at 4 × 10⁵ per well of 6-well plates) were transfected with a total of 3.5−5.5 µg of DNA by the calcium phosphate method. c-Jun-deficient cells (plated at 1 × 10⁶ in 6 wells) were transfected with 5.5 µg of total DNA and 11 µl of JETPEI (Biomol). DNA amount was kept constant in each transfection by adding empty vector pCS3MT. Determination of luciferase and β-galactosidase reporter gene activity was performed as described previously (1, 37).

siRNA Experiments—siRNA duplexes against EGR-1 (Hs_EGR1_7_HP siRNA, SI03078950; sense, 5'-CGUCGUGUGCCACACCGAAdTdT-3'; antisense, 5'--UACGUGUGGCGCCACCGAdGdGdG-3') and negative control siRNA duplex (1022076; sense, 5'--UUUCGGAACGUGUCAGAdTdT-3'; antisense, 5'--ACGUGACGACGUGACGAGAdTdT-3') were obtained from Qiagen. For RNAi experiments, KB cells were transfected with 3 µg of DNA and 6 µl of HiPerFect (Qiagen) per 6 wells. HEK293 cells were transfected using CaPO4. DNA amounts were kept equal by adding empty vector pCS3MT.

DNA Oligonucleotides for ChIP—The following primers were used for ChIP: murine Egr-1 promoter AP-1 site, sense, 5'--gacctggagccgcaaggg-3'; antisense, 5'--tgggttatgagcttcggggc-3'; murine Egr-1 promoter distal three SREs, sense, 5'--gcgtgctgtccctgggtgg-3', and antisense, 5'--tcctcgcgcggaggg-3', human IL8 promoter, sense 5'--aagaactttcctgcattccg-3', and antisense, 5'--ttggttttattatatactccctc-3'; human IL6 promoter, sense 5'--ttcaactagccccac-3', and antisense, 5'--gaagttctagctgttcgctct-3', human CCL2 promoter, sense 5'--ctctggcctccgagtcagttgctgcag-3', and antisense, 5'--gcgagttgaatgttc-3'.

RT-PCR—1 µg of total RNA was prepared as described previously (31) and transcribed into cDNA using Moloney
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A) HEK293IL-1R cells were transiently transfected with empty vector, JNK-MKK7, or a kinase-inactive mutant of JNK-MKK7, JNK-MKK7 kd. 24 h later cells were lysed and expression of JNK-MKK7 fusion proteins was analyzed by antibodies against the Myc epitope tag and by anti-JNK antibodies. Activity of JNK-MKK7 was analyzed in parallel by immune complex protein kinase assay using recombinant GST-c-Jun and by phosphorylation site-specific antibodies against JNK (P-JNK). The lower panel indicates induction and phosphorylation of endogenous c-Jun by JNK-MKK7 as assessed by Western blotting of the same lysates. B, RNA from cells transfected as described in A was analyzed for mRNA expression of EGR-1 by Taqman real-time PCR. Shown is one result out of two independent experiments.

Overexpression of JNK-MKK7 activates endogenous c-Jun and induces EGR-1 mRNA expression. A, HEK293IL-1R cells were transiently transfected with empty vector, JNK-MKK7, or a kinase-inactive mutant of JNK-MKK7, JNK-MKK7 kd. 24 h later cells were lysed and expression of JNK-MKK7 fusion proteins was analyzed by antibodies against the Myc epitope tag and by anti-JNK antibodies. Activity of JNK-MKK7 was analyzed in parallel by immune complex protein kinase assay using recombinant GST-c-Jun and by phosphorylation site-specific antibodies against JNK (P-JNK). The lower panel indicates induction and phosphorylation of endogenous c-Jun by JNK-MKK7 as assessed by Western blotting of the same lysates. B, RNA from cells transfected as described in A was analyzed for mRNA expression of EGR-1 by Taqman real-time PCR. Shown is one result out of two independent experiments.

murine leukemia virus reverse transcriptase (MBI) in a total volume of 20 µl. 1 µl/well of this reaction mixture was used to amplify cDNAs using assays on demand (Applied Biosystems) for murine (Mm00656724_m1) or human EGR-1 (Hs00152928_m1) and murine or human β-actin (Mm0067939_s1, Hs00174103_m1) on an ABI7500 real-time PCR instrument. The threshold value ct for each individual PCR product was calculated by the instrument’s software, and ct values obtained for EGR-1 were normalized by subtracting the ct values obtained for β-actin. The resulting Δct values were then used to calculate relative changes of mRNA expression as ratio (R) of mRNA expression of stimulated/unstimulated cells according to the equation: R = 2−ΔΔct(stimulated)−ΔΔct(unstimulated).

ChIP—Three to five 175-cm² flasks of confluent Mefs or 1–2 flasks of KB cells, treated as described in the figure legends, were used for each condition. Proteins bound to DNA were cross-linked in vivo with 1% formaldehyde in warm PBS. After 1 h (KB cells) or 5 min (Mefs) of incubation at room temperature, this solution was replaced by cold PBS containing 0.125 mM glycine to stop the cross-linking. Then the supernatant was removed, and the cells were washed with and scraped into ice-cold PBS. Cells were collected at 500 × g at 4 °C, washed again in ice-cold PBS, and then lysed in ChIP-RIPA buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA), once more in ChIP-RIPA buffer (cold), once in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) at room temperature, and finally resuspended in 55 µl of elution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1% SDS). Samples were vigorously mixed for 15 min at 25 °C and then centrifuged. 50 µl of supernatant were diluted to 200 µl with TE buffer, including RNase A (50 µg/ml). Similarly, 50 µl of the initial lysate (input samples) were diluted to 200 µl with TE buffer containing 1% SDS and 50 µg/ml RNase A. After 30 min at 37 °C, proteinase K was added (0.5 mg/ml), and both input and immunoprecipitates were incubated for at least 6 h at 37 °C followed by 6 h at 65 °C. Samples were diluted to 500 µl in loading buffer PB (Qiagen), and DNA was purified using QIAquick spin columns (Qiagen) or nucleospin columns (Macherey & Nagel) according to the manufacturer’s instructions. DNA was eluted with TE buffer or sterilized water and stored at −20 °C until further use.

PCR was performed on input and immunoprecipitated DNA using 2.5 units of hotstart Taq polymerase (Qiagen), 1× PCR buffer (Qiagen), and DNA was purified using QIAquick spin columns (Qiagen) or nucleospin columns (Macherey & Nagel) according to the manufacturer’s instructions. DNA was eluted with TE buffer or sterilized water and stored at −20 °C until further use.

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Quantification of ChIP DNA by Real Time PCR—PCR products derived from ChIP were quantitated by real-time PCR using an ABI7500 instrument (Applied Biosystems). The reaction mixture contained 2–3 µl of ChIP or input DNA, 0.5 µM of primers, and 12.5 µl of the Sybr Green Mastermix (Applied Biosystems) or SensiMix DNA kit (Quantace) in a total volume of 25 µl. PCR cycles were as follows: 15 min at 95 °C, 34–36 cycles of 94 °C (20 s), 55–60 °C (20 s), 72 °C (20 s), followed by a final extension reaction at 72 °C for 7 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining using an Biomax TI5 system (Biomax) and the BioDocAnalyse software, version 1.0 (Biomax).

Western Blotting—Cells were lysed either in Triton cell lysis buffer (10 mM Tris, pH 7.05, 30 mM NaPPi, 50 mM NaCl, 1% Triton X-100, 2 mM Na3VO4, 50 mM NaF, 20 mM β-glycerophosphate, and freshly added 0.5 mM phenylmethylsulfonyl flu-
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TABLE 1

| No. of probes present on array/Probes used for calculation | Probe ID | Accession | Gene symbol | Description | JNK-MKK7/ vector | JNK-MKK7/kd | JNK-MKK7/kd vector |
|----------------------------------------------------------|----------|-----------|-------------|-------------|-----------------|--------------|-------------------|
| 2/1                                                      | A_23_P78628 | NM_145185 | MAP2K7      | Mitogen-activated protein kinase kinase 7 | 424.8 | 285.9 | 1.5 |
| 1/1                                                      | A_23_P214080 | NM_001964 | EGR1        | Early growth response 1 | 46.9 | 1.3 | 35.6 |
| 1/1                                                      | A_23_P365738 | NM_015193 | ARC         | Activity-regulated cytoskeleton-associated protein | 3.4 | 0.8 | 4.3 |
| 1/1                                                      | A_23_P49338 | NM_016639 | TNFRSF12A   | Tumor necrosis factor receptor superfamily, member 12A | 4.7 | 1.4 | 3.4 |
| 1/1                                                      | A_23_P46936 | NM_000399 | EGR2        | Early growth response 2 (Krox-20 homolog, Drosophila) | 2.3 | 0.8 | 2.9 |
| 1/1                                                      | A_23_P151895 | NM_003613 | CILP        | Cartilage intermediate layer protein, nucleotide pyrophosphohydrolase | 2.6 | 0.9 | 2.8 |
| 2/1                                                      | A_23_P303087 | NM_002825 | PTN         | Pleiotrophin (hepatic-binding growth factor 8, neurite growth-promoting factor 1) | 3.1 | 1.1 | 2.8 |
| 2/2                                                      | A_23_P215283; A_24_P38290 | NM_003182 | TAC1        | Tachykinin, precursor 1 (substance K, substance F) | 2.7 | 1.0 | 2.7 |
| 1/1                                                      | A_23_P21936 | NM_001561 | TNFRSF9     | Tumor necrosis factor receptor superfamily, member 9 | 2.2 | 0.9 | 2.4 |
| 1/1                                                      | A_23_P150467 | NM_006637 | ORS11       | Offactory receptor, family 5, subfamily I, member 1 | 2.0 | 0.9 | 2.2 |
| 1/1                                                      | A_24_P203315 | NM_182553 | CNJH2       | Cornichon homolog 2 (Drosophila) | 2.9 | 1.3 | 2.1 |
| 1/1                                                      | A_23_P4082 | NM_006584 | CCT6B       | Chaperonin containing TCP1, subunit 6B (2) | 2.2 | 1.0 | 2.1 |
| 2/2                                                      | A_23_P36235; A_23_P153905 | NM_009407 | IER2        | Immediate-early response 2 | 2.4 | 1.1 | 2.1 |
| 1/1                                                      | A_23_P393620 | NM_006528 | TFFP1       | Tissue factor pathway inhibitor 2 | 2.0 | 1.1 | 1.9 |
| 1/1                                                      | A_23_P218463 | NM_013376 | SERTAD1     | SERTA domain containing 1 | 2.0 | 1.1 | 1.8 |
| 1/1                                                      | A_23_P217176 | NM_005139 | ANXA3       | Annexin A3 | 2.3 | 1.3 | 1.8 |
| 2/2                                                      | A_24_P33895; A_23_P34915 | NM_004024 | ATFS        | Activating transcription factor 3 | 2.5 | 1.5 | 1.7 |
| 1/1                                                      | A_23_P43915 | NM_00417 | DISP1       | Dual specificity phosphatase 1 | 2.6 | 1.6 | 1.6 |
| 1/1                                                      | A_23_P48698 | NM_003814 | ADAM20      | A disintegrin and metalloproteinase domain 20 | 2.3 | 1.5 | 1.5 |
| 2/1                                                      | A_23_P255166 | NM_018460 | ARHGAP15    | GTPase-activating protein 15 | 2.2 | 1.5 | 1.5 |
| 1/1                                                      | A_23_P213102 | NM_016081 | PALLED       | Paladin | 2.0 | 1.5 | 1.3 |
| 1/1                                                      | A_23_P421401 | NM_002609 | PDGFRB      | Platelet-derived growth factor receptor, β-polypeptide | 3.3 | 5.2 | 0.6 |

or, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, 1 μg/ml microcystin) or nuclear and cytosolic extracts prepared as described previously (37). Cell extract proteins were separated on 7.5–12.5% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon®, Millipore Corp.). After blocking with 5% dried milk in Tris-buffered saline (TBS) overnight, membranes were incubated for 4–24 h with primary antibodies, washed in TBS, and incubated for 2–4 h with the peroxidase-coupled secondary antibody in 1–5% milk/TBS. Proteins were detected by using the Pierce or Millipore enhanced chemiluminescence system.

**Immune Complex Protein Kinase Assays—**JNK-MKK7 was immunoprecipitated from cell extracts with anti-Myc antibodies, and JNK activity was determined in vitro by radioactive protein kinase assay using recombinant GST-Jun as described previously (5).

**DNA Microarray Experiments—**Total RNA from HEK293-IL1R cells, transfected as indicated in the legend of Table 1, was isolated with the RNeasy kit including “on column” DNase I digestion (Qiagen). 500 ng of total RNA were used to generate Cy3-labeled cRNA with the Amino Allyl MessageAmp™ II with Cy™3 kit (AM1795, Applied Biosystems) according to the manufacturer’s recommendations. Total cRNA yield, fragment length, and labeling efficiency were determined by an Agilent 2100 Bioanalyzer. 3 μg of labeled cRNA mixtures were hybridized individually to the Whole Human Genome Oligo Microarray (G4112A, ID 012391, Agilent Technologies). cRNA fragmentation, hybridization, and washing steps were performed as directed by the manufacturer, described in the One-color Microarray-based Gene Expression Analysis Protocol version 5.0.1.

Hybridized and washed arrays were scanned on an Affymetrix 428 Array Scanner at six different PMT voltage settings (namely 5, 15, 25, 35, 45, 55) to extend the dynamic range of the measurements. Data extraction was performed with the Imagene version 5.0 software (Biodiscovery). All measurements obtained were integrated into one data set per spot by a linear regression-based algorithm (MAVI software, version Pro 2.5.1, MWG Biotech). Normalization of fluorescence intensity (fI) values was performed with the MAVI software according to the following equation: normalized fI = fI / mean (log fI all spots). Spots of poor quality were identified according to manual inspection of Tiff-Images and if one of the following equations were true: (i) CV<sub>signal mean</sub> > 0.20; (ii) SM<sub>global background corrected</sub> / SM<sub>local background corrected</sub> > 1.3; where CV<sub>signal mean</sub> is the coefficient of variance of the signal.
values for all pixels of a spot, and $\frac{SM_{\text{global background corrected}}}{SM_{\text{local background corrected}}}$ is the ratio of a global background-corrected signal mean of a spot compared with the local background-corrected signal mean value. Spots of poor signal-to-noise ratios or bad signal quality were flagged and excluded from further analysis. Ratios of relative mRNA expression were calculated in Excel by dividing the normalized $IJ$ values of the experimental condition by the normalized $IJ$ value of the control condition.

**RESULTS AND DISCUSSION**

To identify genes that are under control of activated JNK, we used a previously characterized JNK-MKK7 fusion protein. As shown before, transient expression of this construct, but not of a kinase-inactive version (JNK-MKK7 kd), resulted in transphosphorylation of JNK by the fused MKK7 and a subsequent strong increase in catalytic activity of JNK as assessed by phosphorylation of recombinant c-Jun in vitro (Fig. 1A). Of note, this construct does not activate endogenous JNKs (Fig. 1A), as shown previously (7). A possible explanation for this phenomenon is that the JNK-MKK7 fusion prevents correct interaction with endogenous scaffolds and upstream kinases of the endogenous JNK pathway (38) such as JIP-1 (39) or β-arrestin (40). However, it modestly increased the amount of c-Jun and strongly phosphorylated endogenous c-Jun (Fig. 1A, lower panel). Hence, the JNK-MKK7 fusion protein provides a highly specific tool to selectively analyze the contribution of c-Jun in the regulation of gene expression. Initially we performed a genome-wide analysis of mRNA expression in response to JNK-MKK7. As shown in Table 1, we found 21 genes up-regulated by JNK-MKK7 by at least 2-fold. Only a small number of genes also required the catalytic activity of JNK-MKK7 indicating that they might be direct targets of JNK-MKK7.

*EGR-1* mRNA expression was increased by 47-fold and was by far the most strongly up-regulated transcript induced by JNK-MKK7. Its induction was almost entirely dependent on active JNK as its up-regulation was absent with the JNK-MKK7-kd fusion protein. To identify genes that are upregulated by JNK, we performed a genome-wide analysis of mRNA expression in response to JNK-MKK7. As shown in Table 1, we found 21 genes up-regulated by JNK-MKK7 by at least 2-fold. Only a small number of genes also required the catalytic activity of JNK-MKK7 indicating that they might be direct targets of JNK-MKK7.

**FIGURE 2.** IL-1-dependent mRNA and protein expression of Egr-1 requires JNK-dependent phosphorylation of c-Jun. A, wild type (wt) embryonic fibroblast cell lines, cells lacking JNK1 and JNK2 genes (JNK1/2−−) or c-Jun (c-Jun−−), or cells that were isolated from mice carrying a c-Jun allele mutated in two of the four JNK phosphoacceptor sites (c-Jun SS63/73AA) were kept in low serum (0.1%) for 48 h. Thereafter, cells were treated for the indicated times with 10 ng/ml IL-1α or were left untreated. Expression values were normalized for β-actin and are shown relative to the unstimulated wild type control cells. B, wild type Mefs were treated for 30 min with 50 μM PD98059 or 20 μM SP600125. Wild type or c-Jun-deficient Mefs were then treated for the indicated times with IL-1α (10 ng/ml), and expression of Egr-1 and c-Jun was analyzed by Western blotting of nuclear extracts. Antibodies against HDAC3 were used to normalize for protein expression. No Egr-1 antigen was found in c-Jun-deficient cells even at the longest ECL exposures.

**FIGURE 3.** Activation of Egr-1 transcription by JNK-MKK7 or by IL-1 requires both a distal AP-1 site and three SREs in the Egr-1 promoter. A, schematic representation of AP-1 and SRE sites in the Egr-1 promoter and enhancer regions. Deletion mutants as described in Ref. 19 are indicated. B, and C, HK293IL-1R cells were transiently transfected with the Egr-1 promoter luciferase constructs shown in A. B, cells were cotransfected with empty vector, JNK-MKK7, or JNK-MKK7 kd. C, cells were stimulated for 4 h with IL-1α (10 ng/ml) or were left untreated prior to lysis. 24 h after transfection cells were lysed, and luciferase activity was determined. Shown are the mean luciferase activities ± S.E. from two independent experiments.
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pathway in genetically altered murine fibroblasts. In wild type fibroblasts, IL-1 induced a 20-fold transient increase in Egr-1 mRNA (Fig. 2A). In cells lacking JNK1/2 genes, mRNA induction by IL-1 was at maximum 1.8-fold, and it was completely lost in cells lacking c-Jun or expressing a phosphorylation-deficient c-JunAA mutant (Fig. 2A). In parallel to the mRNA, EGR-1 protein was induced by IL-1 in wild type fibroblasts (Fig. 2B). This effect was suppressed as expected by the ERK inhibitor PD98059 but also by the JNK inhibitor SP600125 (Fig. 2B). No Egr-1 antigen could be detected in c-Jun-deficient cells (Fig. 2B). These data indicate that induction of Egr-1 expression by proinflammatory stimuli is under major control of the JNK-c-Jun pathway. Moreover, to our knowledge Egr-1 has not previously been identified as a c-Jun target gene, including studies in which c-Jun-deficient fibroblasts were analyzed by a cDNA microarray carrying probes for 15,000 genes (41).

The EGR-1 promoter has been characterized in detail. In particular five SREs that bind serum-response factor and ternary complex factors (TCFs) such as Elk-1 or SAP-1 have been identified in several previous studies. TCFs are phosphorylated and activated by MAPKs JNK, ERK, and p38 (42–48), e.g. our own work has shown that JNK phosphorylates Elk-1 (49). We used a 1.2-kb fragment of the murine Egr-1 promoter and its deletion mutant derivatives as described in Ref. 19 fused to luciferase to locate the cis-elements targeted by JNK-MKK7 (Fig. 3A). The active JNK-MKK7, but not the kinase-dead variant, induced Egr-1 transcription by 29-fold (Fig. 3B). A deletion mutant lacking an upstream AP-1 site was only induced by 13-fold, and two mutants lacking the three distal SREs plus the AP-1 site lost responsiveness to JNK-MKK7 (Fig. 3B). Similarly IL-1 induced Egr-1 promoter activation required both the AP-1 site and the three distal SREs in an additive fashion (Fig. 3C). In HEK293IL-1R cells, the distal AP-1 site was also required for basal promoter activity as its deletion resulted in reduced activity (Fig. 3, B and C). Hence, the Egr-1 promoter integrated signals from the JNK pathway through two regions, the distal AP-1 site and three of the previously characterized five SREs. This result suggested that the JNK-MKK7 pathway might activate Egr-1 transcription by a dual mechanism, one involving c-Jun-mediated activation via the AP-1 site and one involving phosphorylation and activation of SRE-bound proteins such as Elk-1 or SAP-1. Because a role for c-Jun in Egr-1 mRNA expression has not been recognized previously, we analyzed its role in JNK-MKK7-mediated Egr-1 transcription in more detail using murine fibroblasts lacking c-Jun, or cells that were recon-
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![Diagram](image1)

**FIGURE 6.** EGR-1 binds to regulatory regions of cytokines and chemokines in an IL-1-dependent manner. A, sequences for human CCL2, IL6, and IL8 genes were taken from the ENSEMBL data base. 3000 bp upstream of the transcriptional start site of each gene were analyzed by the TRANSFAC®Professional data base (version 10.3). Regions for CCL2 (58, 60), IL6 (51, 59), and IL8 (37, 57) genes that were previously shown to contain regulatory NF-κB sites are illustrated as are EGR-1-binding sites predicted by TRANSFAC. Numbers indicate nucleotides relative to the transcriptional start sites (bent arrow). Horizontal arrows indicate primer chosen for ChIP analysis of these regions to analyze binding of endogenous EGR-1 to ccl2, IL6, and IL8. B, human KB cells were treated for the indicated times with 10 ng/ml IL-1α or were left untreated. After in vivo cross-linking, chromatin was isolated from all cultures and immunoprecipitated with EGR-1 antibodies or control IgG. The genomic fragments associated with immunoprecipitated DNA were amplified by PCR using the primer shown in A. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. In parallel PCRs were performed with input chromatin to control for equal amounts of starting material.

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- **Stimulated with c-Jun expression vectors (Fig. 4A).** Activation of Egr-1 promoter constructs in response to specific activation by JNK-MKK7 was almost completely lost in c-Jun-deficient cells (Fig. 4B, lanes 13–15) or in cells reconstituted with a c-Jun mutant (TAM67) lacking the transactivation domain (Fig. 4B, lanes 7–9). However, Egr-1 transcription in response to MKK7-JNK was restored in cells transfected with a wild type c-Jun expression vector proving the essential role of c-Jun in transcription via c-Jun through the SREs by an indirect mechanism. This assumption was confirmed by analyzing the association of c-Jun with two Egr-1 chromatin fragments covering either the AP-1 site or the three SREs using ChIP. Amounts of PCR products from ChIP experiments were determined by absolute quantitation using real time PCR to account for differences in PCR efficiency. We found IL-1-induced specific and comparable recruitment of c-Jun to both the region harboring the AP-1 site and to the region containing the three distal SREs (Fig. 5B). Enrichment of immunoprecipitated Egr-1 chromatin by c-Jun antibodies was specific compared with ChIP performed with material from c-Jun-deficient cells or by using IgG control antibodies (Fig. 5B). In contrast to c-Jun antibodies, ChIP performed with antibodies against Elk-1 showed strong enrichment around the three distal SREs as expected and little binding of Elk1- to the AP-1 site (Fig. 5B). Elk-1 protein expression was unchanged in wild type and c-Jun−/− Mefs (data not shown).

In summary, according to the experiments shown in Fig. 4 and Fig. 5, c-Jun is recruited directly to a single AP-1 site and indirectly to the three SREs to regulate Egr-1 transcription. This indirect mechanism may involve protein-protein interactions with TCFs such as Elk-1 or SAP-1 or yet to identify other components of the Egr-1 enhancosome.

It was shown previously that Elk-1 is upstream of EGR-1 in the lipopolysaccharide-ERK signaling pathway (19). Our study now extends this transcription factor regulatory network by clearly demonstrating that c-Jun is equally involved in EGR-1 transcriptional regulation. Hence, Elk-1 (or other TCFs), c-Jun, and EGR-1 form a transcription factor network. The rapid mutual control of these immediate early gene transcription factor networks might be required for subsequent cooperative strong transcription of secreted proinflammatory mediators. Indeed, genetic ablation of EGR-1 in mice or suppression by siRNA in human cells has been shown to result in reduced expression of IL-1β, IL-8, CCL2/MCP-1, and MIP-2 in response to amyloid peptides, ischemia/reperfusion, endotoxin, and ovalbumin-induced airway inflammation (12, 22, 52, 53). We therefore searched the upstream regions of some of these genes for potential EGR-1 and Sp-1 sites as both factors recognize highly overlapping gc-rich DNA-binding elements (54, 55). We found a large number of sites, i.e. 104 (for ccl2), 108
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For details see text.

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**FIGURE 7.** RNAi-mediated suppression of EGR-1 inhibits transcriptional regulation of IL-8, IL-6, and CCL2. A, KB cells were transfected with 50 nM siRNA directed against EGR-1 or negative control (neg. ctrl.) siRNA. After 24 h, cells were stimulated with 10 ng/ml IL-1α or were left untreated. Expression of EGR-1 mRNA was analyzed by Taqman real time PCR and is depicted relative to cells treated with the negative siRNA oligonucleotide. B, KB cells were transfected with 50 nm siRNAs as described in A. 0.5 μg of SV-40 β-galactosidase (β-gal), 0.25 μg of pUHC13.3 IL-8 promoter luciferase (luc), or 2.5 μg of pUHC13.3 IL-6 promoter luciferase were co-transfected as indicated. 24 h later cells were stimulated for 3 h with 10 ng/ml IL-1α or were left untreated. Thereafter, cells were lysed, and luciferase activity in cell extracts was determined and normalized for β-galactosidase activity. Shown is the mean normalized luciferase activity ± S.E. from three independent experiments performed in duplicate. C, HEK293IL-1R cells were transfected with 0.5 μg of SV-40 β-galactosidase plus 0.25 μg of pUHC13.3 IL-6 promoter luciferase or 1 μg of CCL2 promoter luciferase (ccl2 2.8/luc) and 100 nM negative control siRNA or 100 nM EGR-1 siRNA as indicated. Reporter gene activity was determined as in B. Shown is the mean normalized luciferase activity ± S.E. from three independent experiments performed in duplicate.

(for il6), or 78 (for il8) bindings matrices for Sp-1 and EGR-1, were predicted by TRANFAC (50, 56) using the position-weighted matrices deposited in its data base. To confirm these predictions experimentally, we chose regulatory regions of all three genes that were previously shown to confer responsiveness to IL-1 or TNF. These regions also contain NF-κB-binding sites, whose importance for inducible regulation of CCL2, IL-6, and IL-8 has been demonstrated by numerous studies (57–59). As shown in Fig. 6A in close proximity to each of these regions we found at least one predicted EGR-1-binding site. IL-1-regulated binding of endogenous EGR-1 to these sites was confirmed by ChIP analysis (Fig. 6B). Moreover, siRNA directed against EGR-1 partially suppressed IL-1-inducible activation of reporter genes containing the regulatory promoter/enhancer regions of IL-8, IL-6, and CCL2 indicating a functional relevance of EGR-1 for IL-1-inducible transcription (Fig. 7).

In summary, we provide novel evidence for a complex c-Jun-mediated mechanism that enhances EGR-1 mRNA expression. Taken together, our results identify this pathway as an unrec-ognized part of a multistep gene regulatory network that controls cytokine and chemokine expression via the IL-1-MKK7-JNK-c-Jun-EGR-1 pathway (Fig. 8). These results merit further investigation as a target for the treatment of inflammatory diseases through inhibition of this novel pathway.
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