MEK1-dependent Delayed Expression of Fos-related Antigen-1 Counteracts c-Fos and p65 NF-κB-mediated Interleukin-8 Transcription in Response to Cytokines or Growth Factors*

Received for publication, June 24, 2004, and in revised form, December 15, 2004
Published, JBC Papers in Press, December 24, 2004, DOI 10.1074/jbc.M407071200

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The transcription factor activator protein 1 (AP-1) is involved in cellular proliferation, transformation, survival, cell death, and immune response (1, 2). AP-1 converts extracellular signals into changes of the expression of target genes, and AP-1 binding sites are found in a large number of genes. AP-1 is not a single protein but a homo- or heterodimer composed of members of the Jun, Fos, and ATF protein families (2). Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, and Fra-related antigen (Fra-1, Fra-2)) proteins act as dimeric transcription factors that can bind to the promoter regions of numerous mammalian genes. Fos and Jun proteins can also dimerize to many other basic leucine zipper proteins such as ATF, C/EBP, Maf, and NF-E2, increasing the number of the potential AP-1 factors that bind to a given AP-1 site (3). Furthermore, activity of AP-1 proteins is controlled at multiple levels, first by changes in gene transcription, mRNA turnover, and protein stability (4), second by interaction with non-basic leucine zipper transcription factors (3, 5), and third by phosphorylation of their transactivation domains by mitogen-activated protein (MAP) kinases (4).

The number of AP-1 proteins and their complex control make it difficult to assess the impact of particular individuals on gene expression. Recently, the usage of genetically altered mice and cells derived from them has unraveled crucial functions for AP-1 as a regulator of cell life and death (6). However, very little information is available from these model systems on the role of AP-1 in inflammation and infection despite the fact that AP-1 binding sites are found in many genes activated during innate (and also adaptive) immune reactions (7). Because of the structural and regulatory complexity of AP-1, at present a prerequisite for defining its role in inflammation is to determine the composition of AP-1 complexes as a function of time and stimulus for individual genes, in particular those that are crucial for the inflammatory process (7). In this regard, a family of secreted proteins called chemokines is exceptionally important, because these factors control the process of leukocyte invasion into injured tissue, a hallmark of inflammation (8).

Interleukin (IL)-8, a prototypic human chemokine, was detected more than a decade ago as the founding member of the chemokine superfamily (9). IL-8 secretion and mRNA synthesis are strongly regulated by more than 100-fold by a plethora of external stimuli (10). A major part of this regulation occurs at the transcriptional level. A relatively small region of the 5′-flanking region of the IL-8 gene (nucleotides -1 to -133) is essential and sufficient for its transcriptional induction by most stimuli (10). This promoter element contains an NF-κB element that is required for activation in all cell types studied as well as a single consensus AP-1 site whose sequence is tgactca (3, 10). Unlike the binding site for NF-κB, the AP-1 site as well as additional sites contained within the IL-8 core promoter, namely those for C/EBP, OCT-1 (11, 12), or for NF-κB-repressing factor (13) are not essential for induction but are required for maximal gene expression (10).

We and others have provided numerous lines of functional evidence that coordinated activation of the MAP kinases ERK, c-Jun NH2-terminal kinase, and p38 is important for IL-8 reg-
ulation in response to proinflammatory cytokines, such as interleukin (IL)-1 (14), viruses (15), bacteria (16–18), and various stress inducers (10). However, the precise molecular mechanisms by which all these three types of MAP kinases regulate onset, duration, or extent of IL-8 mRNA synthesis have been largely elusive. The fact that MAP kinases regulate AP-1 activity suggests that they may induce IL-8 transcription via the single AP-1 site. However, MAP kinase also regulates other steps in the overall process of gene expression such as chromatin remodeling (19) or, as found by us (20, 21), IL-8 mRNA stability. In addition, as for many other AP-1 target genes, there is a gap in the understanding of the actual composition of AP-1 complexes that bind to the endogenous IL-8 gene promoter (7).

Several groups have addressed this point by studying the interaction of nuclear AP-1 proteins with IL-8 promoter-derived oligonucleotides in vitro using band-shift assays (22, 23). However, these experiments cannot explain which AP-1 components interact with the two endogenous, chromatin-embedded alleles of the IL-8 gene in vivo (10).

In addressing these issues we have used the chromatin immunoprecipitation (ChIP) technique to analyze the downstream mechanisms by which the ERK MAP kinase pathway contributes to IL-1-inducible IL-8 gene expression. Hereby, we have focused on the role of c-Fos and Fra-1, two important AP-1 proteins that are expressed in many cell types and whose role in regulation of inflammatory genes has been largely elusive.

In this study, we show that IL-1 up-regulates c-Fos and Fra-1 in an ERK-dependent manner, a process that involves Fra-1 stabilization and ubiquitination. Furthermore, we show that c-Fos and Fra-1 bind to the endogenous IL-8 promoter and provide compelling evidence for a hitherto unrecognized role of Fra-1 in inducible expression of IL-8. Importantly, we find that Fra-1 counteracts the positive regulatory role of c-Fos and p65 NF-κB on IL-8 gene expression. These data provide a mechanism of how c-Fos and Fra-1 enable transcriptional regulation of IL-8 to different levels in response to two physiological inducers of IL-8, namely IL-1 and EGF. Thus, our study establishes an example of how an activated MAP kinase pathway connects to two AP-1 proteins that regulate inflammatory gene expression.

EXPERIMENTAL PROCEDURES

Cells and Materials—KB cells were from the American Type Culture Collection, Manassas, VA. HeLa cells stably expressing the tet-activating KB-9293S cells (HEK293IL-R) were kind gifts of H. Bujard and K. Matsumoto, respectively. Human gingival fibroblasts have been described (24). All cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin. Antibodies against the following proteins or peptides were used in this study: c-Fos (sc-7202), mid pUHC13–3-IL-8pr (nucleotides 1348–1527 of the IL-8 gene) and the corresponding mutants in the NF-κB and AP-1 cis elements have been described in detail (27). HEK293IL-R and HeLa cells (seeded at 5 × 10^4 per well of 6-well plates) were transfected with a total of 5.25 to 6.25 DNA μg by the calcium phosphate method. KB cells (seeded at 1 × 10^5 in 12 wells) were transfected with 8.25 μg of total DNA and 1 μl of JETPEI (Biomol). In all transfections the DNA amount was kept constant by adding empty PC3SMT. Determination of luciferase reporter gene activity was performed as described (27).

ChIP—One 175-cm^2 flask of confluent KB cells treated as described in the figure legends was used for each condition. Proteins bound to DNA were cross-linked in vivo by replacement of the medium with warm PBS including 1% formaldehyde, and after incubation for 1 min at room temperature, this solution was replaced by warm PBS including 0.125 mM glycine to stop cross-linking. Then the supernatant was removed, and the cells were washed in 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-radioimmunoprecipitation assay buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1 μM EDTA, and freshly added 1% aprotinin). Lysates were cleared by sonification (4 × 1 min on ice) and centrifuged at 15,000 × g at 4 °C for 20 min. Supernatants were collected and stored in aliquots at −80 °C for subsequent ChIP. 4–10 μl of antibodies were added to 250–500 μl of lysates, and the mixture was rotated at 4 °C overnight. Then 40 μl of a protein A/G mixture prequillibrated in ChIP radioimmunoprecipitation assay buffer was added to the lysate. Incubation was continued for 1 h at 4 °C. Beads were collected by centrifugation and washed 2 times in 1.4 ml of ChIP-radioimmunoprecipitation precipitation assay buffer, once in high salt buffer (10 mM Tris, pH 7.5, 2 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA), once in ChIP-radioimmunoprecipitation precipitation assay buffer, and once in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and finally resuspended in 55 μl of elution buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.5% SDS). The immunoprecipitates were mixed for 15 min at 30 °C and then centrifuged. 50 μl of supernatant was 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 including 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 15 min at 65 °C and then one wash with 50 μl of TE buffer PB (Qiagen), and DNA was purified using Qiaquick spin columns (Qiagen) according to the manufacturer’s instructions. DNA was eluted with TE buffer 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 μM sense and antisense primer, 2.5–3 μM of template DNA, 0.2 mM dNTPs in a total volume of 50 μl. PCR cycles were as follows: 1 cycle at 95 °C for 15 s, 40 cycles at 95 °C for 1 s, 58 °C for 1 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, visualized by ethidium bromide staining, and fluorescence intensities of bands were quantified using a Biometra T15 system (Biometra) and the BioDocAnalyse software, Version 1.0 (Biometra). Selected ChIP results were validated by performing a second ChIP using a different antibody (Roche Applied Science) as indicated in the legends to Figs. 5 and 7C. The reaction mixture contained 3–4 μl of ChIP or input DNA, 0.5 μM primers, and 4 μl of the light cycler DNA Master SYBR Green 1 kit (Roche Applied Science) in a total volume of 20 μl. PCR cycles were 10 min at 95 °C, 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Melting curve analysis revealed a single PCR product. Serial dilutions of input DNA revealed that PCR reactions were linear from 100 to 0.1 ng and were used to calculate absolute amounts of PCR products by the light cycler software. ChIP primers for the IL-8 gene (accession number M28130) were as follows: IL-8 promoter (sense, nucleotides 1303–1325, 5′-agaagaactttgtgatatcgcc-3′; antisense, nucleotides 1450–1473, 5′-tgctgttatatactataacctac-3′); IL-8 upstream region (sense, nucleotides 407–430, 5′-catagttgctcagagctcag-3′; antisense, nucleotides 519–543, 5′-gtcagggagagttgttatgta-3′).

Reverse Transcriptase-PCR—1 μg of total RNA was prepared as described below and transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a total volume of 40 μl. 5 μl of this reaction mixture were used to amplify cDNAs using assays on demand (Applied Biosystems) for IL-8 (Hs00174103), c-Fos (Hs00177212), Fra-1 (Hs00179076), and β-actin (Hs00607943) and an ABI7500 real time PCR instrument. The threshold cycle (ct) for each individual PCR product was calculated by the instrument software, and ct values obtained for IL-8, c-Fos, and Fra-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 the ratio (R) of mRNA expression of stimulated/unstimulated cells according to the equation, R = 2^{Δc(t)stimulated−Δc(t)unstimulated}.
In Vivo Ubiquitination Assay—This assay was performed essentially as described in Treier et al. (26). Briefly, one 75-cm² flask of cells was transfected with 12.5 µg of pCMV-Tag3H-ubiquitin and expression vectors as indicated in the legend of Fig. 5. The total amount of DNA (37.5 µg) was kept constant by adding empty pcDNA3. After 48 h cells were lysed in 1 ml of lysis buffer (6 mM guanidine·HCl, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0). Lysates were sonicated to shear DNA. Then 900 µl of lysates were incubated with 25 µl of Ni²⁺-agarose beads overnight at room temperature. Beads were collected by centrifugation, washed 3 times in buffer A (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0), and once in buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 6.2), and 1 time in PBS. Beads were collected by centrifugation, and proteins were eluted by boiling them for 5 min at 95 °C in SDS-PAGE sample buffer. For input controls proteins from 100 µl of cell lysates were precipitated by adding 100 µl of 10% trichloroacetic acid. Samples were incubated at 4 °C for 1 h and centrifuged at 15,000 × g at 4 °C for 15 min, washed twice in ethanol, and dried in a Speed Vac. Proteins were redissolved by boiling in SDS-PAGE sample buffer. Purified and input proteins were separated by SDS-PAGE, and Fra-1 and MEK1 were detected by immunoblotting with the appropriate antibodies.

Western Blotting—Cells were lysed either in Triton cell lysis buffer (10 mM Tris, pH 7.05, 0.5 mM NaF, 50 mM NaCl, 1% Triton X-100, 2 mM Na₃VO₄, 50 mM NaF, 20 mM β-glycerophosphate, and freshly added 0.5 mM phenylmethylsulfonyl fluoride, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 400 µM okadaic acid) or in SDS sample buffer, or nuclei and cytosolic extracts were prepared as described (27). Cell extract proteins were separated on 10% SDS-PAGE and electrophoretically transferred in buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 6.2), and 1 time in buffer A (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0), 3 times in buffer A (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 8.0), and once in buffer B (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, pH 6.2). 5% dried milk in Tris-buffered saline overnight, membranes were incubated for 4–24 h with primary antibodies, washed in Tris-buffered saline, and incubated for 2–4 h with the peroxidase-coupled secondary antibody. Proteins were detected by using the Amersham Biosciences enhanced chemiluminescence system.

Coimmunoprecipitation—Cells were transfected or treated as indicated in the figure legends. After 1 day cells were lysed in Triton cell lysis buffer. 2 µg of Fra-1 or HDAC1 antibody was added to 500–1000 µg of cell extract. After 16 h at 4 °C, protein A-Sepharose was added for another h. Pellets were washed 3× in immunoprecipitation buffer (20 mM Tris, pH 7.4, 154 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100). Proteins were eluted with SDS sample buffer and detected by Western blot.

Small Interfering RNA Experiments—Cells were seeded in 24-well plates at 6.5 × 10⁴/ml. At 70% confluency cells were washed 2× in serum-free medium and transfected with 200 nm concentrations of a mixture of 3 double-stranded RNA oligonucleotides directed against Fra-1 using Gene silencer reagent (Peglab) in a total volume of 500 µl. After 4 h, 500 µl of Dulbecco’s modified Eagle’s medium and 10% serum were added. The next day cells were treated as indicated and lysed, and protein expression was determined by Western blot. Small interfering RNA against Fra-1 (accession number X16707) was designed and produced by Eurogentec. Specificity for Fra-1 was confirmed by BLAST search. The sequences of the small interfering RNA oligonucleotides were as follows (duplex 1, 5′-GCCUCUGUGUACACUGAGdTdTT′-3′, 5′-GUACUGUGUAUACUGACUUCCGdTdTT′-3′, duplex 2, 5′-GUACACUGACAUCCGUGGACGdTdTT′-3′, 5′-GACUCAGCUAAUUCCGUCTdTdTT′-3′, duplex 3, 5′-GAUCAGAAAUCACCCGUGCAGdTdTT′-3′), 5′-GAGCCCCACAUUUCUCAAUCdTdTT′-3′).

Oligonucleotide DNA Microarray Experiments—The microarray used in this study was the first version of the Inflammation Array (MWG Biotech) and contained 110 oligonucleotide probes for inflammatory genes that were previously validated by our laboratory (24) as well as 5 “housekeeping” genes. Total RNA from cells treated as indicated in the legend of Table I was purified with a Qiagen RNeasy kit followed by “on column” DNase I digestion (Qiagen). RNA was used to prepare Cy3-labeled cRNA by oligo(dT)₂₀·dT₇·primed double-stranded cDNA synthesis (cDNA synthesis system, Roche Applied Science) followed by in vitro transcription with T7 polymerase (MEGAscript T7 kit, Ambion) as directed by the manufacturer. cRNA yield was determined photometrically. Equal amounts of cRNAs derived from 2.5 µg of total RNA were hybridized individually to microarrays in pre-prepared hybridization solution (MWG Biotech) at 42 °C overnight and then washed sequentially in 2× SSC (1× SSC = 0.15 M NaCl and 0.15 M sodium citrate), 0.1% SDS, 1× SSC, and 0.5× SSC. Hybridized arrays were scanned at maximal resolution on an Affymetrix 428 scanner at variable photomultiplier voltage settings. Fluorescence intensity values from Cy3 channels were processed using Imagene 4.2 software (Biodiscovery). To obtain maximal signal intensities without saturation effects, intensity values from TIFF images were integrated into one value per probe and normalized by the MAVI software (Version Pro 2.5.1, MWG Biotech).

Enzyme-linked Immunosorbent Assay—IL-8 protein concentrations in the cell culture medium were determined using the human IL-8 Duo set kit (R&D Systems) exactly as directed in the manufacturer’s instructions.

### Table I

| identifier (ref.seq.) | gene | ratio of expression (KB epidermal carcinoma cells) | ratio of expression (human gingival fibroblasts) |
|----------------------|------|----------------------------------------------------|--------------------------------------------------|
| NM_012275            | eXX8 | 42.8                                               | 157.8                                             |
| NM_006619            | i8   | 21.2                                               | 74.9                                              |
| NM_006605            | pgk2 | 25.0                                               | 47.9                                              |
| NM_01986              | birc3| 19.8                                               | 23.6                                              |
| NM_004120             | saa1| 12.1                                               | 11.6                                              |
| NM_006609             | eXX8| 19.3                                               | 2.2                                               |
| NM_023908             | ffa1| 10.5                                               | 2.7                                               |
| NM_004443             | sod2| 10.1                                               | 17.9                                              |
| NM_023610             | mmp18| 10.0                                               | 17.9                                              |
| NM_02109             | lama7| 7.0                                                | 14.7                                              |
| NM_00427             | col5| 4.8                                                | 6.6                                               |
| NM_006505             | eex13| 4.4                                                | 3.5                                               |
| NM_006645             | mal2| 4.0                                                | 3.9                                               |
| NM_001051             | birc2| 3.6                                                | 2.2                                               |
| NM_00758             | nkbla| 3.1                                               | 2.7                                               |
| NM_005994             | fib1| 3.1                                                | 2.5                                               |
| NM_002025             | plk2| 2.3                                                | 2.2                                               |
| NM_000964             | gch1| 2.3                                                | 2.4                                               |
| NM_00223             | f33| 2.2                                                | 2.4                                               |
| NM_00241             | f7| 2.0                                                | 2.4                                               |
| NM_006368             | eot1a| 2.0                                               | 2.1                                               |
| NM_006364             | fgt2| 2.0                                                | 2.4                                               |

**Sensitivity of inflammatory genes to MAP kinase inhibition in epidermal carcinoma cells and in primary fibroblasts**

KB epidermal carcinoma cells or human gingival primary fibroblasts cells were left untreated (0) or treated with 2 µM SB203580 (SB), 20 µM SP600125 (SP), 50 µM PD98059 (PD), or all three inhibitors for 30 min and then stimulated with IL-1 (10 ng/ml) for 4 h. Subsequently, total RNA from each sample was used to prepare Cy3-labeled cRNA. cRNAs were hybridized individually to a customized DNA oligonucleotide microarray containing probes for 110 inflammatory genes. Gene expression in cells subjected to the indicated treatments was compared to that of unstimulated cells and is expressed by the indicated ratios of normalized fluorescence intensities. The relative mRNA expression for 22 genes that are induced by at least 2-fold by IL-1 is shown. Details of the microarray experiments are described under “Experimental Procedures.” The complete set of data can be obtained from the corresponding author.
RESULTS

Partial Suppression of IL-1-inducible IL-8 mRNA and Protein Expression by PD98059—Initially we compared the effects of the MAP kinase inhibitors SB203580, SP600125, and PD98059 on IL-1-inducible gene expression in the human epidermal carcinoma cell line, KB, and in human primary fibroblasts. As shown in Table I, we detected 22 genes that were induced by at least 2-fold in both cell types. As expected from the cooperative role of MAP kinases in activation of IL-1-induced genes (7, 10), treatment with individual MAP kinase inhibitors alone or in combination partially suppressed many genes; however, to variable extents. The most strongly induced gene was IL-8 (CCL8). Interestingly, in both cell types the MEK1 inhibitor PD98059, a well characterized and highly specific allosteric inhibitor of the protein kinase MEK1 (28), had the strongest inhibitory effect on IL-8 gene expression compared with SP600125 or SB203580. Kinetic analysis by real time PCR confirmed that PD98059 partially suppressed IL-1-induced IL-8 mRNA expression in KB cells (Fig. 1A) as well as in human gingival fibroblasts and HEK293IL-1R cells (data not shown). PD98059 also partially suppressed the accumulation of secreted IL-8 protein (Fig. 1B). Thus, in three IL-1-responsive cell types PD98059 affected a mechanism that was not essential for IL-1-inducible IL-8 expression but was required to achieve maximal gene expression.

PD98059 Suppresses IL-1-induced mRNA and Protein Expression of c-Fos and Fra-1—We reasoned that the rapid inhibitory effect of PD98059 treatment was very likely to affect IL-1-induced IL-8 transcription via AP-1. We, therefore, tried to identify proteins that bind to the IL-8 promoter and that are affected by PD98059. In parallel to the increase in IL-8 mRNA, IL-1 induces a transient expression of c-fos (Fig. 1C) and of fra-1 mRNA (Fig. 1D). c-Fos mRNA expression peaked at 30 min of IL-1 stimulation, preceding the expression of Fra-1 mRNA, which peaked at around 1 h of IL-1 stimulation (Fig. 1, C and D). Virtually all of the c-Fos and Fra-1 protein was expressed in the nucleus (Fig. 1E). As at the mRNA level, the inducible expression of c-Fos protein preceded that of Fra-1 (Fig. 1E). Of note, both c-Fos and Fra-1 proteins separated by SDS-PAGE showed at least two forms of the proteins with different mobility, suggesting that IL-1 induces substantial posttranslational modifications of both proteins (Fig. 1E). Compared with the other major AP-1 components (c-Jun, JunB, JunD), c-Fos and Fra-1 mRNA and protein expression as well as their post-translational modifications were strongly inhibited by PD98059 (Fig. 1E) or by another MEK1 inhibitor, UO126 (data not shown).

c-Fos and Fra-1 Are Recruited to the Endogenous IL-8 Promoter—We, therefore, designed experiments to determine the effects of PD98059 on the recruitment of c-Fos and Fra-1 proteins to the endogenous IL-8 promoter. For this we amplified in ChIP experiments a fragment of 170 bp that covers the whole known regulatory region of the IL-8 gene including the AP-1 and NF-κB sites, the TATA box region, and the putative polymerase II binding site (Fig. 2A). ChIP conditions were optimized so that this fragment was only amplified after immunoprecipitation with specific antibodies directed at factors that bind to sites within this promoter region, e.g. p65 NF-κB, or...
polymerase II but not with irrelevant antibodies. In addition, no PCR product was obtained with p65 or polymerase II antibodies by using a primer set that covers a non-regulatory region far 5’ within the IL-8 promoter (Fig. 2B). A detailed kinetic analysis revealed that both c-Fos and Fra-1 proteins were induced and recruited to the IL-8 promoter in response to IL-1, c-Fos slightly preceding the maximum of Fra-1 recruitment (Fig. 2C–F). Quantification of Western blot and ChIP results showed that the time-delayed IL-1-inducible recruitment of c-Fos and Fra-1 to the IL-8 promoter exactly mimicked the inducible expression pattern of both proteins (Fig. 2D and F). We also observed some constitutive binding of Fra-1 to the IL-8 promoter (Fig. 2E), whereas there was very little c-Fos binding in unstimulated cells (Fig. 2E). Again, this was reflected at the protein level, as in whole cell lysates we observed a weak constitutive expression of a faster migrating form of Fra-1, whereas c-Fos expression was undetectable (Figs. 1C, 2C, and 3C). Importantly, PD98059 suppressed significantly the IL-1-inducible recruitment of c-Fos and Fra-1 to the endogenous IL-8 promoter by about 30–50% (Fig. 2G and H).

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c-Fos and Fra-1 Have Opposing Effects on IL-8 Transcription—To assess the functional significance of c-Fos and Fra-1 for IL-8 transcription we expressed both proteins in HEK293IL-1R cells together with a luciferase reporter gene construct that contains the regulatory region of the IL-8 promoter as shown in Fig. 2. Expression of c-Fos, but not of Fra-1, induced IL-8 transcription (Fig. 3A). c-Fos synergized in IL-8 transcription when co-transfected with low amounts of a p65...
NF-κB expression construct. Transfection of higher amounts of the p65 NF-κB expression vector masked any synergistic effects with c-Fos due to the strong activity of its transactivation domain (results not shown). In contrast, expression of Fra-1 strongly suppressed IL-8 transcription mediated by c-Fos alone or by c-Fos plus p65 NF-κB. Moreover, c-Fos and p65 NF-κB enhanced IL-8 transcription induced by an active mutant of MEK1, an upstream activator of ERK, whereas Fra-1 inhibited it (Fig. 3B). Similarly, c-Fos induced and Fra-1 inhibited IL-8 transcription in KB cells (Fig. 3C). These data strongly suggest that c-Fos is a positive regulator, whereas Fra-1 is a negative regulator of IL-8 transcription. To further test this hypothesis, we suppressed expression of endogenous Fra-1 in KB cells by RNA interference. As shown in Fig. 3D, suppression of IL-1-induced Fra-1 protein expression (Fig. 3E) resulted in an ~2-fold increase in activity of the cotransfected IL-8 promoter reporter gene. Collectively, these strongly indicate that the MEK1-ERK pathway utilizes c-Fos and Fra-1 to modulate IL-8 transcription. Because c-Fos and Fra-1 serve distinct functions, an activated ERK pathway contributes by a positive as well as by a negative signal to IL-8 mRNA synthesis. This mechanism may serve to modulate the overall IL-8 expression level in response to various external signals.

Fra-1 Interacts with HDAC1 and Is Stabilized and Ubiquitinated in a MEK1-dependent Manner—To search for potential mechanisms of the negative regulatory role of Fra-1, we inves-
HEK293IL-1R cells were transfected with HA-MEK1R4F, FLAG-Fra-1, MYC-HDAC1, and combinations thereof or empty vector control (first lane). A and B, 24 h after transfection cells were lysed, and Fra-1 was immunoprecipitated (IP) from cell extracts. Co-precipitated Myc-HDAC1 was detected by anti-Myc antibodies. Lysates were probed with antibodies against Fra-1, Myc, and HA epitopes to detect expression of Fra-1, MYC-HDAC1, and HA-MEK1R4F, respectively. B, two different exposure times for the Fra-1 Western blot (WB) are shown to indicate the massive increase in Fra-1 protein in the presence of active MEK1R4F. IgGhc and IgGlc; heavy and light chains of immunoglobulins. C, KB cells were treated for 3 h with IL-1 (10 ng/ml) or left untreated. Thereafter cells were lysed, and HDAC1 was immunoprecipitated. Co-precipitated Fra-1 was detected by Western blotting. Expression of Fra-1 and HDAC1 was confirmed by Western blots of lysates (lower panel). D, HEK293IL-1R cells were transfected with 12.5 μg of the expression plasmids for HA-MEK1R4F, FLAG-Fra-1, or His-ubiquitin as indicated. 48 h later cells were lysed in denaturing buffer, precipitated, and redissolved in sample buffer (Input). His-ubiquitinated proteins were purified by adsorption to Ni²⁺-Sepharose (Ni²⁺-pulldown). Proteins were separated by SDS-PAGE, and Fra-1 and MEK1R4F were detected in the input sample and the bound fraction using antibodies against Fra-1 and HA, respectively. Ubᵣ indicates the polyubiquitinated form of Fra-1.

To further assess the impact of PD98059 on the assembly of the preinitiation complex at the IL-8 promoter—To further assess the impact of PD98059 on the putative multiprotein complex that regulates IL-8 transcription.
tion, we analyzed its effects on histone acetylation, on p65 NF-κB, and on assembly of the preinitiation complex. As shown in Fig. 5 in unstimulated cells the overall acetylation level of histones H3 and H4 within the IL-8 promoter region is high and is only weakly induced by IL-1 by about 1.5–2-fold. There is also constitutive binding of HDAC1. In addition, there is a strong transient increase in binding of p65 NF-κB and of TBP, respectively. As a result IL-1 induces a 10-fold increase in polymerase II binding within 30 min. Together with the ChIP experiments shown in Fig. 2, these data suggest that strong polymerase II recruitment requires cumulative binding of c-Fos, Fra-1, p65, and TBP. Cooperative binding and dynamic exchange of factors is facilitated by nucleosome remodeling, as indicated by the substantial histone tail acetylation.

PD98059 weakly suppressed binding of HDAC1 at 8 h of IL-1 stimulation and had no significant effect on the overall histone acetylation level or on p65 NF-κB. However, in addition to preventing binding of c-Fos and Fra-1 (Fig. 2G) PD98059 significantly suppressed binding of TBP of IL-1 stimulation. Finally, PD98059 suppressed recruitment of polymerase II by 30–50% after 3 and 8 h of IL-1-stimulation. Hence, the partial inhibition of c-Fos, Fra-1, TBP, and polymerase II recruitment reflects the partial inhibition of IL-1-induced IL-8 mRNA as observed in the experiments shown in Fig. 1. Collectively, these results provide a mechanism for the downstream action of PD98059 and suggest that c-Fos and Fra-1 serve to fine-tune IL-8 transcription.

Regulation of IL-8 Expression by EGF—To further follow this hypothesis, we investigated the regulation of IL-8 gene expression by EGF, which like IL-1 is also a physiological inducer of IL-8. However, EGF causes only a 10–20-fold increase in IL-8 expression, which is significant but much weaker than that obtained with IL-1 (Fig. 6A). A direct comparison revealed that IL-1 activates transcription from an IL-8 reporter gene construct strongly, more than 20-fold, through the NF-κB and AP-1 cis elements (Fig. 6B), whereas EGF induces IL-8 transcription weakly by about 2–3-fold and mainly through the AP-1 site (Fig. 6B). This difference does not reflect an inadequate response of the cells to EGF as c-Fos and Fra-1 proteins were induced by EGF with the same strength and kinetics compared with IL-1 (compare Fig. 1C with Fig. 7A). As for IL-1, the EGF-induced expression of c-Fos and Fra-1 were suppressed by PD98059 as was their EGF-induced recruitment to the IL-8 promoter (Fig. 7).

In contrast to IL-1, EGF-induced only a weak, delayed recruitment of p65 NF-κB to the IL-8 promoter (Fig. 8) that was

![Fig. 5. Effect of PD98059 on the IL-8 enhanceosome. KB cells were left untreated or pretreated with PD98059 (50 μM) for 1 h. Where indicated cells were stimulated for the indicated times with IL-1 (10 ng/ml). A, histone acetylation (Ac) and recruitment of the indicated proteins were determined by ChIP, and PCR products were resolved by agarose gel electrophoresis and detected by ethidium bromide staining. B, DNA from the ChIP experiment shown in A was also amplified using real-time PCR. Serial dilutions of chromatin inputs were used to quantify absolute amounts of PCR products. Data are expressed as -fold change relative to the unstimulated control. Gray bars indicate additional treatment with PD98059. Shown is one representative out of two experiments.](http://www.jbc.org/)
not affected by PD98059 (Fig. 8). In agreement with its low potency to induce IL-8 expression and p65 NF-κB recruitment, EGF induced only a modest increase in polymerase II binding, which was almost completely inhibited by PD98059 at 0.5 h and 24 h of treatment (Fig. 9, A and B). Finally, we found that the EGF-induced IL-8 secretion was significantly suppressed by PD98059 (Fig. 9C), suggesting that EGF-induced IL-8 gene expression is almost entirely controlled by the ERK pathway via c-Fos and Fra-1. Collectively, the data shown in Figs. 6–9 suggest that EGF activates IL-8 weakly because it mainly utilizes the AP-1 site of the IL-8 promoter and provides a molecular mechanism that explains the weaker ability of EGF compared with IL-1 to induce the endogenous IL-8 gene.

**DISCUSSION**

The transcription factor AP-1 was one of the first mammalian transcription factors to be identified, but its physiological functions are still being uncovered. The precise mechanisms by which AP-1 activates or represses genes in response to external challenge, the target genes that mediate AP-1 function, and the relevance of AP-1 for human disease are still not clear. Furthermore, until recently research on AP-1 has been centered around the role of c-Jun and c-Fos in cell death and survival (1, 2, 6).

**FIG. 6.** Quantitative differences in IL-8 gene expression correlate with differential usage of AP-1 and NF-κB sites in response to IL-1 or EGF. In A, KB cells were left untreated (white bars) or were stimulated for 24 h with IL-1 (10 ng/ml, black bars) or EGF (50 ng/ml, gray bars). IL-8 protein secreted into the cell culture supernatant was determined by specific enzyme-linked immunosorbent assay. Shown are the mean IL-8 levels from three independent experiments ± S.E. In B, HeLa cells were transiently transfected as indicated with 5 μg of empty vector and 0.25 μg of the IL-8 reporter plasmid pUHC13.3.IL-8 promoter luc (wt) or versions mutated in the AP-1, the NF-κB, or both sites. After 43 h cells were left untreated (white bars) or stimulated for 5 h with IL-1 (10 ng/ml, black bars) or EGF (50 ng/ml, gray bars). Then cells were lysed, and luciferase activity was determined. Shown are the mean luciferase activities (RLU) ± S.E. from three independent experiments.

**FIG. 7.** EGF strongly induces c-Fos and Fra-1 proteins and their recruitment to the endogenous IL-8 promoter in a PD98059-dependent manner. KB cells were stimulated for the indicated times with EGF (50 ng/ml). Where indicated cells were treated with PD98059 (50 μM) for 1 h or left untreated. Thereafter cells were lysed in SDS sample buffer (A), proteins were separated by SDS-PAGE, and c-Fos and Fra-1 proteins were detected by Western blotting. In B chromatin from cells treated as in A was prepared, and recruitment of c-Fos or Fra-1 to the endogenous IL-8 promoter was detected by chromatin immunoprecipitation using c-Fos and Fra-1 antibodies as described under "Experimental Procedures." In C, PCR products of c-Fos and Fra-1 ChIP experiments were quantified by real time PCR. Shown is one representative out of two experiments. Gray bars represent treatment with PD98059.

**FIG. 8.** Weak EGF-induced recruitment of p65 NF-κB to the IL-8 promoter. KB cells were treated as described in the legend of Fig. 7, and the recruitment of p65 NF-κB to the IL-8 promoter was determined by ChIP. PCR products were visualized by ethidium bromide staining after gel electrophoresis (A). In B the intensities of bands were quantified, and the results from four ChIP experiments are expressed as mean fold change ± S.E. Gray bars represent treatment with PD98059.

In this study we have focused on the role of AP-1 in regulation of the human chemokine interleukin-8 by the proinflammatory cytokine interleukin-1. Both IL-1 and IL-8 are central...
for the inflammatory response, and elucidating the mechanisms by which IL-1 regulates IL-8 provides a model of general significance for the expression of inflammatory genes. As reported recently, we have not found a role for c-Jun in IL-1-inducible IL-8 secretion as assessed by cell-permeable peptides that inhibit c-Jun NH$_2$-terminal kinase-mediated c-Jun phosphorylation or by RNAi-mediated c-Jun suppression (24).

In this study we have focused on the role of c-Fos and Fra-1 in regulation of the interleukin-8 gene. c-Fos (encoded by fos) and Fra-1 (encoded by fos-like gene 1, fosl1) show homology in their leucine zipper and DNA binding domains, but outside of these domains both proteins show little similarity (31). Both, c-Fos and Fra-1 have long been known as growth factor regulated genes (32–35), and likewise c-Fos was found a long time ago to be induced by the proinflammatory cytokines IL-1 and tumor necrosis factor (36, 37).

Despite this fact, the role of c-Fos in IL-1-mediated gene expression has not been defined. We found that c-Fos is recruited to the endogenous IL-8 promoter in an IL-1-dependent manner, transactivates an IL-8 promoter-driven reporter gene, and synergizes in this effect with p65 NF-kB, a known positive regulator of IL-8 transcription (10). Moreover, IL-1-inducible expression of c-Fos was completely suppressed by the ERK pathway inhibitor PD98059, suggesting that IL-1 induces c-Fos mainly through ERK (Fig. 1). We also found that PD98059 suppressed IL-1-induced shifts in the apparent $M_r$ of c-Fos on SDS-PAGE (Fig. 1). Presumably, this shift is caused by multisite phosphorylation of the carboxyl-terminal transactivation domain of c-Fos as observed by many groups (30, 38–40), and in fact two reports have recently reported that c-Fos is a direct substrate of ERK in isolation and in vivo (30, 40). Phosphorylation potentiates c-Fos-mediated transactivation of AP-1 reporter genes (30, 40). In agreement with these data we show here that selective activation of the ERK pathway by active MEK1 synergizes with coexpressed c-Fos to induce IL-8 transcription (Fig. 3B). Thus, our data clearly show that a MEK1-ERK-c-Fos pathway links the IL-1 receptor to the IL-8 promoter.

In contrast to c-Fos, very little is known about a role of Fra-1 in IL-1 signaling. The induction of Fra-1 by IL-1 occurs mainly in the nucleus and is very strong and slightly delayed compared with that of c-Fos (Fig. 3, 5). A possible explanation for this delay in expression might be that c-Fos is required for cytokine-induced Fra-1 transcription, a mechanism that has been so far only observed in fibroblasts or osteoclasts by conditional activation of c-Fos-ER fusion proteins (41, 42) or during mouse osteoclast differentiation induced by RANKL (42).

As for c-Fos, PD98059 completely suppressed IL-1-induced Fra-1 mRNA and protein synthesis, suggesting that Fra-1 expression is controlled by ERK (Figs. 3 and 4). This finding is in agreement with findings in other systems where PD98059 suppressed Fra-1 expression mediated by the mitogen lysophosphatidic acid or conditional activation of the ERK pathway by RAP or MEK1 mutants, which also activate Fra-1 gene expression (35, 43). Another study recently implied the c-Jun NH$_2$-terminal kinase pathway in tumor necrosis factor-mediated Fra-1 expression (44). Our results clearly show that ERK is essential for IL-1-inducible Fra-1 expression but do not rule out an additional role of other IL-1-induced signaling pathways such as c-Jun NH$_2$-terminal kinase.

Apart from its role in bone metabolism and certain aspects of tumor biology, little is known about Fra-1 functions or about putative Fra-1 target genes (41, 45–48). Fra-1 transgenic mice develop osteosclerosis (49), whereas Fra-1 ablation in mice is embryonic lethal (50). Interestingly, Fra-1 insertion into the c-Fos locus can fully rescue the osteopetrosis phenotype caused by c-Fos gene inactivation in mice (51, 52). In contrast, Fra-1 cannot substitute for c-Fos target genes in mouse embryonic fibroblasts (51). These data suggest that both proteins may have overlapping but also distinct functions.

Controversy exists about the transactivation potential of Fra-1 and the molecular basis for its actions. Several reports have found that Fra-1 lacks an autonomous transcriptional activation domain (41, 46, 48). In agreement with this, Fra-1 repressed an AP-1 reporter gene induced by c-Fos, c-Jun, or TPA, and it was concluded that Fra-1 counteracts AP-1 activation in tumor promotion (53). However, Fra-1 knock-in rescues the c-Fos$^{-/-}$ phenotype in vivo, and it is hard to reconcile how Fra-1 can compensate for loss of c-Fos if it were only a negative regulator of transcription (45, 51). Thus, it is likely that Fra-1 can also positively regulate gene expression. In fact, others have found that GAL4-Fra-1 fusion proteins can be stimulated by ERK pathway activation (25, 54, 55). Furthermore, the transcriptional activity of a tethered Fra-1-c-Jun heterodimer...
was higher than that of a c-Jun homodimer and varied among three distinct promoter constructs tested, suggesting that the capacity of Fra-1 to activate gene transcription is promoter-dependent (56).

We report here that Fra-1 strongly inhibits IL-8 transcription in response to coexpressed c-Fos or p65 NF-κB or both (Fig. 6A). Furthermore, MEK1-induced IL-8 transcription is potentiates by coexpressed c-Fos or p65 NF-κB but strongly inhibited by Fra-1 (Fig. 6B). These data clearly show that Fra-1 is a negative regulator of IL-8 transcription. Thus, apparently IL-1 induces the Fra-1 protein, which in turn limits IL-8 gene expression. Thus, our data support the model of Fra-1 as a negative regulator of gene expression (53) and highlight the importance of analyzing the specific contribution of Fra-1 to expression of individual genes. Our data propose a negative regulatory role for Fra-1 in regulation of a natural chemokine promoter and thereby identify a gene relevant for innate or adaptive immune responses as a bona fide Fra-1 target gene. We also demonstrate that at least for the IL-8 gene, c-Fos and Fra-1 have distinct functions. As these findings contrast with the seemingly functional equivalence of c-Fos and Fra-1 in vivo (45, 51), it is tempting to speculate that in mouse transgenic models differences in c-Fos and Fra-1 function may only become apparent after inflammatory or infectious challenge of the animals.

Fra-1 repression is likely to involve several mechanisms. First, an AP-1 complex containing Fra-1 may displace a transcriptionally active AP-1 heterodimer containing c-Fos at the AP-1 site of the IL-8 promoter. Although we have so far not found recruitment of c-Jun (data not shown), we have preliminary evidence that at the early times of IL-1 stimulation JunB binds to the IL-8 promoter, whereas later an increase in JunD is found.2

Second, we report here that Fra-1 interacts with histone deacetylase 1 (HDAC). The interaction of HDAC1 with Fra-1 may contribute to the overall level of HDAC1 recruitment to the IL-8 gene. In agreement with that we found that PD98059, which suppresses Fra-1 expression, also suppressed HDAC1 binding to the IL-8 promoter after 8 h of IL-1 stimulation. We also observed an interaction of p65 NF-κB with HDAC1 (data not shown), confirming the results of others (29). Of note, HDAC1 is present at the IL-8 promoter already in unstimulated cells. It is, therefore, likely that other factors in addition to Fra-1 and p65 NF-κB contribute to HDAC1 recruitment.

Similar to the interaction of Fra-1 with HDAC1 as reported here, it was shown recently that the interaction of c-Jun with a histone deacetylase mediates gene repression (57). Posttranslational modifications of c-Jun by its upstream activator c-Jun NH2-terminal kinase result in dissociation of the interaction and thereby activate c-Jun-mediated transactivation (57). In the presence of active MEK1 the interaction of coexpressed Fra-1 with HDAC1 was enhanced (Fig. 4). Active MEK1 drastically stabilizes Fra-1 and produced species of higher Mr, detectable on SDS-PAGE (Fig. 4). Equally, IL-1 induced a mobility shift of endogenous Fra-1 that was PD98059-sensitive (Figs. 1 and 2). Both types of experiments indicate that modifications of Fra-1 largely depend on the MEK1-ERK pathway. These modifications may represent phosphorylation of Fra-1, which was recently described in Ras-transformed thyroid cells (55). However, the experiment shown in Fig. 4 provides the first evidence that active MEK1 induces strong polyubiquitination of ectopically expressed Fra-1. This mechanism may help to stabilize the protein. In support of our findings, ERK was recently found to protect Fra-1 against proteasomal degrada-

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2 E. Hoffmann, unpublished data.
IL-8 mRNA synthesis, a positive via c-Fos and a negative via Fra-1. The substance does not affect p65 NF-κB. Hence, on balance PD98059 can only partially suppress IL-1-inducible IL-8 transcription, as exemplified by its partial inhibition of recruitment of key molecules of the preinitiation complex, namely TBP and polymerase II (Fig. 5). As a net effect PD98059 leads to the partial inhibition of IL-1-induced synthesis of IL-8 mRNA and protein that was the starting point for this study (Fig. 1) and cannot suppress IL-1-induced IL-8 mRNA expression completely (Fig. 1A). Another prediction from these experiments was that sole activation of ERK and subsequent c-Fos and Fra-1 expression should not result in maximal IL-8 expression levels. To test the physiological significance of this hypothesis more directly, we analyzed the transcriptional regulation of the IL-8 gene by EGF. Compared with IL-1, EGF is a weak inducer of IL-8 (Fig. 6). In contrast, Fra-1 and c-Fos genes were induced by EGF with a strength and kinetics comparable with that obtained with IL-1 (Fig. 7A). EGF induces c-Fos and Fra-1 recruitment to the endogenous IL-8 promoter followed by a weak recruitment of RNA polymerase II (Figs. 7 and 9). In addition, EGF-induced IL-8 transcription was largely dependent on the AP-1 rather than on the NF-κB site (Fig. 6B). In line with this, EGF failed to cause a strong and rapid recruitment of p65 NF-κB to the endogenous IL-8 promoter (Fig. 8). Finally, the EGF-induced expression of c-Fos and Fra-1, the recruitment of polymerase II, and the activation of the endogenous IL-8 gene were significantly suppressed by PD98059, suggesting that regulation of IL-8 by EGF is almost entirely controlled by the MEK1-ERK-AP-1 pathway (Figs. 7 and 9). Thus, the data in this study provide a model that explains how two different inducers of IL-8, i.e. IL-1 or EGF, activate the gene to different levels within the same cell.

Chemokine-dependent leukocyte recruitment is required during all phases of wound healing (62). The molecular mechanism of IL-8 gene expression described here is likely to be of (patho)physiological importance as extensive leukocyte infiltration during the initial phase of tissue damage requires high levels of IL-8 and other chemokines induced by proinflammatory cytokines such as IL-1, whereas at later stages during wound healing chemokine production may be mainly under the control of locally produced growth factors such as EGF. In this phase lower amounts of leukocytes are likely to be required for tissue remodeling (62).

Collectively the data presented in this study define a hitherto unrecognized important role of Fra-1 in IL-1-mediated gene expression and also demonstrate how the ERK MAP kinase pathway modulates p65 NF-κB-mediated chemokine gene expression via c-Fos and Fra-1. We further demonstrate that an inflammatory gene can be expressed within the same cell to various levels in response to physiological stimuli by combinatorial usage of two signaling pathways (ERK and NF-κB) that modulate the expression and the activity of positive and negative transcriptional regulators (Fig. 10).

The number of signaling pathways that is utilized by infectious or inflammatory stimuli to induce hundreds of inflammatory genes is small, mainly composed of NF-κB and the MAP kinases ERK, c-Jun NH2-terminal kinase, and p38 (7). The time-delayed expression of Fra-1 may, therefore, also represent a "dampening" or "switch off" mechanism relevant for many other inflammatory proteins whose levels of expression are under control of the NF-κB and MAP kinase pathways.

Acknowledgments—We are grateful to Eiisuke Nishida, Bernhard Luecher, Natalie Ahn, Hermann Bujard, and Peter Angel for the gift of valuable reagents and to Matthias Gaestel, Thomas F.Schulz, and Helmut Holtmann for critically reading this manuscript.

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MEK1-dependent Delayed Expression of Fos-related Antigen-1 Counteracts c-Fos and p65 NF-κB-mediated Interleukin-8 Transcription in Response to Cytokines or Growth Factors

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J. Biol. Chem. 2005, 280:9706-9718.
doi: 10.1074/jbc.M407071200 originally published online December 21, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407071200

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