Induction of Interleukin-8 Synthesis Integrates Effects on Transcription and mRNA Degradation from at Least Three Different Cytokine- or Stress-Activated Signal Transduction Pathways

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A hallmark of inflammation is the burst-like formation of certain proteins, initiated by cellular stress and proinflammatory cytokines like interleukin 1 (IL-1) and tumor necrosis factor, stimuli which simultaneously activate different mitogen-activated protein (MAP) kinases and NF-κB. Cooperation of these signaling pathways to induce transcription of IL-8, a prototype chemokine which causes leukocyte migration and activation, was investigated by expressing active and inactive forms of protein kinases. Constitutively active MAP kinase kinase 7 (MKK7), an activator of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway, induced IL-8 synthesis and transcription from a minimal IL-8 promoter. Furthermore, MKK7 synergized in both effects with NF-κB-inducing kinase (NIK). Activation of the IL-8 promoter by either of the kinases required functional NF-κB and AP-1 sites. While NIK and MKK7 did not affect degradation of IL-8 mRNA, an active form of MKK6, which selectively activates p38 MAP kinase, induced marked stabilization of the transcript and further increased IL-8 protein formation induced by NIK plus MKK7. Consistently, the MAP kinase kinase kinase MEKK1, which can activate NF-κB, SAPK/JNK, and p38 MAP kinases, most potently induced IL-8 formation. These results provide evidence that maximal IL-8 gene expression requires the coordinate action of at least three different signal transduction pathways which cooperate to induce mRNA synthesis and suppress mRNA degradation.

Interleukin-8 (IL-8) is a member of the still-growing family of chemokines, cytokines whose main function is to attract and activate leukocytes (2). It plays a significant role in recruiting leukocytes to sites of acute inflammation. On the other hand, excessive amounts of locally produced IL-8 can have deleterious effects (2, 45). Expectedly, therefore, IL-8 gene expression is tightly controlled at several levels (45). IL-8 synthesis, low or undetectable in normal noninflamed tissue, can be induced in vivo as well as in a wide variety of cells in vitro by proinflammatory cytokines such as IL-1 or tumor necrosis factor (TNF), MAP kinases required functional NF-κB and AP-1 sites. While NIK and MKK7 did not affect degradation of IL-8 mRNA, an active form of MKK6, which selectively activates p38 MAP kinase, induced marked stabilization of the transcript and further increased IL-8 protein formation induced by NIK plus MKK7. Consistently, the MAP kinase kinase kinase MEKK1, which can activate NF-κB, SAPK/JNK, and p38 MAP kinases, most potently induced IL-8 formation. These results provide evidence that maximal IL-8 gene expression requires the coordinate action of at least three different signal transduction pathways which cooperate to induce mRNA synthesis and suppress mRNA degradation.

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KINASE INVOLVED IN IL-8 TRANSCRIPTION AND mRNA STABILITY

IONs can be directly activated by MEKK1 (28, 29, 47), a mitogen-activated protein (MAP) kinase kinase kinase which activates the three best-characterized MAP kinases, namely, extracellular regulated kinase (ERK) (26), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (31, 66), and p38 MAP kinase (15, 22, 31). The transcriptional regulator AP-1 is a dimer composed of Fos, Jun, ATF-2, and other family members (reviewed in references 20 and 62). In contrast to NF-κB, AP-1 proteins are usually constitutively bound to their cognate DNA elements. Transcriptional activity of AP-1 proteins is regulated by their abundance, by phosphorylation of transcriptional domains, and by their binding to protein kinases (20, 62). Protein kinases activating AP-1 include the ERKs, SAPK/JNK, p38 MAP kinases (20, 62), and a partially characterized Fox kinase (11).

Despite the rapid progress in identifying stress-induced signaling pathways and, on the other hand, structural elements important in transcriptional activation, there is little information on how different signaling pathways interact with each other in order to mediate a particular biological response, such as expression of a gene like that encoding IL-8. In that context, it is of importance to determine not only how stress kinase pathways cooperate to regulate promoter activity but also how they affect steps other than transcription in the overall process of gene expression.

In this study, we investigated the contribution of NF-κB and stress-activated protein kinase cascades to IL-8 transcription, mRNA stability, and protein formation by overexpressing selective upstream activators for each pathway. We provide evidence for coordinated but distinct function of each of the pathways in IL-8 gene expression.

MATERIALS AND METHODS

Cells and Materials. KB and HEK-293 cells were obtained from the American Type Culture Collection. HeLa cells stably transfected with plasmid pHU51-1 expressing the HIV-1 nuclear matrix protein were a gift from H. Bujard, University of Heidelberg. Cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum. E64 ([trans-epoxysuccinyl]-leucylamido-(4-guanidino)butane), pepstatin, leupeptin, PMSF (phenylmethylsulfonyl fluoride), and all other chemicals were from Sigma; γ-32P-ATP was purchased from Hartmann Analytics. Antiserum SAK14 to the N terminus of NIK, raised in rabbits immunized with the peptide VMEAMYAP-GAPOSAVGOQ KEKL, was a kind gift of Jeremy Saklatvala, Kennedy Institute of Rheumatology, London, England. M2 antibodies against the Flag epitope (M2-agarose beads and Bio-M2) were from Kodak; antibodies 12CA5 against the hemagglutinin (HA) epitope and 9E10 against the C-MyC epitope were from Boehringer Mannheim. Epidermal growth factor (EGF) and horseradish peroxidase-conjugated secondary antibodies against mouse, rabbit, and rat immunoglobulin G (IgG) were from Sigma. Protein A-, protein G-, and glutathione (GSH)-coupled secondary antibodies against mouse, rabbit, and rat immunoglobulins were from Sigma. Protein A– and protein G–agarose beads were from Sigma and Upstate Biotechnology, respectively.

Transfections and preparation of cell extracts. Cells (1 × 106 to 2.5 × 107/well) were seeded into six-well plates. The next day, transfections were performed in triplicate with the calcium phosphate method. KB cells were transfected by the DEAE-dextran (Boehringer Mannheim) method according to the manufacturer’s instructions. In all transfections, DNA amounts were kept constant by adding empty expression plasmids. After 24 h, the medium was changed and cells were incubated further for 24 h. Cells from one triplicate transfection were plated on iced; the medium was removed, and cells were washed once in phosphate-buffered saline and scraped in phosphate-buffered saline. For determination of reporter gene activity, cells were lysed in ice-cold potassium phosphate buffer (100 mM, pH 7.4), containing 0.2% Triton X-100, 1 mg of pepstatin per ml, 1 μg of leupeptin per ml, 1 mM PMSF, 0.5 mM E64, 300 μM PMSE, 0.5 μg of pepstatin per ml, 5 mM dithiothreitol (DTT), 400 mM okadaic acid, 20 mM β-glycerophosphate (whole-cell lysis buffer). The pellet was resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 0.3 mM Na3VO4, freshly added 200 μM leupeptin, 10 μM E64, 300 μM PMSE, 0.5 μg of pepstatin per ml, 5 mM dithiothreitol [DTT], 400 mM okadaic acid, 20 mM β-glycerophosphate). The pellet was resuspended in buffer A containing 0.1% Nonidet P-40. After centrifugation at 10,000 rpm for 5 min at 4°C, supernatants were taken as cytosolic extracts. Pellets were resuspended in buffer B (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.3 mM Na3VO4, 20 mM β-glycerophosphate, 200 μM leupeptin, 0.5 μg of pepstatin per ml, 10 μM E64, 400 mM okadaic acid). After 1 h on ice, nuclear extracts were cleared at 10,000 × g for 5 min at 4°C and supernatants were collected. Protein concentration of cell extracts was determined by the Bradford method, and samples were stored at 20°C.

Immunoprecipitation and Western blotting. One milligram of whole-cell extract protein from cells transfected with plasmids encoding Flag-tagged or Myc-tagged proteins was diluted in 500 μl of immunoprecipitation buffer (20 mM Tris [pH 7.3], 154 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1% Triton X-100). Samples were incubated for 4 h with 20 μl of M2-Flag (M2-agarose beads or with 2 μg of anti-Myc antibody 9E10 to which 20 μl of protein G-Sepharose was added. Beads were spun down three times, washed in 500 μl of immunoprecipitation buffer, and resuspended in 40 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (2% SDS, 25 mM Tris-HCl [pH 6.8], 1% β-mercaptoethanol, 6% glycerol, 0.02% bromophenol blue). Proteins were eluted from the beads by boiling for 5 min, separated by SDS-PAGE on a 7.5 or 10% gel and electroblotted to polyvinylidene difluoride membranes (Immobilon; Millipore). After blocking with 5% dried milk in Tris-buffered saline overnight, membranes were incubated for 4 to 24 h with primary antibodies, washed in Tris-buffered saline, and incubated for 2 to 4 h with the peroxidase-conjugated secondary antibody. Proteins were detected by using the Amersham enhanced chemiluminescence system.

SAPK/JNK assay. The assay was performed as previously described (24, 49). Briefly, 10 μl of whole-cell extract containing 30 μg of protein was incubated with 10 μl of kinase buffer (150 mM HEPES, pH 7.5, 10 mM MgCl2, 60 μM ATP, 4 μCi of [γ-32P]ATP). After 15 min at room temperature, 10 μl of GSH-beads, equilibrated in whole-cell lysis buffer containing 1 mM DTT, was added. Samples were diluted for 30 min at room temperature. Beads were recovered by centrifugation at 10,000 × g for 5 min in 200 μl of whole-cell lysis buffer. Bound GST-Jun was eluted from the beads by boiling for 5 min in SDS-PAGE sample buffer. After centrifugation at 10,000 ×
g for 5 min, supernatants were separated by SDS-PAGE on a 10% gel. Equal recovery of GST-Jun was confirmed by Coomasie staining.

Electrophoretic mobility shift assay (EMSA). A double-stranded oligonucleotide containing the NF-κB consensus sequence (5’-gagagagGG ACTTCCGagaga3’) was end labeled by using [γ-32P]ATP and T4 polynucleotide kinase and purified by gel filtration on S-200 spin columns (Pharmacia). Protein-DNA binding reactions were performed with 5 to 20 μg of whole-cell or nuclear extract protein, labeled oligonucleotide, and 1 μg of poly(dI-dC) in 10 mM Tris (pH 7.4)–10 mM EDTA–0.5% (wt/vol) dextran nonfat milk–0.5 M NaCl–10 mM DTT–50% glycerol in a total volume of 10 μL. After incubation at room temperature for 30 min, protein-DNA complexes were resolved by PAGE on a 4% gel and visualized by autoradiography.

RNA stability measurements. HeLa cells constitutively expressing the tet transactivator protein (14) were seeded into 9-cm-diameter petri dishes (5 × 10^6 cells per dish). The next day, cells were transfected by the calcium phosphate method and in synergy with NIK.

3 activator protein (14) were seeded into 9-cm-diameter petri dishes (5
by endogenous NIK. The extent of IL-8 induction and NF-κB activation by empty pCDNA3 vector reproducibly surpassed that of empty pCS3MT. Therefore, for subsequent experiments NIK and NIK(KK429-430AA) were recloned into pCS3MT.

Levels of IL-8 formed in response to activation of the SAPK/JNK and NF-κB pathways by MKK73E (Fig. 2) and NIK (Fig. 3), respectively, were lower than those in cultures of cells transfected with an expression vector for a constitutively active form of MEKK1 (Fig. 3C). The low induction of IL-8 by NIK could not be ascribed to insufficient activation of NF-κB, since its extent was similar to that induced by MEKK1 (see Fig. 5C). Considering that MEKK1 can activate SAPK/JNK as well as NF-κB pathways, we asked whether both pathways might synergize to induce IL-8. As shown in Fig. 4A, coexpressing NIK and MKK73E induced supra-additive formation of IL-8 protein. This could not be ascribed to increased expression levels of NIK and MKK7 (Fig. 4B), since amounts of both kinases were similar in single and combined transfections (weaker intensity of the NIK band in the cotransfection in the particular experiment shown in Fig. 4B was not reproduced in other experiments [see also Fig. 5 and 7].
MKK7 and NIK selectively activate SAPK/JNK and NF-κB, respectively. Since both NIK and MKK7E triggered IL-8 formation, it was important to determine whether they acted via the same or different downstream effector molecules. Furthermore, since the combined effect of NIK and MKK7E on IL-8 formation was still far below that of MEKK1 (compare IL-8 concentrations in Fig. 3C and 4A), it was of interest to determine whether this was based on different intensities of signals induced. Therefore, activation of signaling mechanisms by MKK7 and NIK alone and in combination, as well as by MEKK1, were assayed. Compared to cells transfected with vector alone, expression of MKK7E resulted in marked activation of SAPK/JNK2 (Fig. 5A and B). No significant influence on SAPK/JNK activity was observed by expressing inactive MKK7A or active or inactive forms of NIK. Cotransfection of NIK did not significantly influence MKK7E-induced SAPK/JNK activation. Of note, MEKK1 did not significantly influence MKK7E-induced SAPK/JNK activation. Of note, MEKK1 clearly is more active than MKK7E in activating SAPK/JNK, suggesting that a more efficient trigger of that pathway, in combination with NIK, would give rise to stronger formation of IL-8. As no other selective activator for SAPK/JNK is available at present, this cannot be tested directly. Determination of NF-κB activity in EMSA, performed in parallel for the same cultures (Fig. 5C), showed that the active form of NIK strongly induced complex formation with the labeled oligonucleotide, while NIK(K429-430AA) as well as both forms of MKK7 were inactive in that respect. Furthermore, the active MKK7E did not affect the extent of NF-κB activation by NIK. Note that MEKK1-induced NF-κB activation is not stronger but comparable to NIK-induced activation. This argues against insufficient NF-κB activation by NIK as an explanation for its low IL-8 induction. Taken together, these results confirm selective activation of the SAPK/JNK pathway by MKK7 and of the NF-κB pathway by NIK, thus arguing against induction of IL-8 by MKK7 through cross-activation of NF-κB.

MKK7 and NIK each require NF-κB and AP-1 cis elements and synergize to activate a minimal IL-8 promoter. To further delineate the mechanisms involved in MKK7-induced IL-8 formation, we studied its effect on the transcriptional activity of a minimal IL-8 promoter, containing the AP-1 and NF-κB binding sequences (43, 64), placed upstream of a luciferase cDNA (Fig. 6A). MKK7E induced a threefold increase in luciferase expression.
activity, comparable to that induced by active NIK (Fig. 6B). In agreement with other studies, the induction of a synthetic promoter consisting of a 5-fold repeat of a consensus NF-κB site by NIK was much more pronounced (about 10-fold [data not shown]), arguing for distinct requirements for induction of the minimal IL-8 promoter. The dominant negative mutant NIK(KK429-430AA) slightly but reproducibly suppressed activity compared to vector-transfected cells (Fig. 6B and C), consistent with its suppression of IL-8 formation (Fig. 3C). The inactive MKK73A did not have a significant effect (Fig. 6B). Coexpression of both NIK and MKK73E had a synergistic effect (Fig. 6C). Consistent with its induction of high levels of IL-8 (Fig. 3C), MEKK1 induced much higher levels of luciferase activity than the active form of MKK7 or NIK (Fig. 6D). Mutation of the AP-1 site or the NF-κB site resulted in a strong decrease in basal activity and in a loss of inducibility by a combination of NIK and MKK73E (Fig. 6D), as well as by each of them alone (not shown). Thus, unexpectedly, each of the kinases assayed requires the presence of both sites for efficient stimulation of transcription. Furthermore, basal activity in this system appears to involve NF-κB activity and both sites as well. Activation of the mutated promoters in MEKK1-transfected cells was also strongly reduced but still clearly discernible. It is not clear at present whether this is due to quantitative differences in SAPK/JNK activation or triggering of additional signaling mechanisms by MEKK1.

Considering the evidence for some basal NF-κB-dependent IL-8 transcription (Fig. 6B and C) and protein formation (Fig. 3C), its role in MKK7-induced promoter activation was tested in a more direct way. As shown in Fig. 7, cotransfection of the dominant negative form of NIK(KK429-430AA) resulted in marked inhibition of the MKK73E-induced luciferase activity. On the other hand, dominant negative MKK73A only marginally interfered with active NIK-induced transcription. These data support a model in which NF-κB-induced activation of IL-8 transcription is enhanced by SAPK/JNK-induced signaling.

Low induction of IL-8 by EGF correlates with insufficient SAPK/JNK activation. The hypothesis that cooperation of the SAPK/JNK and NF-κB pathways is required for maximal IL-8 gene expression is further supported by observations in human KB cells. In these cells, IL-1 induced a more than 100-fold increase in IL-8 secretion (Fig. 8A), as well as strong activation of SAPK/JNK (Fig. 8B) and NF-κB (24). Overexpression of SAPKβ antisense RNA resulted in a strong suppression of IL-1-induced IL-8 secretion (Fig. 8A and reference 24) without affecting activation of NF-κB (24). In the same cells, EGF induced only a 10-fold increase in IL-8 secretion (Fig. 8A). EGF did not activate SAPK/JNK (Fig. 8B). Accordingly, the EGF-induced IL-8 secretion was not decreased in the cell line overexpressing SAPKβ antisense RNA (Fig. 8B). This finding suggests that the extent of IL-8 induction by EGF is limited due to its inability to sufficiently activate SAPK/JNK.

Taken together, the data obtained so far suggest that signals generated by NIK and MKK73E cooperate on the transcriptional level to generate IL-8 formation.

M KK6 contributes to IL-8 induction by stabilizing its mRNA. In addition to activation of transcription, posttranscriptional mechanisms contribute to the induction of IL-8 gene expression (6, 21, 23, 58, 59, 61) and may be regulated by these protein kinase pathways. We therefore investigated the role of NIK and MKK7 in IL-8 mRNA degradation. To compare the half-life of IL-8 mRNA in kinase-activated cells to that in control cells (which express only spurious amounts of the mRNA), it was necessary to transflect cells with a plasmid expressing the IL-8 mRNA. Fusion of a 194-nucleotide fragment of the CAT gene to its 5′ UTR allowed us to distinguish the ectopically expressed mRNA by size from the endogenous IL-8 mRNA induced by active kinases. To avoid the use of a general transcriptional inhibitor like actinomycin D, the cDNA was placed under the control of a tetracycline-regulated promoter, which allows rapid and selective inhibition of transcription (14).

HeLa cells constitutively expressing the tet transactivator

FIG. 4. Synergistic activation of IL-8 secretion by coexpression of NIK and MKK73E. (A) HEK-293 cells were transfected with 5 μg of empty vector, pCS3MT-MKK73E, pCDNAflag3NIK, or both; 48 h later, IL-8 secretion into the cell culture supernatant was determined by ELISA. Shown are means ± SEM from three independent experiments performed in triplicate. (B) Expression levels of MKK7 and NIK from one experiment were analyzed by immunoprecipitation (IP) from 1 mg of cell extract protein followed by Western blotting using antibodies against the Myc and Flag epitope tags, respectively (IgG hc, IgG heavy chain).
were transiently transfected with the CAT–IL-8 plasmid together with empty vector or with expression vectors for the different active forms of kinases. Following inhibition of transcription by adding the tetracycline analogue doxycycline, the CAT–IL-8 mRNA rapidly decayed in cells cotransfected with empty vector (half-life of \( \approx 20 \) min [Fig. 9]). Cotransfection of plasmids encoding active forms of MKK7 or NIK did not affect RNA degradation. In sharp contrast, cotransfection with MEKK1 resulted in pronounced stabilization of the RNA (half-life of \( \approx 80 \) min [Fig. 9]). In addition to activation of NF-κB and SAPK/JNK pathways, MEKK1 has been shown to activate p38 MAP kinase through the MAP kinase kinase MKK4/SEK1 (15, 22). MKK4/SEK1 also activates SAPK/JNK (66). For that reason, we tested the involvement of p38 MAP kinase in IL-8 mRNA degradation by using an active form of the p38-activating kinase MKK6, MKK6E2E. MKK6 specifically activates p38 MAP kinase but not ERK or SAPK/JNK MAP kinases (50). Expression of MKK6E2E increased the stability of the CAT–IL-8 mRNA comparable to that induced by MEKK1 (Fig. 9). Similar results were obtained with authentic IL-8 mRNA lacking the CAT cDNA insertion, when the amount of endogenous IL-8 mRNA (which comigrates with it in Northern blots) was subtracted (data not shown). Thus, the p38 MAP kinase pathway contributes to induction of IL-8 synthesis by stabilizing its mRNA.

In agreement with this finding, coexpression of the active MKK6E2E strongly enhanced NIK- and MKK7-induced IL-8 protein secretion while only moderately enhancing transcription. The kinase-inactive mutant MKK6K82A had no effect (Fig. 10).

Our data suggest that rapid accumulation of high levels of IL-8 transcript, a prerequisite for massive production of the protein, involves the combined effects of the SAPK/JNK and NF-κB pathways on IL-8 promoter activity and the mRNA-stabilizing effect of the p38 MAP kinase pathway.

**DISCUSSION**

Leukocyte recruitment and migration toward sites of trauma or infection is essential for innate and adaptive immune reactions. It is initiated by a family of extracellular signaling molecules, termed chemokines (2), of which IL-8 was among the first to be cloned. Control of chemokine production is a crucial step in regulating leukocyte infiltration and hence the intensity of an inflammatory process. This is reflected in the fact that IL-8 is low or absent under normal conditions but highly inducible by a wide range of extracellular stimuli, such as the proinflammatory cytokines IL-1 and TNF (5, 21, 45).

While the IL-8 gene contains a well-characterized promoter region, information on postreceptor events triggered by inflammatory cytokines to activate transcription of IL-8 is lacking. Furthermore, only limited information is available on the contribution of posttranscriptional mechanisms to IL-8 formation. In this report, we show that three distinct protein kinase cascades cooperate on different mechanistic levels to induce IL-8 expression. Appropriate forms of the upstream activators NIK, MKK7, and MKK6 were used to selectively activate the NF-κB, SAPK/JNK, and p38 MAP kinase pathways, respectively.

Transient ectopic expression of the NF-κB inducing kinase NIK was sufficient to induce secretion of IL-8 (Fig. 3 and 4) and transcription from a minimal IL-8 promoter (Fig. 6). These results complement previous data in which deletion or mutation of binding sites for NF-κB abolished responsiveness of an IL-8 promoter to IL-1, TNF, or other stimuli. However, the extent to which transfected NIK induces IL-8 expression is low compared to its strong activation of NF-κB (Fig. 3). NIK activates NF-κB as strongly as MEKK1 (Fig. 5C), by activating IKKs to comparable extents (28, 29, 47, 56). Yet MEKK1 induces a much stronger expression of IL-8 (Fig. 3 and 6). This observation suggests that additional MEKK1-activated pathways contribute to IL-8 induction.
SAPK/JNK are part of another MEKK1-activated pathway. NIK did not activate JNK2 in our experiments (Fig. 5), which is in agreement with two recent reports showing that NIK failed to activate coexpressed JNK1 (48, 56). Importantly, a gain-of-function mutant of the upstream activator of the SAPK/JNK pathway, MKK73E, was as effective as NIK in inducing IL-8 secretion (Fig. 4) and transcription (Fig. 6 and 7). This observation is not totally unexpected, since we previously reported that the SAPK/JNK pathway provides an essential signal for IL-1-induced IL-8 formation in KB cells (24). While AP-1 represents a major nuclear target for SAPK/JNK in general, previous studies have disagreed as to the importance of the AP-1 site in the IL-8 promoter. In contrast to the NF-κB site, which is essential, the AP-1 site was dispensable in some studies (45, 64), contributed only partially to IL-8 transcription (1, 16, 25, 30, 36, 43, 44, 46, 57, 64). In support of this model, simultaneous triggering of NF-κB and SAPK/JNK by NIK and MKK7 resulted in synergistic activation of IL-8 transcription and secretion (Fig. 4 and 6). MEKK1 was still far more effective in inducing IL-8 transcription and secretion than the combined NIK and MKK73E. This correlates with a much stronger activation of SAPK/JNK by MEKK1 than by MKK73E (Fig. 5B). MEKK1 activates SAPK/JNK through stimulation of both MKK7 and MKK4 (17, 27, 32, 56, 65). The combination of MKK4 and MKK7 might result in stronger activation of the SAPK/JNK pathway and consequently IL-8 gene expression than was achievable with the active MKK7 mutant alone. However, it is also possible that MEKK1 activates a third pathway enhancing IL-8 expression.

The IL-8 promoter provided a model with which to study the relative contribution of NIK- and MKK7-induced pathways to activation of a natural promoter containing a single NF-κB site and a single AP-1 site. We found that NIK and MKK7 acted through separate immediate downstream events, since NIK did not activate SAPK/JNK and MKK7 did not activate NF-κB (Fig. 5). Mutational analysis of the IL-8 promoter showed that NIK and MKK73E each required functional AP-1 and NF-κB sites for IL-8 transcriptional activation (Fig. 6). These data suggest that signals from the NF-κB and the SAPK/JNK pathways converge at the same sites on the IL-8 promoter. Two observations indicate that the MKK7 signal may serve to further enhance transcription which is activated by NF-κB, rather than inducing transcription independently: First, MKK7-in-

FIG. 6. MKK73E and NIK each require NF-κB and AP-1 cis elements and synergize to activate a minimal IL-8 promoter. (A) Schematic representation of the minimal IL-8 promoter cloned 5′ of the luciferase cDNA in pUHC13-3-IL-8pr. (B) HEK-293 cells were cotransfected with 0.25 μg of pUHC13-3-IL-8pr and with 5 μg of pCS3MT-MKK73E, pCS3MT-MKK73A, pCS3MT-NIK, or pCS3MT-NIK(KK429-430AA), 0.1 μg of pFcMEKK1 plus 4.9 μg of pCS3MT, or pCS3MT alone; 48 h after transfection, cells were lysed and luciferase (luc.) reporter gene activity was determined as described in Materials and Methods. Results are expressed as relative light units (RLU); mean ± SEM from four independent experiments performed in triplicate; P < 0.01 for comparing vector versus MKK73E and NIK. (C) HEK-293 cells were cotransfected with 0.25 μg of pUHC13-3-IL-8pr and either pCS3MT-NIK or pCS3MT-NIK(KK429-430AA) (0.5 μg of each), 5 μg pCS3MT-MKK73E alone, or a combination thereof as indicated. Amounts of DNA were kept equal by adding empty pCS3MT. Reporter gene activity (mean ± SEM from three independent experiments) was determined as for panel B. (D) HEK-293 cells were cotransfected with 0.25 μg of pUHC13-3-IL-8pr (open bars) or of mutants thereof in which the AP-1 (black bars) or NF-κB (hatched bars) sites were mutated and a combination of pCS3MT-MKK73E (5 μg) plus pCS3MT-NIK (0.5 μg) or pFcMEKK1 (0.1 μg) plus pCS3MT (4.9 μg). Reporter gene activity (mean ± SEM from four independent experiments) was determined as for panel B. For each experiment, equal expression of protein kinases was confirmed by Western blotting (not shown).
duced transcription is inhibited by coexpression of dominant negative NIK (Fig. 7). Second, basal IL-8 transcription is also inhibited by dominant negative NIK, indicating that some NF-κB activity is involved. Basal NF-κB activity may be necessary to observe transcriptional activation by MKK7. On the other hand, neither basal nor NF-κB-activated transcription was sensitive to coexpression of dominant negative MKK7, arguing against basal activity of that pathway.

These data can be explained by a model in which NIK induces translocation of the NF-κB dimer to the IL-8 promoter, where it binds in close proximity to AP-1 proteins. Activated SAPK/JNK molecules bound to AP-1 may phosphorylate NF-κB subunits or other regulatory components in addition to phosphorylating AP-1. This could lead to enhanced IL-8 promoter activity.

In that model, the SAPK/JNK pathway is used by the cell to boost IL-8 transcription initiated by NF-κB. Support for a crucial role of the SAPK/JNKs in IL-8 (and IL-6) formation comes from experiments in which IL-1-induced cytokine secretion was strongly reduced by inhibiting SAPK/JNK, but activation of NF-κB was unimpaired (24). The fact that EGF, a poor activator of SAPK/JNK, induced only low levels of IL-8 secretion also supports the notion that SAPK/JNK is required for the formation of this cytokine. EGF is also a weak activator of NF-κB (23a).

Several studies have suggested that IL-8 mRNA stabilization may be induced by IL-1 or TNF, but the signaling pathways involved have not been identified (6, 21, 58, 59, 61). By using an inducible expression system which allows rapid transcriptional shut off, we found no effect of NIK or MKK7 on IL-8 mRNA degradation. However, an active mutant of MKK6, a specific activator of p38 but not SAPK/JNK or ERK MAP kinases (50), stabilized the IL-8 mRNA (Fig. 9). These data define a novel function for MKK6, namely, regulating IL-8 mRNA stability. During further analysis of this effect (62a), we also observed MKK6-induced stabilization of IL-6 mRNA and of β-globin-reporter mRNAs carrying AU-rich sequences of different cytokine mRNAs in their 3′ UTRs. An unrelated transcript (of the CAT gene) was not affected. This finding suggests that AU-rich elements are involved in the observed regulation. Recently it was shown that the SAPK/JNK pathway regulates the stability of the IL-2 (8) and IL-3 (42) mRNAs. JNK2 (M KK7) and MEKK1, but not MKK6, enhanced IL-2 mRNA stability, suggesting that the SAPK/JNK pathway was involved (8). In discordance with the latter results, MKK7 did not affect IL-8 mRNA decay in our study. We do not know whether this discrepancy is related to the difference in the transcripts and/or the cell types studied (T cells versus epithelial cells). In support of our results, it was recently shown that

FIG. 7. MKK73E-induced activation of the IL-8 promoter is suppressed by coexpression of dominant negative NIK. HEK-293 cells were transfected with 5 μg of pCS3MT-MKK73E, pCS3MT-MKK73A, pCS3MT-NIK, or pCS3MT-NIK (KK429-430AA) in the indicated combinations together with pUHC13-3-IL-8pr. Where required, empty pCS3MT was added to keep total DNA amounts constant. (A) At 48 h after transfection, cells were lysed and luciferase reporter gene activity was determined as for Fig. 6 (mean ± SEM from three independent experiments); (B) 100 μg of lysate proteins from one experiment was separated by SDS-PAGE and Western blotted to confirm equal expression of Myc-epitope tagged protein kinases MKK7 and NIK. RLU, relative light units.

FIG. 8. Weak induction of IL-8 secretion by EGF correlates with its lack of SAPK/JNK activation. Human KB epithelial cells stably transfected with vector (KB-vector) or with antisense RNA to SAPKβ (KB-SAPKβas64, clone 64) as described in detail in reference 24 were left untreated (control [C]) or stimulated for 24 h with IL-1α (10 ng/ml) or with EGF (50 ng/ml). (A) IL-8 concentrations in the cell culture supernatant were determined by ELISA (means ± SEM from two independent experiments performed in duplicate). (B) The cells were stimulated with IL-1α (10 ng/ml) or EGF (50 ng/ml) or left untreated for 15 min and lysed, and SAPK/JNK activity was determined as described for Fig. 1. Shown is a result representative for three independent experiments.
the p38 MAP kinase inhibitor SB203580 suppressed IL-1- or lipopolysaccharide-induced stabilization of cyclo-oxygenase II (9, 52) and IL-6 (40) mRNAs. In summary, our data suggest that NIK- and MKK7-dependent pathways cooperatively regulate IL-8 transcription, whereas a third protein kinase cascade involving p38 MAP kinase regulates IL-8 mRNA stability. Thus, high expression of IL-8 requires at least three distinct protein kinase cascades (Fig. 10). Stimuli which are capable of activating NF-κB, SAPK/JNK, and p38 MAP kinase cascades, such as TNF, IL-1, or the upstream kinase MEKK1, consequently result in maximal IL-8 production and secretion.

In conclusion, our results provide a striking example of usage of three signal transduction pathways for regulating the expression level of an endogenous gene.

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