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The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism

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Stabilization of mRNAs contributes to the strong and rapid induction of genes in the inflammatory response. The signaling mechanisms involved were investigated using a tetracycline-controlled expression system to determine the half-lives of interleukin (IL)-6 and IL-8 mRNAs. Transcript stability was low in untreated HeLa cells, but increased in cells expressing a constitutively active form of the MAP kinase kinase kinase MEKK1. Destabilization and signal-induced stabilization was transferred to the stable β-globin mRNA by a 161-nucleotide fragment of IL-8 mRNA which contains an AU-rich region, as well as by defined AU-rich elements (AREs) of the c-*fos* and GM-CSF mRNAs. Of the different MEKK1-activated signaling pathways, no significant effects on mRNA degradation were observed for the SAPK/JNK, extracellular regulated kinase and NF-κB pathways. Selective activation of the p38 MAP kinase (=SAPK2) pathway by MAP kinase kinase 6 induced mRNA stabilization. A dominant-negative mutant of p38 MAP kinase interfered with MEKK1 and also IL-1-induced stabilization. Furthermore, an active form of the p38 MAP kinase-activated protein kinase (MAPKAP K2 or MK2) induced mRNA stabilization, whereas a negative interfering MK2 mutant interfered with MAP kinase kinase 6-induced stabilization. These findings indicate that the p38 MAP kinase pathway contributes to cytokine/stress-induced gene expression by stabilizing mRNAs through an MK2-dependent, ARE-targeted mechanism.

Keywords: cytokines/kinase cascade/MAPKAP kinase-2/mRNA degradation/signal transduction

Introduction

The response of higher organisms to external insult, inflicted by infectious microorganisms, toxic agents, UV-light and other causes of non-specific tissue damage, comprises processes aimed at elimination or neutralization of the cause of damage, metabolic adaptation and repair. The highly coordinated changes in gene expression of cells participating in these processes are elicited by the damaging agents (Ulevitch and Tobias, 1995; Tyrrell 1996), as well as by pro-inflammatory cytokines like tumor necrosis factor (TNF) or interleukin (IL)-1, which are formed immediately and orchestrate the response (Tracey and Cerami, 1994; Saklatvala *et al.*, 1996; Dinarello, 1998). Both cytokines and external stressors act through signaling mechanisms that diverge into different pathways, including the NF-κB pathway (Baldwin, 1996) and the extracellular regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and p38 MAP kinase (=SAPK2) pathways (Karin, 1998; Widmann *et al.*, 1999). MAP kinase kinase kinases like MEKK1, situated upstream in the functionally hierarchical organization of these kinase cascades, are able to initiate signaling through several of these pathways (Lange-Carter *et al.*, 1993; Yan *et al.*, 1994; Lee *et al.*, 1997; Guan *et al.*, 1998; Nemoto *et al.*, 1998; Yujiri *et al.*, 1998).

For each of the signaling pathways evidence has been accumulated that demonstrates its contribution to activation of gene transcription (Baldwin, 1996; Karin *et al.*, 1997). On the other hand, little information exists on their role in controlling gene expression at the post-transcriptional level. It has been known for many years that such regulatory mechanisms contribute strongly to gene induction in inflammation and stress. Known examples are the activation-induced release of translational blockade upon induction of TNF in monocytic cells (Han *et al.*, 1990; Gueydan *et al.*, 1999) or upon activation of T cells (Mao *et al.*, 1992; Abraham, 1998), regulation of ferritin translation by cytokines (Weiss *et al.*, 1997) and stabilization of mRNAs with high basal degradation rate (Shaw and Kamen, 1986). The latter feature aids in limiting RNA levels in the uninduced state and is essential for rapid increases in RNA amounts subsequent to induction.

AU-rich sequences have been identified as crucial *cis* elements mediating RNA instability. In 1986 Shaw and Kamen reported destabilization of a long-lived reporter RNA by inserting into its 3′-untranslated region (UTR) the AU-rich element (ARE) of GM-CSF (Shaw and Kamen, 1986). Since then AREs have been identified in many short lived and rapidly inducible RNAs, in particular those encoding cytokines and proto-oncogene products (for a review see Chen and Shyu, 1995), but also in transcripts of other proteins, indicating the importance of their regulatory function in many biological processes. The structure–function relationship of these sequences has been analyzed in detail (e.g. Lagnado *et al.*, 1994; Chen *et al.*, 1995; Zubiaga *et al.*, 1995; Xu *et al.*, 1997), which allowed them to be grouped in three categories with differences in their apparent features of degradation (Chen

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and Shyu, 1995; Xu *et al.*, 1997). Several putative *trans*-acting factors that affect RNA stability by interacting with AREs have been purified and some of them cloned. There is a large body of correlative evidence for the destabilizing function of the AUF1/hnRNP D0 proteins (Zhang *et al.*, 1993; Kajita *et al.*, 1995; DeMaria and Brewer, 1996; Sirenko *et al.*, 1997). Most recently it has been shown that ectopic expression of AUF1 leads to rescue of the inactivation of ARE-mediated decay by hemin in K562 cells, supporting an *in vivo* RNA destabilizing role for AUF1 (Loflin *et al.*, 1999). The zinc-finger protein tristetraprolin has been identified as an ARE-binding protein with destabilizing activity on TNF mRNA (Carballo *et al.*, 1998). Proteins of the *elav* family have been observed to bind selectively to AU-rich sequences (Ma *et al.*, 1996). By overexpression of the wild-type protein or of its antisense RNA, evidence has been obtained for a stabilizing function of the ubiquitously expressed human *elav*-like protein HuR on the mRNA of vascular-endothelial growth factor (Levy *et al.*, 1998), as well as on reporter mRNAs containing defined AREs (Fan and Steitz, 1998; Peng *et al.*, 1998).

Several examples point to stabilization of otherwise labile RNAs under conditions of their induction, which contributes to a rapid increase in their amounts. Phorbol ester treatment increased the half-life of GM-CSF mRNA (Shaw and Kamen, 1986; Bickel *et al.*, 1990). Transcript stabilization of interleukin-3 was induced with calcium ionophore in a murine mast cell line (Wodnar-Filipowicz and Moroni, 1990). In T cells, formation of IL-2 upon stimulation of the T cell receptor is enhanced by a simultaneous co-stimulatory signal through CD28, which results in stabilization of IL-2 transcripts (Lindsten *et al.*, 1989). CD28 signaling is required for activation of the SAPK/JNK pathway in T cells (Su *et al.*, 1994). Indeed, a recent report demonstrates T cell activation-induced stabilization of IL-2 mRNA through this pathway (Chen *et al.*, 1998).

The cytokines IL-6 and IL-8 bear AU-rich sequences in their 3′-UTRs. Both mediators are induced by pro-inflammatory cytokines, stress and infectious agents. IL-8 is a member of the chemokine family of proteins (Baggiolini, 1998). Its main function is to support the extravasation of leukocytes, induce their chemotactic movement towards sites of infection and activate their defense functions. The long half-life of IL-8 mRNA under conditions of its induction by several agents (Kowalski and Denhardt, 1989; Kasahara *et al.*, 1991; Stoeckle, 1991; Tobler *et al.*, 1992; Chaudhary and Avioli, 1996; Villarete and Remick, 1996) suggests that message stabilization is involved in the rapid accumulation of high levels of the transcript, a prerequisite for massive formation of IL-8 protein. IL-6 is a multifunctional cytokine that promotes B-cell growth and differentiation, and stimulates acute phase protein synthesis in liver (for review see Akira *et al.*, 1993). Evidence for stabilization of its mRNA in response to pro-inflammatory cytokines has been presented (Elias and Lentz, 1990; Ng *et al.*, 1994).

In our attempts to analyze the function of different cytokine-activated signaling mechanisms in the induction of IL-6 and IL-8 formation in epithelial cells, we previously demonstrated a critical role for the SAPK/JNK pathway (Krause *et al.*, 1998). Selective activation of this pathway can induce some transcriptional activation and synergizes with NF-κB-induced transcription (Holtmann *et al.*, 1999). To investigate the contribution of message stabilization to the burst of IL-6 and IL-8 formation induced by cytokines and stress factors, we studied the effect of different stress/cytokine-activated kinases on the half-life of IL-8 and IL-6 mRNAs, as determined by their expression under the control of a heterologous, tetracycline-controlled promoter. We demonstrate that (i) the short basal half-life of transcripts for these cytokines is strikingly prolonged by expression of a constitutively active form of MEKK1; (ii) an AU-rich region of the IL-8 transcript, as well as the AREs of the c-*fos* and GM-CSF transcripts, are sufficient to confer both rapid basal turnover and inducible stability to a reporter RNA; and (iii) a constitutively active mutant of MAPKAP kinase 2 (MK2) (Stokoe *et al.*, 1992a), a p38 MAP kinase-activated protein kinase (Freshney *et al.*, 1994; Rouse *et al.*, 1994), induces mRNA stabilization, whereas a kinase-dead mutant of it interferes with stabilization. These data provide evidence that the p38 MAP kinase pathway regulates ARE-based mRNA degradation via its downstream effector molecule MK2.

Results

Stabilization of IL-8 and IL-6 mRNAs by the MAP kinase kinase kinase MEKK1

Strong and rapid induction of cytokines in response to proinflammatory cytokines like TNF and IL-1, as well as different forms of stress, is achieved in part by an increase in half-life of the transcripts (Elias and Lentz, 1990; Ng *et al.*, 1994; Leverkus *et al.*, 1998; Levy *et al.*, 1998). In HeLa cells we observed rapid induction of IL-8 mRNA by these stimuli (not shown). To delineate the roles of different signaling pathways in IL-8 mRNA stabilization, activators of those pathways were transiently expressed in intact cells. We first expressed an active (N-terminally truncated) form of the MAP kinase kinase kinase MEKK1 (MEKK1-Δ), which has been shown to potently activate signaling pathways also activated by TNF and IL-1, namely the NF-κB (Lee *et al.*, 1997; Nemoto *et al.*, 1998), SAPK1/JNK (Yan *et al.*, 1994; Yujiri *et al.*, 1998); ERK (Lange-Carter *et al.*, 1993; Yujiri *et al.*, 1998) and p38 MAP kinase pathways (Guan *et al.*, 1998). Transient expression of MEKK1-Δ indeed was sufficient to induce a strong and dose-dependent increase in IL-8 mRNA (Figure 1A). Initial results obtained in experiments applying actinomycin D to inhibit transcription indicated a long half-life of endogenous IL-8 transcripts in MEKK1-Δ-transfected cells (Figure 1A). Yet, a comparison with the half-life in unstimulated cells was hampered by their extremely low content of IL-8 mRNA. Furthermore, concern has been raised about the use of general inhibitors of transcription like actinomycin D to estimate RNA turnover in intact cells (Chen *et al.*, 1995; Kessler and Chasin, 1996; Peng *et al.*, 1996), as actinomycin D clearly can interfere with mRNA degradation.

To overcome these problems, IL-8 mRNA was expressed using a regulatable promoter system. HeLa cells constitutively expressing the tetracycline-sensitive transactivator (tTA) (Gossen and Bujard, 1992) were transfected with a plasmid expressing the IL-8 cDNA under the control of the tTA-responsive promoter. A

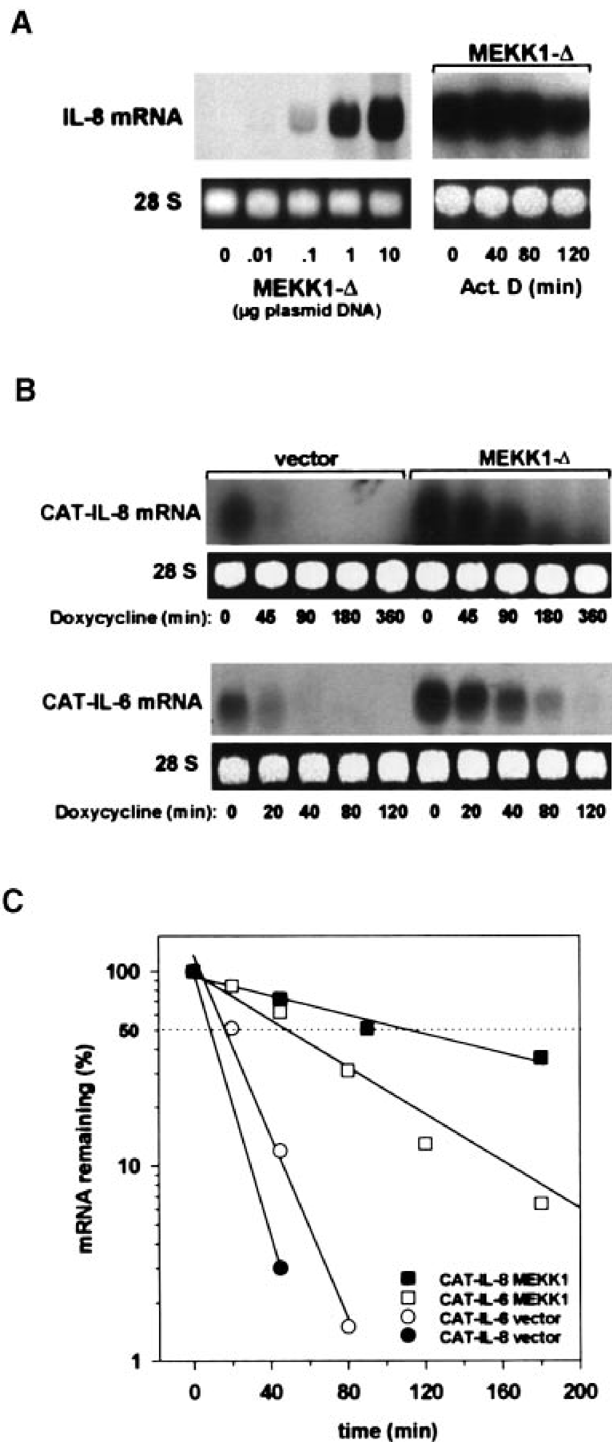

Fig. 1. MEKK1-induced increase in mRNA stabilization. (**A**) Northern blot analysis for IL-8 mRNA was performed on total RNA (10 µg/sample) isolated from HeLa cells after transient tranfection with an expression plasmid for MEKK1-Δ at the amounts indicated (left panel), or with 10 µg of the plasmid, followed the next day by incubation with actinomycin D (5 µg/ml) for the times indicated (right panel). (**B**) HeLa-tTA cells were cotransfected with pUHD10-3-CAT-IL-8 or pUHD10-3-CAT-IL-6 and empty vector or MEKK1-Δ expression vector. tTA-dependent transcription was stopped by addition of doxycycline (3 µg/ml). At the indicated times thereafter, total RNA was isolated and hybrid mRNAs detected by Northern blotting (for details see Materials and methods). Ethidium bromide staining of the 28S rRNA is shown to allow comparison of the RNA amounts applied. (**C**) Results were quantified by a video analyzer system and are expressed in percentage of the mRNA amount at the time of doxycycline addition (= 0 min).

fragment of the CAT cDNA was inserted 5′ of the IL-8 cDNA to enable distinction of the transcripts from endogenous IL-8 mRNA. Upon tetracycline-induced shut-off of transcription the CAT–IL-8 mRNA disappeared rapidly, indicating a very short transcript half-life in unstimulated cells (Figure 1B). Cotransfection with MEKK1-Δ induced a marked increase in transcript stability ($t_{1/2} \approx 110$ min, versus <15 min in control cells). Similarly, a CAT–IL-6 mRNA was rapidly degraded in vector-cotransfected cells ($t_{1/2} \approx 20$ min) but was considerably more stable in MEKK1-Δ-cotransfected cells ($t_{1/2} \approx 50$ min). In additional experiments (not shown) exogenous IL-8 and IL-6 mRNAs lacking the CAT insertion showed comparably short half-lives in vector-cotransfected cells and long half-lives in MEKK1-Δ-cotransfected cells (the amount of endogenous IL-8 message induced by MEKK1-Δ transfection was subtracted in those experiments). Furthermore, rapid basal degradation was also observed for a constitutively expressed IL-6 mRNA, ruling out that this was a consequence of transfection conditions.

Transfer of instability/signal-induced stabilization to a stable reporter mRNA by 3′-UTR-derived mRNA sequences

AU-rich sequences in the 3′-UTRs of many cytokine and proto-oncogene mRNAs have been identified as the *cis* elements responsible for their rapid degradation (Chen and Shyu, 1995). The 3′-UTR of the IL-8 transcript contains several scattered AUUUA motifs, including a group of four (with two of them clustered), starting at position 1050, and one motif in an A- and U-rich environment starting at position 1153 (see scheme in Figure 6). A fragment of the IL-8 cDNA (nucleotides 972–1310) that contains both these regions was inserted in sense and antisense orientation into the 3′-UTR of a rabbit β-globin genomic DNA construct expressed under the control of the tTA-responsive promoter. In accordance with previous studies (Xu *et al.*, 1998), the β-globin RNA was very stable (Figure 2A). Insertion of the 3′-UTR region of IL-8 in antisense orientation had no significant effect. In contrast, β-globin transcripts containing the IL-8 RNA region in sense orientation were rapidly degraded. The half-life was comparable with that of IL-8 mRNA, varying between 13 and 24 min in different experiments (e.g. Figures 2–5). Thus IL-8 3′-UTR sequences between nucleotides 972 and 1310 are sufficient to confer instability. Most importantly, co-expression of MEKK1-Δ induced a strong increase in half-life of the hybrid mRNA, showing that the region of the IL-8 RNA inserted was also sufficient to confer signal-induced stabilization. It should be noted that an RNA-stabilizing mechanism is suggested also by increased steady-state levels of hybrid RNA in the kinase-transfected cells. That increase was not due to enhanced transcription from the tTA-dependent promoter, since amounts of β-globin mRNA did not differ between kinase-transfected and control cells. Differences in steady-state RNA levels in accordance with different RNA half-lives were noted throughout this study and in some figures for presentation purposes are compensated by different exposure times of the autoradiographs.

To investigate further the selectivity of the stabilization mechanism, β-globin constructs containing nucleotides 767–1022 of the IL-6 3′-UTR (with AU-rich sequences

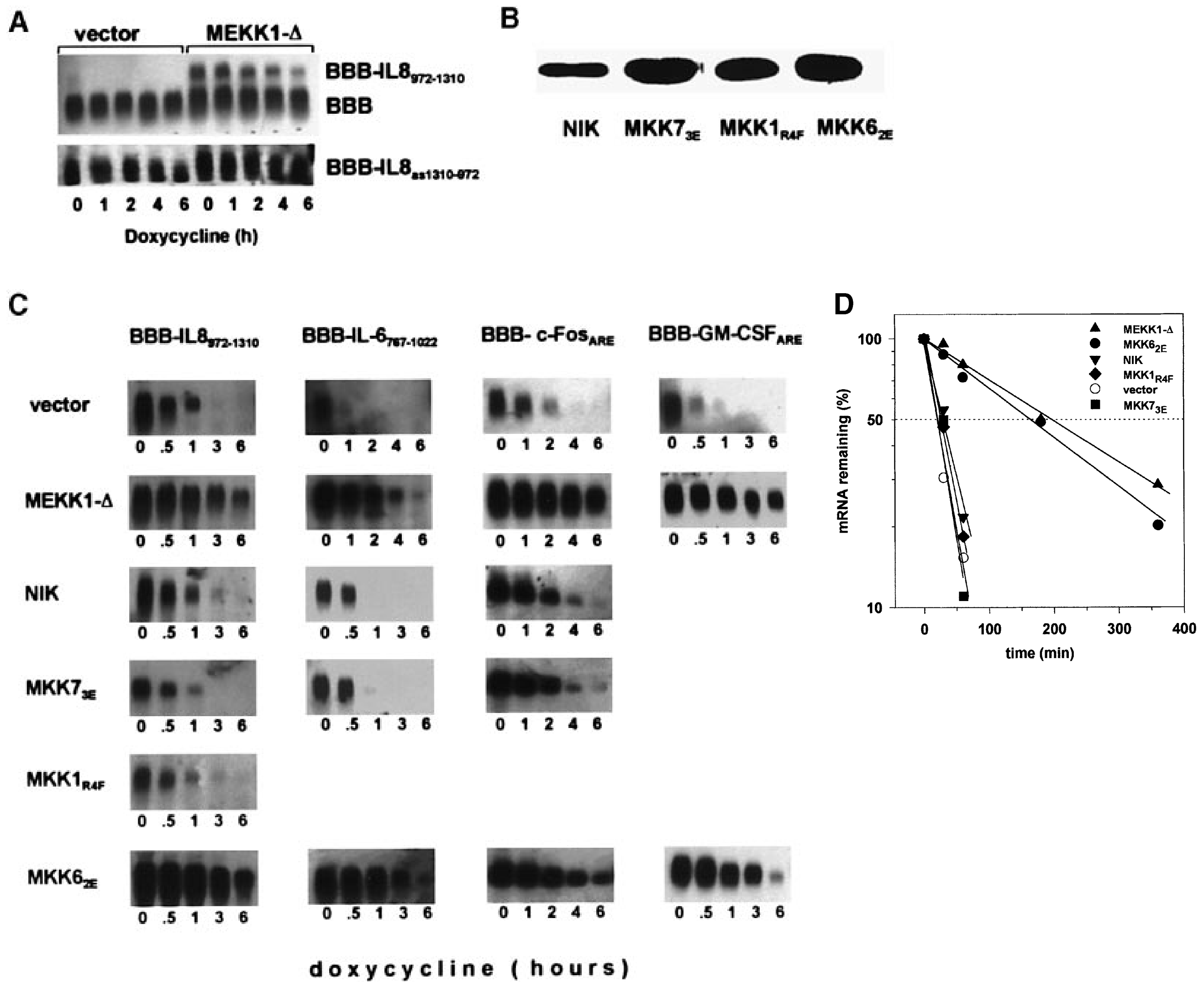

Fig. 2. Destabilization and signal-induced stabilization of a β-globin reporter mRNA containing 3′-UTR-derived sequences of IL-8 or IL-6, or the AREs of c-*fos* or GM-CSF. (**A**) HeLa-tTA cells were cotransfected with empty vector or expression plasmids for MEKK1-Δ, and with plasmids for tTA-dependent expression of β-globin mRNA without insertion (BBB) or containing a region of the IL-8 3′-UTR in sense (BBB–IL-$8_{972–1310}$) or antisense orientation (BBB–IL-$8_{as1310–972}$). Following addition of doxycycline (3 μg/ml) for the times indicated, RNA was isolated and Northern blots hybridized to a β-globin probe. (**B**) HeLa-tTA cells were cotransfected with ptet-BBB-IL-$8_{972–1310}$ and expression plasmids for MEKK1-Δ, NIK, $MKK7_{3E}$, $MKK1_{R4F}$ or $MKK6_{2E}$, or empty vector as control. Expression of the kinases was controlled by Western blots employing antibodies against the epitope tags. (**C**) mRNA amounts of BBB–IL-$8_{972–1310}$ and of β-globin containing a region of the IL-6 3′-UTR (BBB–IL-$6_{767–1022}$), the c-*fos* ARE (BBB-c-fos_{ARE}) or the GM-CSF ARE (BBB-GM-CSF_{ARE}) were determined in total RNA from cells transfected with kinase cDNAs as in (B) and incubated for the times indicated with doxycycline. Equal loading of RNA was controlled by ethidium bromide-staining of 28S rRNA (not shown). (**D**) Quantification of the results obtained for ptet-BBB–IL-$8_{972–1310}$ was performed as in Figure 1C.

therein), or the AREs of c-*fos* and GM-CSF, previously well characterized regulatory RNA elements (Chen *et al.*, 1995; Xu *et al.*, 1997), were transfected. The RNAs derived from all three constructs showed characteristics similar to the β-globin–IL-8 hybrid RNA, i.e. rapid degradation in vector-cotransfected cells and marked stabilization in the MEKK1-Δ-cotransfected cells (Figure 2C). Thus expression of MEKK1-Δ induces stabilization of various ARE-containing transcripts, suggesting a general regulatory mechanism.

Involvement of the p38 MAP kinase pathway in MEKK1-induced mRNA stabilization

MEKK1 can initiate signaling along the different MAP kinase cascades and the NF-κB pathway. Epitope-tagged constitutively active forms of kinases that selectively activate the different pathways were ectopically expressed to probe them for inducing mRNA stabilization. Expression was verified by Western blotting (Figure 2B). Wild-type NF-κB inducing kinase (NIK) (Malinin *et al.*, 1997), a constitutively active mutant of the SAPK/JNK-activating kinase MKK7 ($MKK7_{3E}$) (Holland *et al.*, 1997), and a constitutively active mutant of the ERK-activating kinase MKK1 ($MKK1_{R4F}$) (Mansour *et al.*, 1994), had no effect on stability of the β-globin–IL-$8_{972–1310}$ RNA (Figure 2C). All three kinases are functionally active in these cells, inducing transcription from a minimal IL-8 promoter (not shown). Therefore the results argue against involvement of the NF-κB, SAPK/JNK and ERK pathways in mRNA stabilization. In contrast, expression of a constitutively active mutant of MKK6 ($MKK6_{2E}$) resulted in pronounced message stabilization, comparable in degree to that induced

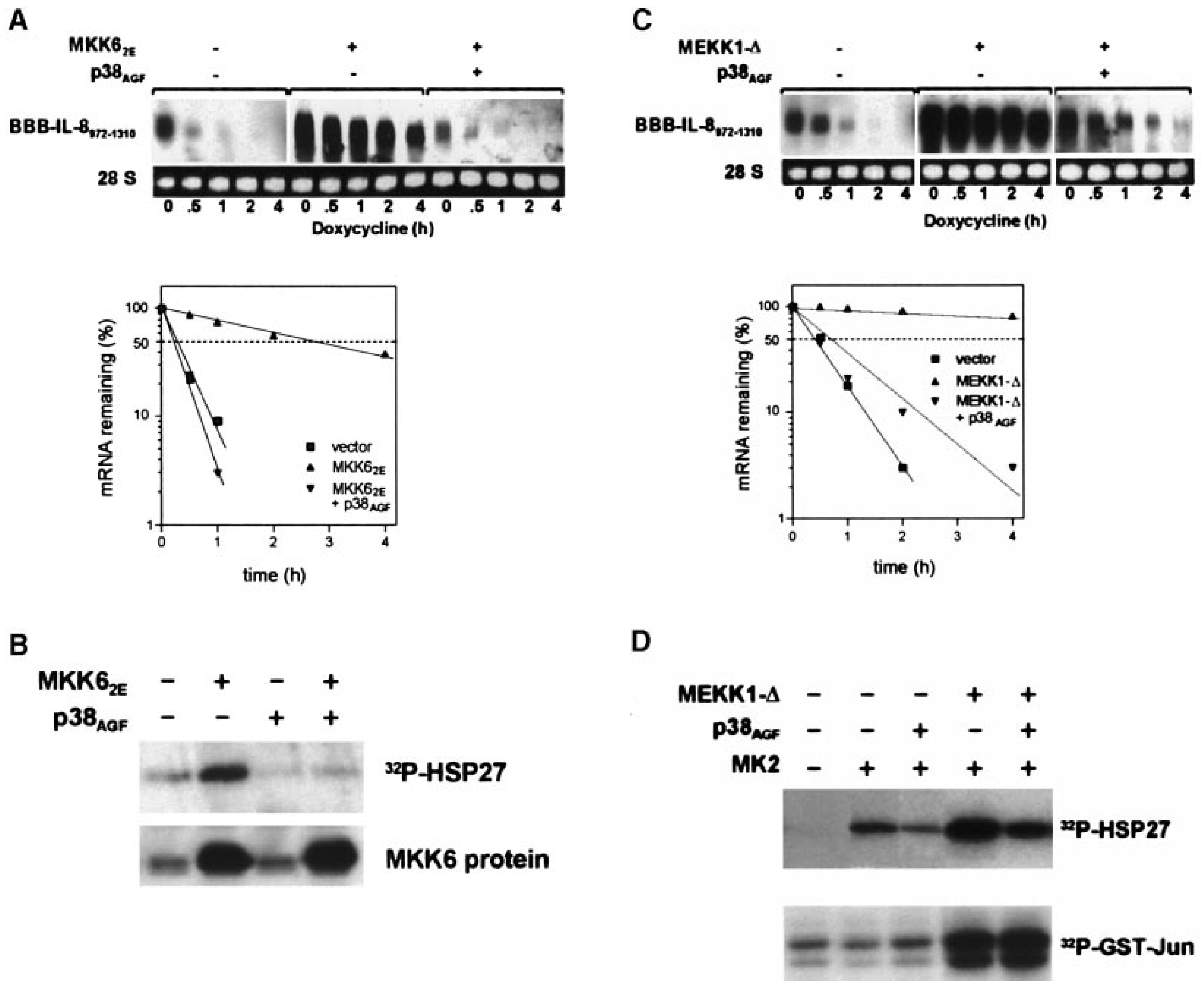

Fig. 3. Inhibition of MKK6 and MEKK1-induced mRNA stabilization by a dominant-negative mutant of p38 MAP kinase. HeLa cells were cotransfected with ptet-BBB–IL-8$_{972–1310}$ and empty vector, expression vectors for dominant-negative p38 MAP kinase (p38$_{AGF}$) and (**A** and **B**) MKK6$_{2E}$ or (**C** and **D**) MEKK1-Δ as indicated. (A and C) Cells were incubated with doxycycline for the times indicated. Isolation of total RNA, Northern blot analysis with a β-globin probe and quantification of the results were performed as in Figure 2. (B) Endogenous MK2 was immunoprecipitated from cell lysates and its kinase activity assayed *in vitro*, using HSP27 as substrate (Stokoe *et al.*, 1992b) (for details see Materials and methods). The amount of MKK6 protein was determined by Western blotting using specific antibodies. (D) Activation of MK2 by MEKK1-Δ was determined as in (C), except that ectopically co-expressed myc-tagged MK2 was immunoprecipitated to increase sensitivity of the assay. SAPK/JNK activity was determined in parallel by immunoprecipitating the enzymes and determining their kinase activity against GST–Jun as described in Materials and methods.

by MEKK1-Δ ($t_{1/2} \approx$ 170 versus 200 min). Similarly to β-globin–IL-8$_{972–1310}$ mRNA, expression of MKK6$_{2E}$ but not NIK and MKK7$_{3E}$ stabilized β-globin–IL-6$_{767–1022}$ and β-globin–c-fosARE mRNAs (Figure 2C). Furthermore, a β-globin–GM-CSFARE mRNA was also stabilized by MKK6$_{2E}$, suggesting that these transcripts are affected by the same mechanisms. A kinase-inactive mutant of MKK6 did not increase RNA stability (not shown). As MKK6 has been found to selectively activate the p38 MAP kinase pathway (Raingeaud *et al.*, 1996), the results suggest involvement of this pathway in mRNA stabilization.

To test this hypothesis further, MKK6$_{2E}$ was co-expressed with a dominant-negative mutant of p38 MAP kinase, p38$_{AGF}$. Its co-expression strongly interfered with the MKK6-induced stabilization of β-globin–IL-8$_{972–1310}$ mRNA, resulting in a half-life comparable to that in cells transfected with vector alone (Figure 3A). The dominant-negative effect of the p38 MAP kinase mutant on signaling was confirmed by assaying the activity of the p38 MAP kinase-activated protein kinase MK2. MKK6$_{2E}$-induced activation of MK2, determined by phosphorylation of its substrate HSP27, was inhibited by cotransfection of p38$_{AGF}$ (Figure 3B). Western blot analysis showed that this was not due to decreased levels of MKK6$_{2E}$ protein. In further support for a role of the p38 MAP kinase pathway in mRNA turnover, co-expression of the dominant-negative p38 MAP kinase mutant also strongly impaired MEKK1-Δ-induced mRNA stabilization (Figure 3C). This could not be ascribed to a broad non-specific effect of the p38 MAP kinase mutant, since its expression only suppressed MEKK1-Δ-induced activation of the p38 MAP kinase pathway, whereas MEKK1-Δ-induced SAPK/JNK activation remained unaffected (Figure 3D). These observations indicate that MEKK1

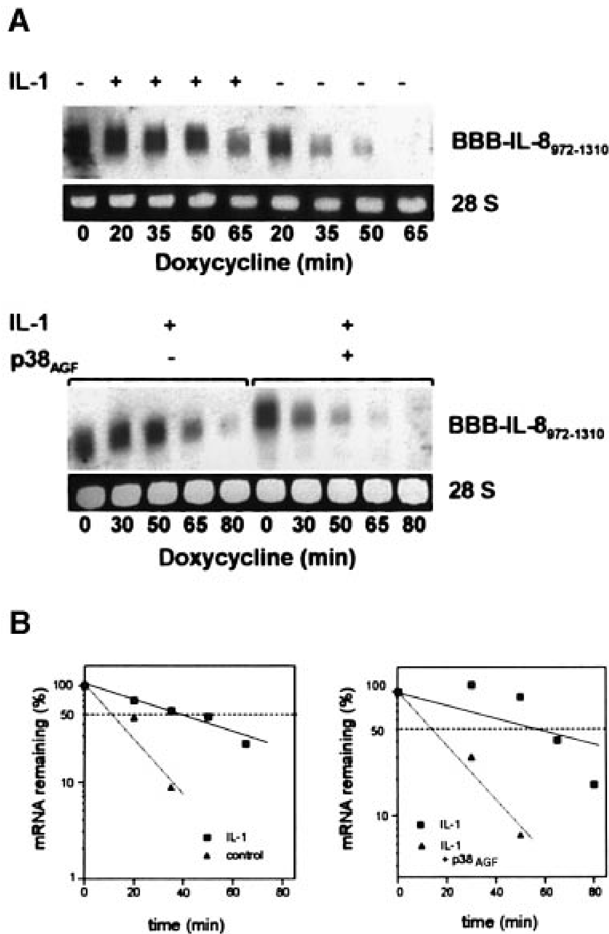

Fig. 4. IL-1-induced stabilization of a β-globin–IL-8 hybrid RNA and its inhibition by dominant-negative p38 MAP kinase. (**A**) HeLa-tTA cells cotransfected with ptet-BBB-IL-$8_{972–1310}$ and empty vector or a plasmid encoding dominant-negative p38 MAP kinase received IL-1 (1 ng/ml) and doxycycline (3 µg/ml) simultaneously. (**B**) RNA was isolated at the times indicated thereafter and hybridized to a β-globin probe. Equal loading of RNA was controlled by ethidium bromide-staining of 28S rRNA. Results were quantified as in Figure 1C.

affects mRNA metabolism in the HeLa cells mainly through the p38 MAP kinase pathway.

Involvement of the p38 MAP kinase pathway in IL-1-induced mRNA stabilization

The inhibitory effect of dominant-negative p38 MAP kinase on signal-induced mRNA stabilization was applied to probe directly for involvement of the mechanism investigated above in IL-1-induced gene expression. Treatment of the cells with IL-1 induced an ~3-fold stabilization of the β-globin–IL-$8_{972–1310}$ mRNA ($t_{1/2}$ = 40–58 min versus 13–18 min in four experiments) (Figure 4). This effect appears limited to a short time period following IL-1 exposure (note the short time intervals, chosen according to results of pilot experiments). In cells cotransfected with the construct encoding dominant-negative p38 MAP kinase, IL-1-induced mRNA stabilization was largely abrogated. Taken together, these results point to contribution of the p38 MAP kinase pathway to extracellular signal-induced gene expression by an ARE-targeted mRNA-stabilizing mechanism.

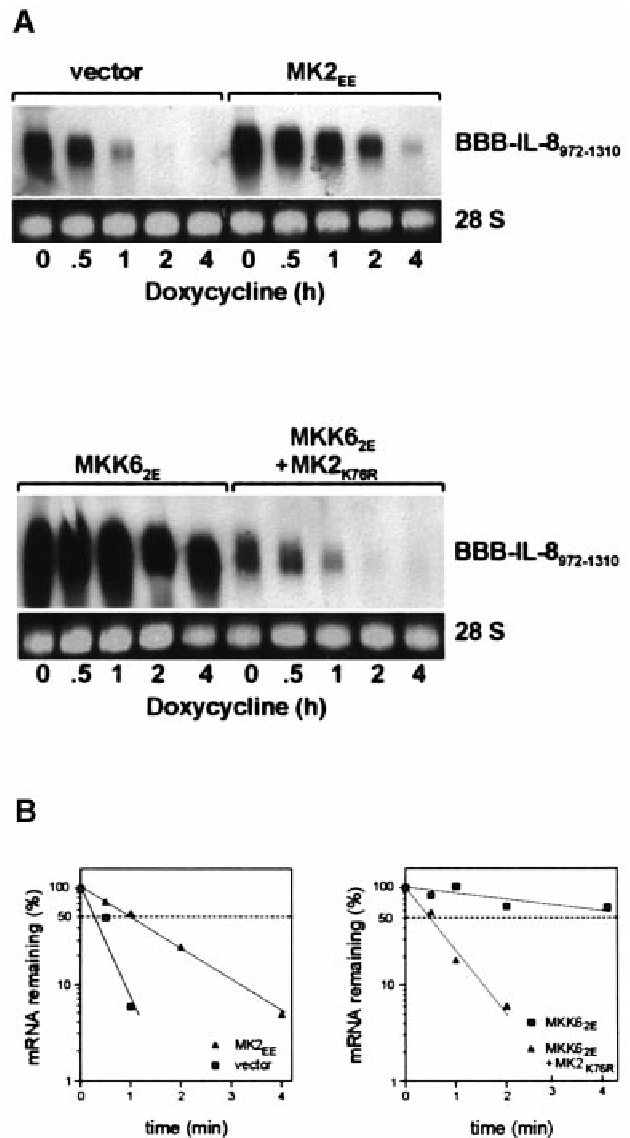

Fig. 5. Effect of mutants of the p38 MAP kinase-activated protein kinase MK2 on mRNA stability. (**A**) In HeLa-tTA cells BBB–IL-$8_{972–1310}$ was expressed alone or together with an active mutant of MK2 ($MK2_{EE}$), or of MKK6 ($MKK6_{2E}$), or with $MKK6_{2E}$ and a negative interfering mutant of MK2 ($MK2_{K76R}$). (**B**) Upon incubation with doxycycline for the times indicated, BBB–IL-$8_{972–1310}$ mRNA was detected by Northern blot analysis and the results quantified as in Figure 1C.

mRNA-stabilizing function of the p38 MAP kinase-activated protein kinase MK2

Among several kinases and transcription factors which are targets for p38 MAP kinases, the protein kinase MK2 has been shown to be phosphorylated and activated by them (Freshney *et al.*, 1994; Rouse *et al.*, 1994). Co-expression of a constitutively active mutant of MK2 ($MK2_{EE}$) (Engel *et al.*, 1995) increased the half-life of β-globin–IL-$8_{972–1310}$ mRNA (Figure 5), strongly suggesting that p38 MAP kinase-induced mRNA stabilization occurs via activation of its substrate MK2. The involvement of MK2 as the downstream effector in p38 MAP kinase-induced mRNA stabilization was further tested by employing a kinase-dead negative interfering MK2 mutant where the conserved lysine in the ATP-binding subdomain II is replaced by arginine ($MK2_{K76R}$). The mRNA-stabiliz-

A

IL-8 mRNA — coding region

	destabilization	signal-induced stabilization
BBB-IL-8 972-1310	+	+
BBB-IL-8 1048-1076	-	n.d.
BBB-IL-8 1150-1168	-	n.d.
BBB-IL-8 1048-1200	-	n.d.
BBB-IL-8 972-1200	+	+
BBB-IL-8 972-1048	-	n.d.
BBB-IL-8 972-1132	+	+
BBB-IL-8 1310-972as (antisense)	-	n.d.
BBB-(AUUU)5A	-	n.d.

972-auguugugaggacauguggaagcacuuuaaguuuuuucaucauaacauaaauuauuuuc
1031-aaguguaacuuauuaaccuauuuauuauuuauguauuuauuuaagcaucaaauauuugug
1091-caagaauuuggaaaaauagaagaugaaucauugauugaauag-1132

B

vector | MKK6 2E

BBB-IL-8 972-1200; 28 S
BBB-IL-8 1048-1076; 28 S
BBB-IL-8 1047-1200; 28 S
BBB-IL-8 972-1049; 28 S
BBB-IL-8 972-1132; 28 S
BBB-(AUUU)5A; 28 S

0 .5 1 2 4 0 .5 1 2 4
Doxycycline (h)

Fig. 6. Localization of regulatory regions within the IL-8 mRNA. (**A**) Scheme of IL-8 mRNA (hatched bars, AU-rich regions; underlined sequences, AUUUA motifs). Fragments expressed in β-globin hybrid RNAs (by inserting the corresponding cDNA into the *Bgl*II site of the β-globin construct) are outlined. Their effect and that of the synthetic sequence $(AUUU)_5A$ on stability of the hybrid RNAs are indicated (n.d., not determined due to long basal half-life). (**B**) HeLa-tTA cells transiently cotransfected with plasmids encoding the indicated hybrid RNAs and empty vector or $MKK6_{2E}$ expression vector were incubated with doxycycline for the times indicated, followed by Northern blot analysis employing a β-globin RNA probe.

ing effect of active MKK6 was strongly diminished by co-expression of the negative interfering mutant (Figure 5). This result demonstrates a novel function of MK2 in the regulation of mRNA stability.

Localization of the regulatory cis element of IL-8 mRNA

To analyze further the structural requirements of destabilization/signal-induced stabilization, shorter parts of the IL-8 cDNA fragment encompassing nucleotides 972–1310 were introduced into the β-globin reporter construct and the stability of the resulting RNAs was tested (Figure 6). The two AU-rich sequences within that region (nucleotides 1048–1076 and 1150–1168), either alone or within one large fragment (nucleotides 1048–1200), were not able to destabilize the reporter RNA. However, when a fragment extending further 5′ (nucleotides 972–1200) was inserted, destabilization and signal-induced stabilization were conferred to the reporter RNA, indicating that an essential regulatory element is located between nucleotides 972 and 1048. Notably, introducing that region alone into the β-globin transcript was not sufficient to destabilize (BBB–IL-$8_{972–1048}$ in Figure 6). Only when that region was combined with the first AU-rich sequence (BBB–IL-$8_{972–1132}$ in Figure 6), were destabilization and signal-induced stabilization observed. The first AU-rich region (nucleotides 1048–1076) contains four AUUUA motifs but lacks nonamers of UUAUUUA(U/A) (U/A), previously described as the minimal destabilizing sequences (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995). To find out whether this caused the lack of destabilization, we introduced the sequence $(AUUU)_5A$ into the β-globin mRNA. That sequence, which is also found in the 3′ part of the GM-CSF ARE, alone was ineffective in destabilizing the β-globin mRNA within the time period assayed (Figure 6). This result indicates that in addition to an AU-rich region a second region, which may lack AUUUA motifs, is mandatory for the observed regulation.

Discussion

Post-transcriptional mechanisms play an important role in the tight control of gene expression. In this report we analyzed the effects of cytokine/stress-induced signaling pathways on the half-life of IL-8 and IL-6 mRNAs. Both transcripts are rapidly degraded in unstimulated cells (Figure 1). Expression of a constitutively active form of MEKK1, a MAP kinase kinase kinase that can be stimulated by IL-1 and TNF (Nemoto *et al.*, 1998), markedly stabilized those transcripts (Figure 1), as well as reporter RNAs containing 3′-UTR sequences of IL-6 and IL-8 (Figure 2). MEKK1 is an upstream activator of several

cytokine/stress-activated signaling pathways. Stabilization was induced also upon activation of one of these pathways, involving the p38 MAP kinase, by expressing its selective activator MKK6. Correspondingly, a dominant-negative form of p38 MAP kinase interfered with MKK6-induced, MEKK1-Δ-induced and IL-1-induced stabilization (Figures 3 and 4). Finally, an active form of MK2, a substrate kinase of p38 MAP kinase, induced stabilization, whereas its dominant-negative mutant interfered with MKK6-induced stabilization (Figure 5). Together, these results suggest a signal transduction pathway by which IL-1 and possibly other external stimuli induce mRNA stabilization through subsequent activation of MEKK1, MKK6, p38 MAP kinase and MK2.

Our results do not rule out, however, that in IL-1-activated cells other activators of p38 MAP kinase in addition to MKK6 function. Recent evidence suggests that MEKK1 results in p38 MAP kinase activation through MKK4 rather than MKK6 (Guan *et al.*, 1998). Consistent with these results, a dominant-negative form of MKK6 did not interfere with MEKK1-Δ-induced mRNA stabilization (data not shown). MKK6 was nevertheless used in this study, since it selectively activates the p38 MAP kinases, unlike MKK4 which also activates the SAPK/JNKs.

No change in mRNA stability was observed by us upon selective activation of the NF-κB pathway by NIK (Malinin *et al.*, 1997), of the SAPK/JNK pathway by constitutively active MKK7 (Holland *et al.*, 1997), or of the ERK pathway by constitutively active MKK1 (Mansour *et al.*, 1994). In a study carried out in parallel on transcriptional regulation of the IL-8 gene functionality of these kinases was confirmed by the observation that transfection of each of their active mutants alone activated transcription from a minimal IL-8 promoter (Holtmann *et al*., 1999). In those experiments MKK73E was a relatively weak activator of SAPK/JNK when compared with MEKK1-Δ. It cannot be fully excluded that stabilization can be induced by a more efficient activator of the SAPK/JNK pathway. However, the strong interference of dominant-negative p38 MAP kinase with MEKK1-Δ-induced stabilization and the observation that a dominant-negative form of MKK7 did not interfere with stabilization (not shown) argue against a role of SAPK/JNK in it.

The ERK and SAPK/JNK pathways have been shown to participate in mRNA stabilization in other studies. In a recent report Karin and coworkers demonstrated a stabilizing effect on IL-2 mRNA upon expression of MEKK1 in T cells (Chen *et al.*, 1998). Their results clearly differ from those obtained in this study, as they could ascribe stabilization to activation of the SAPK/JNK pathway. Furthermore, the stabilization required 5′ untranslated sequences in addition to 3′ untranslated sequences of the IL-2 transcript. The latter could not be replaced by other ARE-containing sequences, pointing to a different, more transcript-specific mechanism. At present it is not clear whether this is related to specific properties of the IL-2 mRNA or to differences in the way different cell types (T-lymphocytes versus epithelial cells) control RNA stability. Results supporting an mRNA-stabilizing role for the SAPK/JNK family of kinases have also been presented in a study investigating the turnover of IL-3 mRNA in mast cells (Ming *et al.*, 1998). A possible contribution of the p38 MAP kinase was not fully excluded in those experiments. Interestingly, stabilization of IL-8 mRNA has been observed in response to a rise in cAMP in human colonic epithelial cells (Yu and Chadee, 1998). The sequence involved in this effect has been shown to reside in the 3′-UTR but was not localized further, thus excluding direct comparison with elements involved in MKK6-induced stabilization. Based on the use of PD98059, an inhibitor of the ERK-activating MAP kinase kinase MKK1, activation of the ERK pathway has been suggested to increase stability of muscarinic receptor mRNA (Lee and Malek, 1998). Taken together, these studies suggest that stabilization of mRNAs can be achieved by different signaling events, which correspond to the condition/stimulus of rapid induction of synthesis of the respective proteins.

Several reports based on the use of selective inhibitors point to involvement of the p38 MAP kinase pathway in IL-8 induction, without, however, discerning between transcriptional and post-transcriptional mechanisms (Manthey *et al.*, 1998; Matsumoto *et al.*, 1998). Our results suggest that these observations are at least in part explained by p38 MAP kinase-induced mRNA stabilization. Recently stabilization of IL-6 and cyclo-oxygenase mRNAs has been shown to be inhibited by the SB203580 compound at concentrations of ~1 μM, which selectively inhibit p38 MAP kinase (Miyazawa *et al.*, 1998; Ridley *et al.*, 1998; Dean *et al.*, 1999). Here we demonstrate a role of the p38 MAP kinase pathway in regulation of mRNA stability by an approach that is independent of the use of inhibitors and allows both selective activation and inhibition of signaling pathways by employing active and dominant-negative kinase mutants. Using this approach we were able to identify MK2 as the most downstream effector molecule involved in p38 MAP kinase-mediated RNA stabilization known at present. Only a few substrates for MK2 have been identified so far. MK2 phosphorylates the nuclear transcription factors CREB (Tan *et al.*, 1996; Iordanov *et al.*, 1997), ATF-1 (Tan *et al.*, 1996) and SRF (Heidenreich *et al.*, 1999), indicating a role in transcriptional regulation. Recently, evidence was obtained that MK2 is predominantly located in the nucleus and rapidly translocates to the cytoplasm upon stress in a p38 MAP kinase-dependent manner (Ben-Levy *et al.*, 1998; Engel *et al.*, 1998). Interestingly, the constitutively active mutant used in our study has been shown to localize exclusively in the cytoplasm (Engel *et al.*, 1998), which may indicate that phosphorylation of a cytoplasmic substrate is involved in mRNA stabilization.

The short half-lives of a number of proto-oncogene- and cytokine gene-encoded mRNAs depend on AU-rich regions in their 3′-UTR, many of which contain reiterations of the sequence motif AUUUA. The IL-8 mRNA has a long 3′-UTR which contains several AUUUA motifs in AU-rich regions. We studied how fragments of the IL-8 3′-UTR, chosen according to these criteria, affect RNA degradation. A fragment encompassing nucleotides 972–1310 indeed conferred instability and MKK6-induced stabilization to a β-globin reporter mRNA. While the lack of destabilization upon insertion of several other fragments into the reporter construct (Figure 6) clearly demonstrates that the observed phenomena are selective, the required structural determinants are not fully clear at present. Sequences between 972 and 1310 contain two AU-rich

regions. Since a 5′ shortened fragment in which both these regions were preserved was devoid of destabilizing activity, an important regulatory RNA element is located between nucleotides 972 and 1048. This element lacks AUUUA motifs; it does not destabilize on its own but in cooperation with the proximal AU-rich region, which contains four AUUUA motifs. In analogy, the sequence $(AUUU)_5A$, which represents the 3′ part of the GM-CSF ARE, is ineffective in destabilization (Figure 6), whereas together with the 5′ part of the GM-CSF ARE it is very effective (Figure 2C). In a previous study it was shown that the AUUUA pentamers in the 5′ part could be mutated without abolishing destabilization (Xu *et al.*, 1997). In that study similar observations were made with the synthetic ARE sequence $UU(AUUU)_3AUU$: while it was ineffective in destabilizing alone, efficient destabilization was observed when a 54 nucleotide region of the c-*fos* 3′-UTR (distinct from its ARE) was placed 5′ to it.

The region comprising nucleotides 972–1048 of IL-8 mRNA, the 5′ part of the GM-CSF ARE and the 54 nucleotide region of the c-*fos* transcript appear to support a destabilizing function of a short ARE sequence. While all three regions are rich in A + U (71, 100 and 62%, respectively), their essential structural features and exact function remain to be elucidated. Of note, others (e.g. Zubiaga *et al.*, 1995; Myer *et al.*, 1997) have shown that insertions of shorter ARE sequences can destabilize. As the p-B vectors used by them already contain the c-*fos*-derived 54 nucleotide sequence 5′ to the insertions, their results are compatible with those of Xu *et al.* (1997) and of the present report.

While it is not clear whether the more 3′ located AUUUA-containing region of the IL-8 transcript can also contribute to destabilization, presently nucleotides 972–1132 represent the minimal region that mediates destabilization and signal-induced stabilization. Further analysis of the structural requirements for the observed regulation is required. Importantly, since we did not observe destabilization and lack of signal-induced stabilization for any of the RNA regions tested so far, it remains unclear whether both effects reside in the same structural feature or depend on different regulatory regions, with destabilization being just a prerequisite for stabilization to come into effect. Furthermore, the similarity of consequences induced by inserting 3′-UTR sequences of IL-8, IL-6 and the AREs of c-*fos* and GM-CSF into the β-globin mRNA indicates a broader role of the regulatory mechanism investigated here. Their common structural features need to be defined to identify other transcripts regulated by the p38 MAP kinase/MK2 pathway and appreciate the relevance of that regulation.

Materials and methods

Plasmids

The human cDNAs for IL-8 (nucleotides 20–1545, DDBJ/EMBL/GenBank accession No. Y00787) and IL-6 (nucleotides 1–1118, DDBJ/EMBL/GenBank accession No. M14584) were amplified by RT–PCR with primer pairs containing 5′ *Xba*I sites followed by (IL-8) 5′-TCAGAGACAGCAGAGCAC-3′ (sense), 5′-GTTAAAAATATAAAG-CCTTGTA-3′ (antisense), or by (IL-6) 5′-ATTCTGCCCTC GAGC-CCACC-3′ (sense), 5′-AAATGCCATTTATTGGTATAAA-3′ (antisense), and cloned into the pUHD10-3 plasmid containing the tTA-regulated promoter (Gossen and Bujard, 1992, kindly provided by Hermann Bujard). To generate pUHD10-3-CAT-IL-6 and pUHD10-3-CAT-IL-8 plasmids, a 196-nucleotide CAT cDNA fragment was PCR-amplified from the CAT gene block (Stratagene) with the *Eco*RI-flanked primers 5′-ATCACTGGATATACCACCGT-3′ (sense) and 5′-GCATTCATCAGGCGGGCAA-3′ (antisense) and inserted into the *Eco*RI site of the vector 5′ of the IL-8 or IL-6 cDNA. Sequences were confirmed by automated DNA sequencing. To express β-globin–IL8, β-globin–IL-6 and β-globin–$(AUUU)_5A$ hybrid RNAs, fragments of IL-6 and IL-8 cDNAs were PCR-amplified or (short fragments) prepared by overlapping primer annealing as described (Winzen *et al.*, 1996), and cloned into the *Bgl*II site of the β-globin 3′-UTR in the ptet-BBB vector (Xu *et al.*, 1998). Sequences of the primers are available upon request. Plasmids ptet-BBB-c-fosARE and ptet-BBB-GM-CSF ARE have been described elsewhere (Xu *et al.*, 1998). Plasmid pFC-MEKK1 encoding amino acids 360–672 of MEKK1 was obtained from Stratagene. Plasmid pCS3MT-MKK73E, encoding myc-tagged constitutively active MKK7 [amino acids serine 271, serine 275 and threonine 277 in wild-type MKK7 (Holland *et al.*, 1997) mutated to glutamic acid], was kindly provided by Pamela Holland. A *Bam*HI–*Xho*I fragment of pCDNA3flag-NIK (Malinin *et al.*, 1997, kindly provided by David Wallach) was subcloned into the *Bgl*II site of pCS3MT to generate pCS3MT-NIK. The cDNA of human MKK6 was amplified from KB cell RNA by RT–PCR and cloned into the *Kpn*I site of plasmid peVHA (Krause *et al.*, 1998) which adds an N-terminal HA epitope tag. Serine 207 and threonine 211 in MKK6 were mutated to glutamic acid according to Raingeaud *et al.* (1996) to generate peVHA-MKK62E. To express dominant-negative p38 MAP kinase (Raingeaud *et al.*, 1995), RT–PCR-generated cDNA was inserted into pCS3MT vector and threonine 180 and tyrosine 182 mutated to alanine and phenylalanine, respectively. Mutations were performed using the Quick Change™ kit (Stratagene). pMCL-HAMKK1R4F, encoding constitutively active MKK1 (ΔN3/S218E/S222D, Mansour *et al.*, 1994), was kindly provided by Nathalie G.Ahn. The wild-type and constitutively active forms of MK2 were expressed using the plasmids pcDNA3mycMK2WT and pcDNA3-mycMK2T205E,T317E, respectively (Engel *et al.*, 1995). The plasmid coding for the negative interfering mutant of MK2 was constructed using the mutagenic primer 5′-CCTGGAGCATCCTTAGGGCGAATT-3′, the Transformer™ site-directed mutagenesis kit (Clontech) and the plasmid pGEX-5X3-MK2-Δ3B (Engel *et al.*, 1995) as template. The mutated *Bst*EII–*Not*I fragment of pGEX-5X3-MK2-Δ3B was subcloned into *Bst*EII–*Not*I cut pcDNA3mycMk2WT (Engel *et al.*, 1995) to generate pcDNA3mycMK2K76R. The expression plasmid for glutathione *S*-transferase (GST)–Jun (amino acids 1–135) was a kind gift of James R.Woodgett. GST–Jun and recombinant HSP27 were produced in bacteria and purified using standard methods.

Cells and transfections

HeLa-tTA cells constitutively expressing the tetracycline-sensitive transactivator protein (Gossen and Bujard, 1992) were obtained from David Wallach by kind permission of Hermann Bujard, and cultured in Dulbecco's modified Eagle's medium complemented with 5% fetal calf serum. Transient transfections were performed by the calcium phosphate method. Briefly, cells (5×10^6 per dish) were seeded into 9-cm Petri dishes. The next day calcium phosphate–DNA precipitate suspensions containing between 16 and 24 μg DNA (the amount of DNA was kept constant within an experiment by adding empty vector as required) were added for 5 h, followed by addition of medium containing glycerol (10% v/v) for 3 min. After extensive washing with phosphate-buffered saline and further incubation for 2 h the cells were trypsinized and seeded into 25 cm^2 culture flasks. The next day doxycycline (3 μg/ml, ICN) was added to stop transcription from the tTA-controlled promoter, and total RNA isolated at different time points thereafter (see below). To assure equal transfection efficiency within samples of a given kinetics of RNA degradation, the corresponding flasks were seeded from one transfected Petri dish.

RNA isolation and Northern blot

Cells were scraped and total RNA prepared using the Qiagen RNA extraction kit according to the manufacturer's instructions. RNA (10 μg) was separated by denaturing 1% agarose gel electrophoresis in 20 mM MOPS pH 7.0, 1 mM EDTA, 5 mM Na acetate, 6.8% formaldehyde. RNA was blotted onto nitrocellulose Hybond-N membranes (Amersham) by capillary transfer. Specific RNAs were detected by hybridization to digoxygenin-labeled RNA probes as described by the manufacturer (Boehringer Mannheim). Specific probes consisted of β-globin antisense RNA transcribed from Bluescript vector containing an *Eco*RI fragment of ptetBBB, and of IL-8 and IL-6 antisense RNAs transcribed from

Bluescript vector containing the PCR-generated cytokine cDNA (see above). Quantification was performed with a video imaging system (GelDoc100 system/Molecular Analyst program, Bio-Rad) on autoradiographs with exposure times adjusted to linear signal intensity.

Western blot analysis

Expression of transfected kinase cDNAs was controlled by Western blotting as described elsewhere (Krause *et al.*, 1998). Briefly, proteins were separated on SDS–PAGE and electrophoretically transferred to PVDF membranes (Immobilon^R, Millipore). The membranes were blocked with 5% dried milk in Tris-buffered saline (TBS) overnight, followed by incubation for 4–24 h with antibodies against the tag epitopes used, or rabbit antibodies RAW3 against MKK6 (kindly provided by Jeremy Saklatvala). Blots were washed in TBS, and incubated for 2–4 h with peroxidase-coupled second antibody. Proteins were detected by using the Amersham enhanced chemiluminescence system.

Immune complex protein kinase assays

Cells were lysed in 10 mM Tris pH 7.05, 30 mM $NaPP_i$, 50 mM NaCl, 1% Triton X-100, 2 mM Na_3VO_4, 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, 10 mM *para*-nitrophenyl phosphate, 400 nM okadaic acid. After 10 min on ice, lysates were cleared by centrifugation at 10 000 *g*. Lysate protein (250–500 μg) was diluted in 500 μl immunoprecipitation (IP) buffer (20 mM Tris, pH 7.3, 154 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100). To determine the activity of endogenous MK2 or SAPK/JNK, or of ectopically expressed MK2, samples were incubated with 2 μl of rabbit antibodies raised against GST–MK2 Δ3B (Schultz *et al.*, 1997), or against JNK2 (SAK9, kindly provided by Jeremy Saklatvala), or with 2 μg of monoclonal antibody 9E10 against the Myc-epitope (Boehringer Mannheim), respectively, for 2–4 h at 4°C. Then the antibodies were adsorbed to protein A–Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. The beads were washed three times in 500 μl of IP buffer and resuspended in 10 μl of IP buffer. Twenty microliters of kinase buffer (150 mM Tris pH 7.4, 30 mM $MgCl_2$, 60 μM ATP, 4 μCi [γ-^{32}P]ATP) containing GST–Jun or HSP27 (1–2 μg) were added. After 15 min at room temperature assays were stopped by adding SDS–PAGE sample buffer and boiling for 5 min. Following centrifugation at 10 000 *g* for 5 min supernatants were analyzed by SDS–PAGE. Phosphorylated GST–Jun and HSP27 were detected by autoradiography.

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