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Analysis of the Mechanism Regulating the Stability of Rat Macrophage Inflammatory Protein-2 mRNA in RBL-2H3 Cells

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Abstract Using rat peritoneal neutrophils, the complete nucleotide sequence of rat macrophage inflammatory protein-2 (MIP-2) mRNA including 5′untranslated region (UTR) and 3′UTR was determined (GenBank Accession number, AB060092). It was found that the MIP-2 mRNA has a 70 bp 5′UTR, a 303 bp coding region and a 728 bp 3′UTR which contains adenylate/uridylate (AU)-rich areas defined as AU-rich elements (AREs). Site-directed mutagenesis studies using the tetracycline-sensitive transactivator protein-expressing rat basophilic leukemia cells (RBL-2H3-TO cells) revealed that MIP-2 mRNA mutants which lack the 3′UTR are more stable than MIP-2-wild-type (wt) mRNA. A MIP-2 mRNA mutant in which some mutations were introduced to the ARE was also stable. The stability of MIP-2 mRNA was low in untreated RBL-2H3-TO cells, but it increased in the antigen-stimulated immunoglobulin E (IgE)-sensitized cells. The antigen-induced MIP-2 mRNA stabilization was counteracted by the highly specific p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 and the MAPK/ERK kinase (MEK-1) inhibitor PD98059. These findings indicate that ARE is the *cis*-element which mediates the rapid decay of MIP-2 mRNA, and the antigen stimulation stabilizes MIP-2 mRNA and the p38 MAPK and p44/42 MAPK pathways are involved in the antigen-induced stabilization of MIP-2 mRNA. J. Cell. Biochem. 90: 976–986, 2003.

Key words: MIP-2; AU-rich element; mRNA stability; p38 MAPK; p44/42 MAPK; RBL-2H3 cells

Chemokines are engaged in the infiltration of leukocytes at the inflammatory site and each chemokine has cell-specific chemotactic activity. For example, MCP-1 is specific for monocytes [Leonard and Yoshimura, 1990], RANTES and eotaxin are for eosinophils [Kameyoshi et al., 1992; Jose et al., 1994], and interleukin (IL)-8 is for neutrophils [Yoshimura et al., 1987] and monocytes [Abrams et al., 2003]. In humans, IL-8 is the major chemokine for neutrophils, but in rats, the major chemoattractant for neutrophils is not the IL-8 counterpart but gro-gene products/melanoma-growth-stimulating-activity-related factors [Watanabe et al., 1993]. Rat cytokine-induced neutrophil chemoattractants (CINCs) are the gro-gene products consisting of CINC-1, CINC-2α, CINC-2β, and CINC-3 [Nakagawa et al., 1994]. CINC-3 is also known as macrophage inflammatory protein-2 (MIP-2) and leucocyte-derived neutrophil chemotactic factor-2 (LDNCF-2) [Tanabe et al., 1994, 1995c]. In the rat air pouch-type allergic inflammation model, MIP-2 (CINC-3, LDNCF-2) plays an important role in neutrophil infiltration [Tanabe et al., 1994, 1995a,b,c; Xiao et al., 1997]. Previously, we reported that staurosporine, generally used as a non-specific protein kinase inhibitor [Tamaoki et al., 1986], stimulates MIP-2 production in rat peritoneal neutrophils [Xiao et al., 1999]. Therefore, it is possible that neutrophils infiltrating the pouch fluid in the rat air pouch-type allergic inflammation model produce MIP-2.

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We also reported that the staurosporine-induced MIP-2 production in rat peritoneal neutrophils is related to the expression and stabilization of its mRNA [Xiao et al., 1999]. Recently, it has been reported that several cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) have *cis*-elements in the 3′ untranslated regions (UTRs) of their mRNA which functionally regulate the mRNA stability [Guhaniyogi and Brewer, 2001]. It is also reported that mitogen-activated protein kinase (MAPK) signaling pathways are involved in regulating the stability of mRNA for IL-2 [Chen et al., 2000], IL-6, and IL-8 [Winzen et al., 1999]. However, the regulatory mechanism of MIP-2 mRNA stability has not yet been elucidated. Therefore, in this study, we first determined the complete nucleotide sequence of MIP-2 including 5′UTR and 3′UTR using rat peritoneal neutrophils. Then, we analyzed the mechanism of MIP-2 mRNA stability using MIP-2 mRNA-expressed RBL-2H3 cells, a cell line from rat basophilic leukemia cells having mucosal mast cell characteristics, because mast cells play significant roles in the development of allergic inflammation in the air pouch-type inflammation model in rats [Ohuchi et al., 1985]. In RBL-2H3 cells, p38 MAPK, p44/42 MAPK, and c-jun N-terminal kinase (JNK), which belong to the MAPK family, are activated by antigen stimulation after sensitization with immunoglobulin E (IgE) [Hirasawa et al., 2000]. In this study, we aimed to clarify the participation of the *cis*-elements in the regulation of MIP-2 mRNA stability, and clarify whether the antigen stimulation stabilizes MIP-2 mRNA through MAPK signaling pathways in RBL-2H3 cells.

MATERIALS AND METHODS

Preparation of Rat Peritoneal Neutrophils and RNA Isolation

Rat peritoneal neutrophils were prepared as described previously [Xiao et al., 1999]. Briefly, male rats of the Sprague–Dawley strain, specific pathogen-free, weighing 120 ~ 140 g (Charles River Japan, Inc., Kanagawa, Japan) were intraperitoneally injected with 40 ml of Ca^{2+}-free Krebs–Ringer solution containing 1% (w/v) casein (Wako Pure Chemical Ind., Osaka, Japan). After 16 h, peritoneal neutrophils were harvested and washed twice with Ca^{2+}-free phosphate-buffered saline (PBS, pH 7.4). The cells were then suspended at a concentration of 1×10^7 cells/ml in RPMI-1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% (v/v) fetal bovine serum (FBS, Flow Laboratories, North Rydge, NSW, Australia) and staurosporine (64 nM) (Kyowa Medex, Tokyo, Japan), before being incubated for 4 h at 37°C. Total RNA was then isolated by acid guanidinium–phenol–chloroform extraction [Chomczynski and Sacchi, 1987], and the yield of RNA extracted was determined spectrophotometrically.

Cloning of the Full-Length MIP-2 cDNA

For the cloning of the full-length MIP-2 cDNA, 3′RACE and 5′RACE were performed. For 3′RACE, 1 μg of RNA extracted from rat peritoneal neutrophils was reverse-transcribed in 20 μl of buffer (50 mM Tris-HCl, 75 mM KCl, and 3 mM $MgCl_2$, pH 8.3) containing 5 μM of the primer 5′-GGACTCGAGAATTCGATGC-TTTTTTTTTTTTTTTTT-3′, 200 U of reverse transcriptase from moloney murine leukemia virus (M-MLV, Gibco BRL, Gaithersburg, MD), 0.5 mM deoxyribonucleotide triphosphates (dNTP, Pharmacia Biotech, Uppsala, Sweden), and 10 mM dithiothreitol. The polymerase chain reaction (PCR) was performed using KOD-plus–(Toyobo, Osaka, Japan) and the reverse transcription (RT) product as a template. The primers used for PCR amplification were 5′-CGGGATCCAGAATGGCCCCTCCCA-CT-3′ (sense) and 5′-GCTCTAGAGGACTCGA-GAATTCGATGCT-3′ (antisense), containing *Bam*H I site and *Xba* I site overhangs, respectively. PCR was performed for 30 cycles; 30 s denaturation at 94°C, 30 s annealing at 58°C, and 90 s extension at 72°C, using a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer Cetus, Norwalk, CT). After amplification, the PCR product was digested with *Bam*H I (New England Biolabs, Beverly, MA) and *Xba* I (New England Biolabs), before being cloned into a *Bam*H I–*Xba* I-cut pUC19 plasmid (Takara Shuzo, Shiga, Japan) and transformed into DH5α cells (Takara Shuzo). DNA was purified with a Qiagen Midi Kit (Qiagen, Valencia, CA) and sequenced using a DyeDeoxy™ Terminator Cycle Sequencing Core Kit (Perkin Elmer Japan Co., Chiba, Japan). For 5′RACE, a 5′-full RACE Core Set (Takara Shuzo) was used according to the manufacturer's protocol. Briefly, RT was performed using the 5′-phosphorylated

reverse primer 5′-GTTCTTCCTTTCCAG-3′, and RT product was ligated with T4 RNA ligase (Takara Shuzo). The 5′ fragment of MIP-2 was then amplified using the following primers, 5′-GCTCTAGAAAGGCAAGGCTAACTGA-3′ (sense) and 5′-CGGGAATTCGAGGAGCAG-GACCAGTA-3′ (antisense), containing *Xba* I and *Eco*R I overhangs, respectively, which would allow the resulting product to be cloned in *Xba* I–*Eco*R I (New England Biolabs)-digested pUC19 plasmids (Takara Shuzo). The PCR product was then cloned and sequenced.

Plasmids

To generate pTRE2-MIP-2-wild-type (wt) and each of the deleted-MIP-2 mRNA expression plasmids, the rat cDNA for MIP-2 was amplified by PCR with the primer pairs shown in Table I, terminating in a *Bam*H I (sense primer) or *Xba* I (antisense primer) recognition sequence, respectively. PCR was performed for 30 cycles; with a 30 s denaturation at 94°C, 30 s annealing at 58°C, and 100 s extension at 72°C. Each amplified MIP-2 cDNA fragment was digested with *Bam*H I and *Xba* I and subsequently inserted into *Bam*H I–*Xba* I-cut pTRE2 plasmids (CLONTECH, Palo Alto, CA). To generate the pTRE2-MIP-2-ARE-mutant (ARE-mut.), enzymatic inverse PCR (EIPCR) [Stemmer and Morris, 1992] was performed using pTRE2-MIP-2-wt as a template. PCR was performed using the primer pairs containing a *Bsa* I recognition sequence (Table I). The PCR conditions were as follows; 1st amplification: 1 min denaturation at 94°C, 1 min annealing at 50°C, and 5 min extension at 68°C (three cycles), while the 2nd amplification was: 1 min denaturation at 94°C, 1 min annealing at 55°C, and 5 min extension at 68°C with sequential 3 s increase at each extension step (35 cycles). After amplification, the PCR product was digested with *Bsa* I (New England Biolabs) and subsequently ligated using Ligation High (Toyobo). Each MIP-2 mRNA expression plasmid was transfected into DH5α cells and purified with a Quantum Prep® Plasmid Maxiprep Kit (Bio-Rad Laboratories, Hercules, CA) for mammalian transfection.

Transient Expression of Each MIP-2 mRNA and Isolation of Total RNA

RBL-2H3 cells, a cell line from rat basophilic leukemia cells, were obtained from American Type Culture Collection (Manassas, VA), and the tetracycline-sensitive transactivator protein-expressing RBL-2H3 cells (RBL-2H3-TO cells) were prepared by transfection with the pTet-Off plasmid (CLONTECH) using the electroporation method. They were transiently transfected with each MIP-2 mRNA expression plasmid (10 μg) by the electroporation method and cultured in Eagle's minimal essential medium (EMEM) (Nissui Pharmaceutical Co., Ltd.) containing 10% (v/v) FBS. After 24 h incubation, the medium was changed and cells were further incubated for 24 h at 37°C. Transcription was then inhibited by adding actinomycin D (Wako Pure Chemical Ind.) to the cell culture medium (1 μg/ml). Actinomycin D was dissolved in dimethylsulfoxide (DMSO)

TABLE I. Primers Used for Generating Wild-Type (wt)-MIP-2 and Each of the Mutant MIP-2 mRNA Expression Plasmids

Plasmids	Sequences
wt	
Sense	5′-CGGGATCCACTGCACCTCTGGGCCT-3′
Antisense	5′-GCTCTAGAGGACTCGAGAATTCGATGCT-3′
Δ688-1101	
Sense	5′-CGGGATCCACTGCACCTCTGGGCCT-3′
Antisense	5′-GCTCTAGAGCATCACCTTCCAACT-3′
Δ3′UTR	
Sense	5′-CGGGATCCACTGCACCTCTGGGCCT-3′
Antisense	5′-GCTCTAGATCAGTTAGCCTTGCCTT-3′
Δ5′UTR	
Sense	5′-CGGGATCCAGAATGGCCCCTCCCACT-3′
Antisense	5′-GCTCTAGAGGACTCGAGAATTCGATGCT-3′
Δ5′UTR, 3′UTR	
Sense	5′-CGGGATCCAGAATGGCCCCTCCCACT-3′
Antisense	5′-GCTCTAGATCAGTTAGCCTTGCCTT-3′
ARE-mut.	
Sense	5′-TCTCTCGAGGTCTCATATATG TATGTATGTATGTATTTTCAGTGC-3′
Antisense	5′-CTACACCTGGTCTCATATATA CATACATAGATAAACTCAGACAGC-3′

(Wako Pure Chemical Ind.) (10 μg/μl) and 0.5 μl of this solution was added to the medium. The final concentration of vehicle in the medium was 0.01% (v/v). Total RNA was isolated at various time points after the addition of actinomycin D using an AquaPure RNA Isolation Kit (Bio-Rad).

Treatment of RBL-2H3-TO Cell with Drugs

The transiently transfected cells were cultured at 37°C for 24 h in EMEM containing 10% (v/v) FBS. After a 24 h incubation, the medium was changed and the cells were further incubated for 24 h at 37°C in medium containing 0.1% (v/v) conditioned medium of a dinitrophenol (DNP)-specific IgE-producing hybridoma (kindly supplied from Dr. Kazutaka Maeyama, Ehime University, Ehime, Japan). After washing with EMEM, the cells were preincubated with or without the p38 MAPK inhibitor SB203580 (Calbiochem Novabiochem Japan, Tokyo, Japan) or the MEK inhibitor PD98059 (Calbiochem Novabiochem Japan) for 10 min before the antigen challenge. These drugs were dissolved in DMSO and an aliquot of the solution was added to the medium. The final concentration of DMSO was adjusted to 0.1% (v/v). Control medium contained the same amount of DMSO. After washing, the cells were incubated for the periods indicated in medium containing actinomycin D (1 μg/ml) in the presence or absence of the antigen DNP-conjugated human serum albumin (DNP-HSA) (50 ng/ml) and SB203580 (3 μM) or PD98059 (10 μM). Total RNA was then isolated using an AquaPure RNA Isolation Kit (Bio-Rad).

Northern Blotting

RNA (20 μg) was separated by electrophoresis at 100 V for 3 h on a 1% (w/v) agarose gel containing 1.7% (v/v) formaldehyde in the buffer (20 mM MOPS, 1 mM EDTA, 5 mM sodium acetate, and 5% (v/v) formaldehyde, pH 7.0). The agarose gel was stained with ethidium bromide to show the level of 28S rRNA. RNA was blotted onto a nitrocellulose Hybond-N^+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL) by capillary transfer. MIP-2 mRNA was detected by using AlkPhos Direct (Amersham Pharmacia Biotech) according to the manufacturer's protocol. To generate a MIP-2-specific oligonucleotide probe, the coding region of MIP-2 cDNA was amplified by PCR using the following primer pairs; 5′-CGGGATCCAGAATGGCCCCTCCCACT-3′ (sense) and 5′-GCTCTAGATCAGTTAGCCTTGCCTT-3′ (antisense). A pTRE2-MIP-2-wt plasmid was used as a template, and PCR was performed for 30 cycles; with a 30 s denaturation at 94°C, 30 s annealing at 58°C, and 30 s extension at 72°C. The blots were probed with this MIP-2-specific oligonucleotide probe. Hybridization was carried out at 55°C for 16 h and MIP-2 mRNA was detected by autoradiography. The levels of MIP-2 mRNA and 28S rRNA were quantified by scanning densitometry, and ratio of the MIP-2 mRNA density vs. the 28S rRNA density in each point was calculated.

Statistical Analysis

Statistical analysis was performed on densitometry data. Significance was determined by Student's *t*-test.

RESULTS

Determination of Complete Rat MIP-2 mRNA Nucleotide Sequence

To determine the complete nucleotide sequence of rat MIP-2 mRNA, 3′RACE and 5′RACE were carried out. As shown in Figure 1, the sequence analysis revealed that MIP-2 mRNA has a 70 bp 5′UTR, a 303 bp coding region and a 728 bp 3′UTR (GenBank Accession number AB060092).

Construction of wt and Each Mutant MIP-2 mRNA Expression Plasmids

Using various pairs of primers, we cloned each pTRE2 plasmid that expresses wt MIP-2 mRNA or the deleted-MIP-2 mRNAs, MIP-2-Δ688-1101, -Δ3′UTR, -Δ5′UTR, and -Δ5′UTR, 3′UTR mRNAs (Fig. 2). The pTRE2 plasmid which expresses MIP-2-ARE-mut. mRNA, a mutated AUUUA pentamer in AU-rich element (ARE) to AUGUA, was also constructed (ARE-mut. in Fig. 2) by the EIPCR method.

ARE as a cis-Element Mediating Rapid MIP-2 mRNA Decay

RBL-2H3-TO cells were transiently transfected with each MIP-2 mRNA expression plasmid and incubated as described in "Materials and Methods." Prior to transfection with the plasmids, MIP-2 mRNA was not detected under the experiment condition (data not shown). Total RNA was then isolated at various time periods after the inhibition of transcription by

1 ACUGCACCUCUGGGCCUCCAGCAAGCUCCCUCCUGUGCUCAAGACUCCAACCACUCUUUG

61 GUCCAGAGCC**AUGGCCCCUCCCACUCGCCAGCUCCUCAAUGCUGUACUGGUCCUGCUCCU**

121 **CCUGCUGGCCACCAACCAUCAGGGUACAGGGGUUGUUGUGGCCAGUGAGCUGCGCUGUCA**

181 **AUGCCUGACGACCCUACCAAGGGUUGACUUCAAGAACAUCCAGAGCUUGACGGUGACCCC**

241 **UCCAGGACCCCACUGCGCCCAGACAGAAGUCAUAGCCACUCUUAAGGAUGGUCAUGAAGU**

301 **UUGUCUCAACCCUGAAGCCCCCUUGGUUCAGAGGAUCGUCCAAAAGAUACUGAACAAAGG**

361 **CAAGGCUAACUGA**CCUGGAAAGGAAGAACAUGGGCUCCUGUACCUCAACGGGCAGAAUCA

421 AAGAGAAAAGAAACAAACUGCACCCAGGAAGCCUGGAUCGUACCUGAUGUGCCUCGCUGU

481 CUGAGUUUAUCUAUUUAUUUAUAUAUGUAUUUAUUUAUUUAUUUUCAGUGCCUAGAUGUU

541 GUUACAUUUACUAUGAUAUUUAAAGAUAUGCAUUGGCCAGCUCACUGUAGUAUCUUAAGA

601 GGUCAUUUUAAUAUGUUGAAGUUUAUUGUAAUAAUGUUCAAUGUGUUCAGUCAGCAUUAU

661 UUUACUUAUGUAGUUGGAAGGUGAUGCAUUUUUAAAUCUAUAUUUAUUACUUUCUGGGGG

721 GGGAGGGGGAGUUGGGUACUGACUACACCACCUCCACACUGUGAUAGAGAUUGGGGAUGA

781 GGGGGGUGGGGGGGCAAACAGACGCAGUCAGAGGGCUUUCAAGGCAGGACUGUGCCUGUC

841 CACGUCAUUUUCUGUAAGCCCCGAGAAGGGCGGGACGACUGUUAUUUCUGUCUCCGUGUU

901 UCUACACUAUGUGUACAACAUUUCUGAUGCUGAAUGUUCAACAAUCGUAAUGUGAAUAUC

961 CCCUGGACAUUCUAUGUCUUCUCUGUAAGGCACAGUGCCUCGUUUAGCAAUUGUUUUGUC

1021 AUGCUUUCUCAUGUCUUGAAGUGGGGACAUUUAUUUAUUCAUGUACUUUUACAAAUAACA

1081 AAAAAAAUAAAAAUUUUUACU

Fig. 1. Complete sequence of rat macrophage inflammatory protein-2 (MIP-2) mRNA (GenBank Accession number AB060092). Rat peritoneal neutrophils (1×10^7 cells) were incubated for 3 h at 37°C in 10 ml of RPMI-1640 medium containing 10% (v/v) FBS and staurosporine (64 nM). After incubation, the total RNA was isolated and reverse transcription (RT) was performed. The 3′RACE and 5′RACE were then carried out and the MIP-2 mRNA nucleotide sequence was determined. The coding region of MIP-2 mRNA is indicated by the bold characters. MIP-2 mRNA has a 70 bp 5′UTR (1–70), a 303 bp coding region (71–373), and a 728 bp 3′UTR (374–1101). The 3′UTR has the AU-rich element (ARE) (underlined) which contains the clusters of AUUUA motifs (circled).

actinomycin D. Northern blot analysis was carried out using a probe specific to the MIP-2 coding region. It was demonstrated that MIP-2-Δ3′UTR mRNA, MIP-2-Δ5′UTR, 3′UTR mRNA, and MIP-2-ARE-mut. mRNA were more stable than MIP-2-wt mRNA, MIP-2-Δ688-1101, and MIP-2-Δ5′UTR (Fig. 3). These findings indicate that the ARE in the 3′UTR is a *cis*-element mediating the rapid decay of MIP-2 mRNA.

Stabilization of MIP-2 mRNA by the Antigen Stimulation

We next examined the effect of the antigen stimulation on the stabilization of MIP-2 mRNA in RBL-2H3-TO cells. The cells were transiently

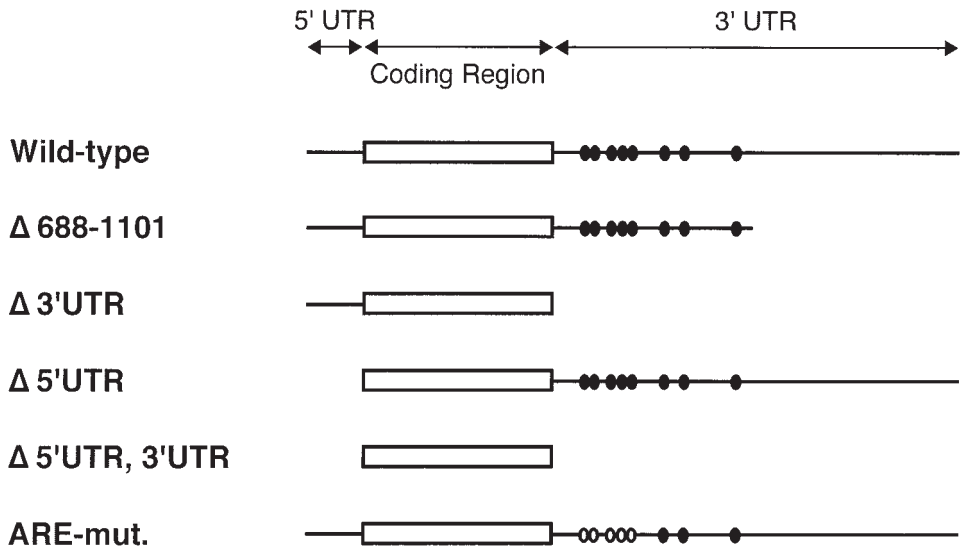

Fig. 2. Schematic representation of wild-type (wt) and mutant MIP-2 transcripts. Coding region (open box); 5′ and 3′UTRs (straight lines); AUUUA motifs (closed circles); and AUGUA pentamers (open circles).

transfected with MIP-2-wt mRNA and incubated for 48 h at 37°C in EMEM containing 10% (v/v) FBS and 0.1% (v/v) conditioned medium from the DNP-specific IgE-producing hybridoma. The cells were then washed and incubated at 37°C for 0, 1, 2, and 4 h in medium containing actinomycin D (1 μg/ml) in the presence or absence of the antigen DNP-HSA (50 ng/ml). As shown in Figure 4, the MIP-2-wt mRNA level was rapidly decreased in the non-stimulated cells, while it was stable in the antigen-stimulated cells.

Involvement of p38 MAPK and p44/42 MAPK in the Antigen-Induced Stabilization of MIP-2 mRNA

We then examined whether the activation of p38 MAPK and p44/42 MAPK is involved in the antigen-induced stabilization of MIP-2 mRNA. The IgE-sensitized cells were preincubated for 10 min at 37°C in the presence or absence of the p38 MAPK inhibitor SB203580 (3 μM) or the MEK inhibitor PD98059 (10 μM), before being washed and further incubated at 37°C for 0, 1, 2, and 4 h in medium containing actinomycin D (1 μg/ml) in the presence or absence of the antigen DNP-HSA (50 ng/ml) together with SB203580 (3 μM) or PD98059 (10 μM). In the presence of SB203580 (3 μM) or PD98059 (10 μM), the antigen-induced stabilization of MIP-2-wt mRNA was counteracted (Fig. 4). U0126, another MEK inhibitor at 10 μM, also counteracted the stabilization of MIP-2-wt mRNA (data not shown). These findings indicate that p38 MAPK and p44/42 MAPK promote the stabilization of MIP-2 mRNA expressed in the antigen-stimulated RBL-2H3-TO cells.

DISCUSSION

Chemokines play critical roles in the development and resolution of inflammatory reactions [Rollins, 1997; Baggiolini, 1998; Luster, 1998]. Stabilization of mRNA for the inflammatory cytokines is thought to contribute to the strong and rapid induction of genes in the inflammatory response [Winzen et al., 1999]. In the present study, we determined the complete nucleotide sequence of rat MIP-2 mRNA including 5′UTR and 3′UTR (GenBank Accession number AB060092). We also found that *cis*-element mediates the rapid decay of MIP-2 mRNA. MIP-2 mRNA has a 70 bp 5′UTR (nucleotides 1–70), a 303 bp coding region (nucleotides 71–373), and a 728 bp 3′UTR (nucleotides 374–1101) (Fig. 1). In common with other proinflammatory cytokine mRNAs such as those encoding TNF-α, IL-1, IL-6, IL-8, and GM-CSF [Caput et al., 1986; Chen and Shyu, 1995], it was found that MIP-2 mRNA also has an ARE with repeats of the so-called destabilizing motif AUUUA (Fig. 1). Since ARE has been identified as a crucial *cis*-element mediating mRNA instability [Chen and Shyu,

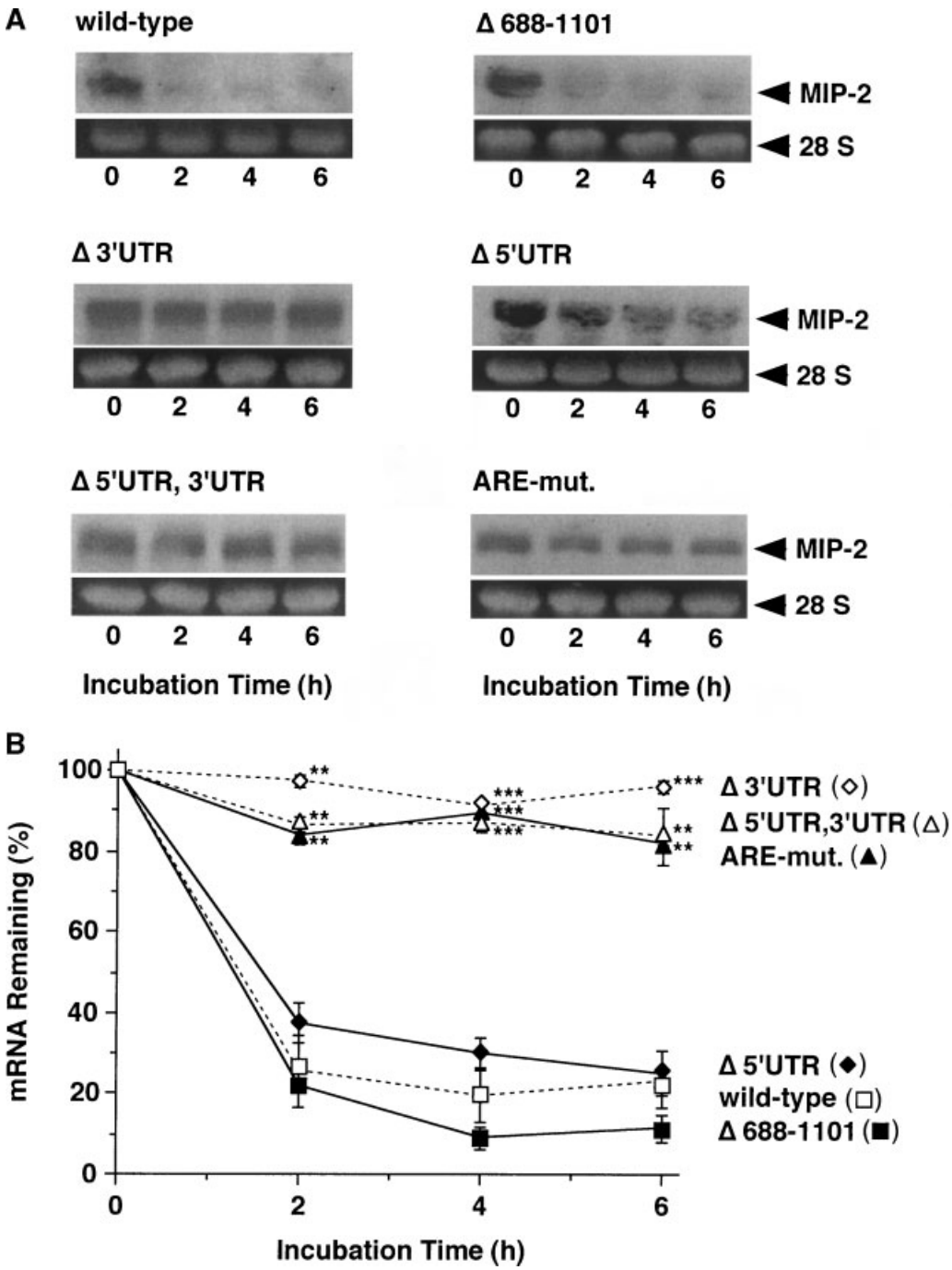

Fig. 3. Identification of a *cis*-element mediating the rapid decay of MIP-2 mRNA. **A**: RBL-2H3-TO cells were transiently transfected with 10 μg of each expression plasmid for MIP-2-wt (wild-type), MIP-2-Δ688-1101 (Δ688-1101), MIP-2-Δ3′UTR (Δ3′UTR), MIP-2-Δ5′UTR (Δ5′UTR), MIP-2-Δ5′UTR, 3′UTR (Δ5′UTR, 3′UTR), or MIP-2-ARE mutant (ARE-mut.), and incubated for 48 h at 37°C in Eagle's minimal essential medium (EMEM) containing 10% (v/v) FBS. Total RNA was isolated 0, 2, 4, and 6 h after the addition of actinomycin D (1 μg/ml) and the levels of each MIP-2 mRNA were analyzed by Northern blotting with a MIP-2-specific antisense riboprobe. The ethidium bromide-stained 28S rRNA is also shown to allow a comparison of the RNA amounts applied. The results shown are representatives of three independent experiments. **B**: The density ratio of each MIP-2 mRNA to 28S rRNA were determined and the mean value at time 0 is set to 100%. Values are the means with SEM from three independent experiments. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. wt MIP-2 mRNA group (wt, □).

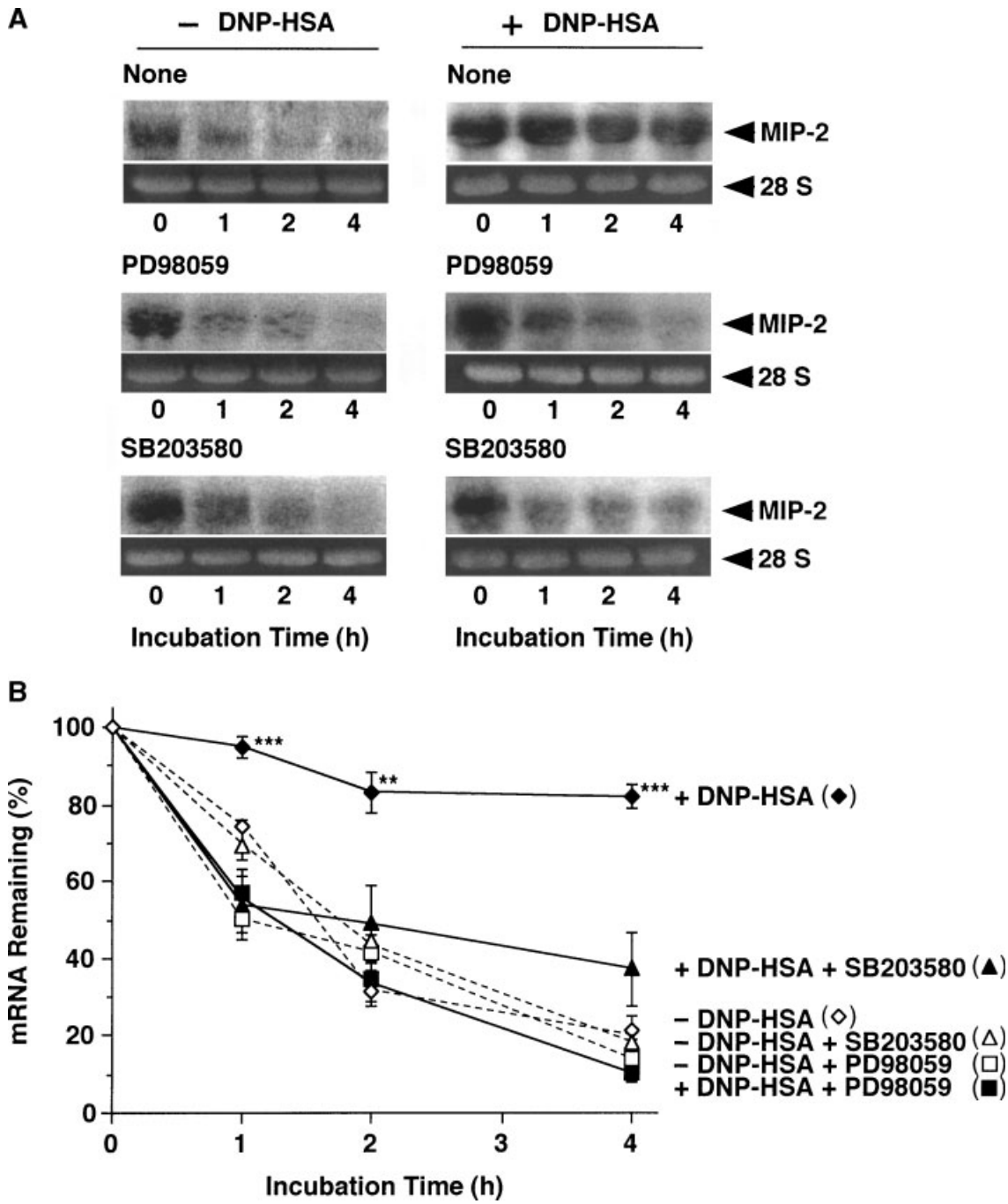

Fig. 4. Involvement of p38 and p44/42 mitogen-activated protein kinase (MAPK) activations in the stabilization of MIP-2 mRNA. **A**: RBL-2H3-TO cells were transiently transfected with pTRE2-MIP-2-wt plasmid (10 μg) and incubated for 48 h at 37°C in EMEM containing 10% (v/v) FBS and 0.1% (v/v) conditioned medium from a dinitrophenol (DNP)-specific IgE-producing hybridoma. After washing, the cells were incubated for 10 min at 37°C in medium in the presence or absence of SB203580 (3 μM) or PD98059 (10 μM). Actinomycin D (1 μg/ml) with or without DNP-human serum albumin (HSA) (50 ng/ml) was then added to the medium and the cells were incubated for the periods indicated. Total RNA was then isolated and Northern blot analysis for the levels of MIP-2 mRNA was carried out. The ethidium bromide-stained 28S rRNA is also shown to allow a comparison of the RNA amounts applied. The results shown are representatives of three independent experiments. **B**: The density ratios of MIP-2 mRNA to 28S rRNA were determined and the mean value at time 0 is set to 100%. Values are the means with SEM from three independent experiments. Statistical significance: $^{**}P < 0.01$, $^{***}P < 0.001$ vs. without antigen group (−DNP-HSA, ◇).

1995], it was conceivable that MIP-2 mRNA is labile and that the stability of MIP-2 mRNA is regulated by ARE in a similar fashion to other short lived mRNAs.

As shown in Figure 3, the MIP-2-wt mRNA was labile in RBL-2H3-TO cells. MIP-2-Δ3′UTR mRNA and MIP-2-Δ5′UTR, 3′UTR mRNA were more stable than MIP-2-wt mRNA, while MIP-2-Δ688-1101 mRNA and MIP-2-Δ5′UTR mRNA were similar to MIP-2-wt mRNA. These findings indicate that the rapid decay of MIP-2 mRNA is regulated not by the 5′UTR but by the 3′UTR, and the 3′UTR region after ARE (nucleotides 688–1101) does not participate in this rapid decay. Furthermore, since the MIP-2-ARE-mut. mRNA having mutations introduced to ARE was stable (Fig. 3), it is suggested that the *cis*-element mediating the rapid decay of MIP-2 mRNA is the ARE in the 3′UTR.

It was demonstrated that the antigen stimulation of the IgE-sensitized RBL-2H3-TO cells stabilized MIP-2 mRNA (Fig. 4). Therefore, it is possible that the IgE-sensitized mast cells in the air pouch-type allergic inflammation model participate in the production of MIP-2 and contribute to neutrophil infiltration in this model [Tanabe et al., 1994, 1995a,b,c; Xiao et al., 1997]. Furthermore, we suggested that p38 MAPK and p44/42 MAPK play important roles in mediating the antigen-induced MIP-2 mRNA stabilization, because the p38 MAPK inhibitor SB203580 and the MEK inhibitor PD98059 lowered the antigen-induced retardation of MIP-2 mRNA decay (Fig. 4). Actually, p38 MAPK and p44/42 MAPK are activated by the antigen challenge in RBL-2H3 cells [Hirasawa et al., 2000]. It has been reported that the stability of some labile mRNAs is regulated by MAPKs. For example, IL-2 mRNA is regulated by JNK [Chen et al., 1998] and mRNAs for IL-6 and IL-8 are regulated by p38 MAPK [Winzen et al., 1999]. The p38 MAPK has been reported to play a crucial role in the stabilization of mRNA for cyclooxygenase 2 [Lasa et al., 2001] and for IL-8 and IL-6 [Winzen et al., 1999] through ARE. Although it has been suggested that p44/42 MAPK does not participate in the stability of p27 mRNA through ARE [Millard et al., 2000], recent studies demonstrate that p44/42 MAPK is also involved in the regulation of TNF-α mRNA stabilization [Rutault et al., 2001]. Our study has demonstrated that not only p38 MAPK but also p44/42 MAPK is involved in the antigen-induced stabilization of MIP-2 mRNA. Previously, we reported that p38 MAPK and p44/42 MAPK pathways are involved in staurosporin-induced production of MIP-2 in rat peritoneal neutrophils [Xiao et al., 1999].

There have been many studies identifying RNA-binding proteins that exhibit sequence specificity to ARE [Hel et al., 1996; Myer et al., 1997; Wilson and Brewer, 1999]. At least two families of ARE-binding proteins have been well characterized. One is the embryonic lethal abnormal vision (Elav) family. This family consists of at least four members (elr A–D), one of which, called HuR (elr A), is widely expressed and has been shown to bind ARE and regulate the stability of ARE-containing mRNAs [Fan and Steitz, 1998; Ford et al., 1999]. Another is the heterogeneous nuclear ribonucleoprotein family [Wagner et al., 1998]. One of the family members called AUF1 is a well-characterized RNA-binding protein. HuR is correlated with mRNA stabilization, while AUF1 appears to destabilize the target mRNAs [Loflin et al., 1999]. Further experiments are necessary to analyze the mechanism by which p38 MAPK and p44/42 MAPK participate in controlling the stability of MIP-2 mRNA through ARE and to clarify what kinds of RNA-binding proteins are involved in the destabilization/stabilization of MIP-2 mRNA.

In conclusion, we have determined the complete nucleotide sequence of MIP-2 mRNA including 5′UTR and 3′UTR and demonstrated that the ARE is the *cis*-element which mediates the rapid decay of MIP-2 mRNA. Furthermore, the antigen stimulation stabilized MIP-2 mRNA in IgE-sensitized RBL-2H3-TO cells, and that the p38 MAPK and p44/42 MAPK signaling pathways are involved in the antigen-induced stabilization of MIP-2 mRNA.

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