**p38α Stabilizes Interleukin-6 mRNA via Multiple AU-rich Elements**

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**Abstract**

AU-rich elements (AREs) in the 3′-untranslated region (UTR) of unstable mRNA dictate their degradation or mediate translational repression. Cell signaling through p38α MAPK is necessary for post-transcriptional regulation of many pro-inflammatory cytokines. Here, the cis-acting elements of interleukin-6 (IL-6) 3′-UTR mRNA that required p38α signaling for mRNA stability and translation were identified. Using mouse embryonic fibroblasts (MEFs) derived from p38α+/+ and p38α−/− mice, we observed that p38α is obligatory for the IL-1-induced IL-6 biosynthesis. IL-6 mRNA stability is promoted by p38α via 3′-UTR. To understand the mechanism of cis-elements regulated by p38α at post-transcriptional level, truncation of 3′-UTR and the full-length 3′-UTR with individual AUUUA motif mutation placed in gene reporter system was employed. Mutation-based screen performed in p38α+/+ and p38α−/− mouse embryonic fibroblast cells revealed that ARE1, ARE2, and ARE5 in IL-6 3′-UTR were targeted by p38α, and truncation-based screen showed that IL-6 3′-UTR-(56–173) was targeted by p38α to stable mRNA. RNA secondary structure analysis indicated that modulated reporter gene expression was consistent with predicted secondary structure changes.

Interleukin-6 (IL-6) is a multifunctional cytokine produced by lymphocytes, macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, and keratinocytes (1). IL-6 expression was induced by a variety of stimuli, including interleukin-1 (IL-1), tumor necrosis factor (TNF), platelet-derived growth factor, and lipopolysaccharide (LPS) (1). Evidence exists for the role of IL-6 in various diseases, including inflammation and cancer. For example, constitutive overexpression of IL-6 by synovial tissues of rheumatoid patients has been reported (2) and the role of IL-6 in human malignancy is most clearly established in multiple myeloma (3) where monoclonal antibodies directed against IL-6 enhance the effectiveness of chemotherapy in this disease. There is also evidence that IL-6 acts as an autocrine growth factor in a number of human epithelial malignancies, including renal, lung, and prostate cancer (4–6). To clarify the mechanism involved in the abnormal expression of IL-6, it is imperative to investigate the mechanism of the IL-6 gene expression under physiological conditions.

The production of IL-6 is under transcriptional and post-transcriptional control (7). The transcriptional regulation of IL-6 expression has been thoroughly investigated. IL-1-responsive element was mapped within both the murine and human IL-6 promoter region, whereas regulation at the post-transcriptional level has not been as thoroughly investigated (8,9).
transcriptional mechanisms controlling pre-mRNA splicing and maturation, as well as mRNA transport, turnover, and translation, critically influence gene expression programs in mammalian cells. Central to the post-transcriptional regulatory events is the interaction of RNA with RNA-binding proteins that influence their splicing, localization, stability, and association with the translation machinery (10–12). Many ribo-nucleoprotein complexes that govern mRNA stability and translation in response to various stimuli (e.g., developmental, stress-inducing, immune, and proliferative) are composed of transcripts that bear adenine and uridine (AU)-rich elements (AREs) in the 3′-untranslated region (-3′-UTR) and the RNA-binding proteins (ARE-RNA-binding proteins) (13,14). Many ARE-RNA-binding proteins have been described that regulate the stability of target mRNAs, their translation, or both processes: AU-binding factor 1, tristetraprolin, K homology splicing-regulatory protein, butyrate response factor-1, the Hu protein family (HuR, HuB, HuC, and HuD), T-cell-restricted intracellular antigen-1, and the T-cell-restricted intracellular antigen-1-related protein TIAR (15–22).

In recently years, significant information has accumulated relative to the role of AREs of pro-inflammatory gene as the targets of the mitogen-activated protein kinase (MAPK) p38 pathway, and many of the effects appear to be mediated by its substrate, MAPK-activated protein kinase 2 (MK2) (23,24). It has been shown that p38 MAPK/MK2 cascade is involved in regulating mRNA stability via 3′-UTRs of TNF, IL-8, granulocyte macrophage-colony stimulating factor, Cox-2 and vascular epidermal growth factor mRNA (25–27). In the MK2 knockout mouse strain, LPS-induced expression of IL-6 was blocked at both protein and mRNA levels, while expression of both TNFα and interferon γ was blocked at the protein but not the mRNA level (24). For IL-1-induced IL-6 biosynthesis, the role of p38 is involved in vivo at post-transcriptional level is poorly understood.

Some p38α inhibitor and overexpression data indicate that p38α is involved in the regulation of IL-6 production at the post-transcriptional level (26). However, no cis-elements in IL-6 3′-UTR targeted by p38α have been conclusively identified. To avoid some of the potential artifacts associated with inhibitor and overexpression experiments, we have performed the analysis of IL-6 production in stable fibroblast cell lines derived from p38α+/+ and p38α−/− embryos. We found that p38α is critical for the IL-1-induced IL-6 production and mRNA stability of IL-6 is promoted by p38α via the IL-6 3′-UTR. Deletion and mutation analysis identified three ARE elements that require p38α signaling, and IL-6 3′-UTR-(56–173) is critical for p38α to promote mRNA stability. We have further analyzed the secondary structure of IL-6 3′-UTR and mutants by using a computational approach. Interestingly, the data indicated that p38α targeting IL-6 mRNA required the secondary structure of wild-type 3′-UTR.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Mouse embryo fibroblasts (MEFs) were derived from p38α+/+ and p38α−/− mice. The establishment of MEFs has been previously described (1). Cells were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml), and incubated at 37 °C in 5% CO2. The MEFs were transfected with Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer’s protocol.

**Reagents**

IL-6 enzyme-linked immunosorbent assay kit and recombinant mouse and recombinant mouse IL-1β and TNFα were purchased from R&D Systems. Lipopolysaccharide from *Escherichia coli* strain 0127:B8 was purchased from Sigma. Dual-Luciferase Reporter Assay System was purchased from Promega. SB203580 was from Calbiochem. Actinomycin D was from

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Invitrogen. Assays-on-Demand Gene Expression Products (mIL-6 and mGAPDH) and Taq
Man Universal PCR Master Mix were from Applied Biosystems.

Plasmids

pcDNA3-p38α and p38AF were gifts from J. Han (Scripps Institute). IL-6 3′-UTR-(1–403) were amplified from the mIL-6 cDNA built on pCR2.1 by PCR using primers terminating in XbaI recognition sequences: forward, 5′-CCTCTAGATGTGCGTTATGCCTAAGCA-3′; reverse, 5′-CCTCTAGAGTTTGAAGACAGTCTAAACAT-3′ and were ligated in the unique XbaI site of the pGL3 promoter vector (Promega Corp.). Deletion constructs and ARE mutants were generated from the full-length 3′-UTR as the template by PCR. The forward primer for 3′-UTR-(1–70) was the same as that for the full length; the reverse primer was 5′-CCTCTAGAAACAAGGAT-3′. The forward primer for 3′-UTR-(56–173) was 5′-CCTCTAGAGTTTGAAGACAGTCTAAACAT-3′; the reverse primer was 5′-CCTCTAGAGTTTGAAGACAGTCTAAACAT-3′. The forward primer for 3′-UTR-(172–403) was 5′-GGACTCTAGACTTTAAGTTAATTTTAG-3′; the reverse primer was the same as for the full length. All ARE mutants were generated by PCR-directed mutagenesis with anchor primers and mutagenic primers. The forward mutagenic primers were: M1, 5′-TTAAGGGATGATAATTTAATAAG-3′; M2, 5′-TTAAGGGATGATAATTTAATAAG-3′; M3, 5′-TTAAGGGATGATAATTTAATAAG-3′; M4, 5′-TTAAGGGATGATAATTTAATAAG-3′; M5, 5′-TTAAGGGATGATAATTTAATAAG-3′. The reverse mutagenic primers were: M1, 5′-TATCAATCCCTTAAAATAATTAAAATAG-3′; M2, 5′-TATCAATCCCTTAAAATAATTAAAATAG-3′; M3, 5′-TATCAATCCCTTAAAATAATTAAAATAG-3′; M4, 5′-TATCAATCCCTTAAAATAATTAAAATAG-3′; M5, 5′-TATCAATCCCTTAAAATAATTAAAATAG-3′. All plasmids were sequenced to verify authenticity.

Luciferase Assay

Luciferase activity was determined using a luciferase assay system, following the manufacturer’s protocol (Promega, Madison, WI). Briefly, cell monolayers in 12-well plates were removed by scraping into 200 μl of reporter lysis buffer. Cells were vortexed, and cellular debris was removed by centrifugation (30 s at 12,000 ×g). Luciferase activity was measured using a luminometer (LMaxII 384, Molecular Devices). A Renilla luciferase reporter vector was included in every experiment for transfection efficiency control. Relative luciferase activity was determined and normalized to Renilla luciferase activity.

Northern Blot

RNA was prepared from actively growing cells by using the TRIzol reagent (Invitrogen) as specified by the manufacturer. 10 μg of RNAs was separated in 1.25% Seakem® gold-agarose, transferred onto a nylon membrane (GeneScreen Plus, Dupont), and cross-linked by UV treatment (Stratalinker apparatus). The membrane was hybridized in ULTRA Hyb solution (Ambion), using 32P-labeled firefly luciferase probe (nucleotides 491–1049). The mRNA levels were normalized by 28 S RNA.

Reverse Transcription-PCR and Quantitative Real-time PCR

IL-6 mRNA expression was analyzed by reverse transcription-PCR and quantitative real-time PCR. First strand cDNA was synthesized from RNA (600 ng) using SuperScript® III reverse transcriptase (Invitrogen). First strand cDNA was used for PCR with specific oligonucleotide primers for mIL-6: forward, 5′-ATGAAGTTTCTCTCAGGAAGACT-3′; reverse, 5′-
CACTAGGTTTGCCGAGTAGATCTC-3'. Quantitative real-time PCR with primers were designed by Applied Biosystems (mIL-6, mm00446190; mGAPDH, mm99999915) using an ABI7500 thermocycler, and the gene expression was analyzed by using 7500 System SDS v1.4 software.

RNA Secondary Structure and Analysis

RNA secondary structure analysis was performed using the M-fold program (28). The folding temperature was fixed at 37 °C.

Statistical Analysis

Student t tests were utilized to obtain individual p values using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, CA.

RESULTS

p38α Mediates IL-1 Receptor-induced IL-6 Production

To investigate the in vivo function of p38α in the mechanism of IL-6 production, MEFs were prepared from p38α+/+ and p38α−/− mouse embryos and stimulated with IL-1β, Escherichia coli LPS and TNFα for 24 h. IL-6 content from culture supernatant was analyzed by enzyme-linked immunosorbent assay (Fig. 1A). Remarkably, p38α−/− MEFs incubated with IL-1β showed significantly decreased amounts of IL-6 production compared with p38α+/+ MEFs. E. coli LPS, and TNFα did not induce the dramatic differences of IL-6 production between wild-type and p38α−/− MEFs.

To confirm that this defect was caused by p38α deficiency rather than genetic mutations associated with IL-1R signaling, wild-type p38α and kinase-dead p38 (AF) cDNAs were transiently transfected into MEFs, and IL-6 production induced by IL-1β measured. Results indicate that wild-type p38α but not p38AF restored the IL-6 production in p38α−/− MEFs (Fig. 1B). In contrast, overexpression of p38α in p38α+/+ MEFs produced IL-6 in amounts similar to those of parental control cells. As expected, overexpression of kinase-dead p38AF decreased the IL-6 production in p38α+/+ MEFs. Interestingly, IL-6 production in p38α−/− MEFs was also decreased by p38AF over-expression. These data strongly indicate that p38α is critical for IL-1R-induced IL-6 production.

p38α Stabilizes Endogenous IL-6 mRNA

Previous data have indicated that the stability of IL-6 mRNA is regulated by MK2 via an AU-rich 3′-UTR of the IL-6 mRNA (29). To confirm whether p38α signaling was required for IL-6 mRNA turnover, the stability of endogenous IL-6 mRNA transcripts were monitored by actinomycin D pulse chase. After 24 h of IL-1β treatment with or without SB203580 (p38α inhibitor), actinomycin D was added to p38α+/+ MEFs, and IL-6 mRNA levels were measured at the indicated time points. The addition of SB203580 resulted in a rapid decrease in IL-6 mRNA level (Fig. 2A). The density was measured and shown in Fig. 2B. The analysis revealed that SB203580 decreased IL-6 mRNA t½ from 3.7 to 1.7 h. Importantly, the stability of endogenous IL-6 mRNA was compared in the p38α+/+ and p38α−/− MEFs. After MEFs were stimulated by IL-1β for 24 h, actinomycin D was added, and IL-6 mRNA levels were measured by real-time PCR. Fig. 2C shows a relative shorter half-life in p38α−/− MEFs (t½ = 1.9 h) compared with p38α+/+ cells (t½ = 4.6 h) indicating that IL-6 mRNA is stabilized by p38α.

p38α Targets IL-6 3′-UTR to Promote mRNA Stability

Because AREs in the 3′-UTR of many cytokine mRNAs are responsible for both translational control and mRNA stability, we examined whether the ARE-containing 3′-UTR of IL-6 is
sufficient to confer p38-dependent stabilization to a reporter mRNA. The full length of 3′-UTR of IL-6 contains five AREs with no overlapping pentanucleotide AUUUA core motifs. Instead, the AUUUA motifs are scattered throughout the IL-6 3′-UTR where both the human and mouse IL-6 3′-UTR exhibit high homology to each other (30).

To clarify the functional role, the full-length 3′-UTR was inserted into the 3′-UTR of the luciferase reporter gene and transiently transfected into p38α+/+ and p38α−/− MEF cells. The mRNA stability of luciferase reporter transcript was measured following addition of actinomycin D. As shown in Fig. 3 (A and B), IL6-3′-UTR was sufficient to elicit rapid luciferase mRNA decay in p38α−/− MEFs with a half-life of 8.6 h compared with p38α+/+ MEFs (t1/2 = 13 h). In contrast, luciferase mRNA lacking IL-6 3′-UTR was stable during a 5-h period in p38α+/+ and p38α−/− MEFs. These data indicate that p38α stabilized mRNA via IL-6 3′-UTR.

Proximal AREs Contain Elements Targeted by p38α

To investigate the role of individual AUUUA motifs in mediating post-transcriptional control regulated by p38α, a series of luciferase reporter-gene constructs, containing the various regions of IL-6 3′-UTR and the motifs mutated from AUUUA to AGGGA, were generated (Fig. 4A) and transiently expressed in MEFs. The luciferase activity of the full-length 3′-UTR and all truncations was compared first in the individual cell type. For the p38α+/+ MEFs, when the full-length 3′-UTR-(1–403) was placed in the reporter message, luciferase activity decreased more than 30% compared with luciferase alone (Fig. 4B). These data indicate that the 3′-UTR contains the elements that confer mRNA stability and/or translational efficiency. The pGL3-IL-6ARE-(56–173) truncation reporter includes the two proximal AREs (ARE1 and ARE2), whereas the three distal AREs (ARE3, ARE4, and ARE5) are included in pGL3-IL-6 3′-UTR-(127–403). The decreased expression was lost in both pGL3-IL-6 3′-UTR-(56–173) and pGL3-IL-6 3′-UTR-(172–403) compared with the full-length 3′-UTR-(1–403), suggesting that multiple control elements were operative. Whereas in the p38α−/− MEFs, the luciferase activity of the full-length 3′-UTR decreased more than 50% compared with luciferase alone and the significant decreased expression still occurred in the presence of both proximal and distal truncations. This result indicated that p38α was involved when these multiple elements mediated post-transcriptional control. When the truncation pGL3-IL-6 3′-UTR-(1–70), which includes none of the AREs was inserted in the reporter message, luciferase activity in both wild-type and p38α−/− MEFs was comparable with the control. These data indicate that the decreased expression only occurred in the presence of AUUUA motifs.

To confirm the involvement of p38α clearly, a further comparison of luciferase activity between p38α+/+ and p38α−/− MEFs was analyzed. Again, for the full-length IL-6 3′-UTR-(1–403), pGL3-IL-6 3′-UTR-(56–173), and pGL3-IL-6 3′-UTR-(172–403), the luciferase activity in p38α−/− MEFs decreased significantly compared with p38α+/+ cells (p < 0.01).

As shown in Fig. 4D, p38α enhanced luciferase mRNA stability via the full length of IL-6-3′-UTR. To confirm the cis-elements targeted by p38α, the luciferase reporter mRNA stability of truncated reporter constructs was examined in p38α+/+ and p38α−/− MEF. As shown in Fig. 5, IL-6 3′-UTR-(56–173) elicited the rapid luciferase mRNA decay in p38α−/− MEFs (t1/2 = 3.5 h), whereas the mRNA contained with IL-6 3′-UTR-(172–403) appeared to be more stable compared with 3′-UTR-(56–173) (t1/2 = 6.1 h) indicating that p38α targets the 3′-UTR-(56–173) region to stabilize IL-6 mRNA.

To estimate the influence of the individual AREs, AUUUA motifs were mutated individually and transiently expressed in MEFs. In p38α+/+ MEFs, the luciferase activity of Mu3 increased significantly compared with full-length 3′-UTR (p < 0.05) but still was significantly lower than that of control (p < 0.05). For the Mu5, the luciferase activity decreased another 20% compared

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with full-length 3′-UTR. This result indicated that ARE3 included the major repressive element, whereas ARE5 bears an enhancive element. In the p38α+/− MEFs, the luciferase activity of Mu1–4 increased significantly compared with full-length 3′-UTR, whereas Mu5 was comparable.

Again, the luciferase activity of all mutants between p38α+/+ and p38α−/− MEFs was compared. After the individual ARE motifs mutated, the significant difference due to the p38α deficiency was lost for M1, M2, and M5, whereas the luciferase activity of M3 and M4 were still significantly lower in p38α−/− MEFs compared with p38α+/+ cells suggesting that ARE1, -2, and -5 were the elements targeted by p38α.

Secondary structure prediction of IL-6 3′-UTR and mutants. Although RNA-binding proteins bind RNA in a sequence-specific manner, previous studies have indicated that RNA secondary structure plays a critical role in defining 3′-UTR RNA-protein interaction. Binding sites are often located in single-stranded RNA region (31). Using M-fold, the secondary structures of IL-6 3′-UTR and mutants were predicted (Fig. 6). For wild-type 3′-UTR, ARE2 and ARE5 comprised the sequence to form the external loop structures (LoopA and LoopD), whereas ARE3 and ARE4 formed the internal loop structures (LoopB and LoopC). ARE1 formed the stem adjacent to the external LoopA.

Mutation of ARE1 and ARE2 abolished external LoopA, and mutation of ARE5 abolished external LoopD, whereas mutation of ARE3 and ARE4 had a relatively minor effect on the secondary structure. Consistent with minor changes in secondary structure, mutation of ARE3 and ARE4 to luciferase reporter gene assay did not abolish the effect of p38α. This indicates that the secondary structure of wild-type 3′-UTR is necessary for the p38α targeting.

DISCUSSION

TNFα remains the best demonstrated cytokine regulated by the p38α MAPK pathway at post-transcriptional level. The tight control of TNFα expression is achieved by regulating mRNA stability, translational initiation, and polyadenylation. During this process, trans-acting factors that bind to TNFα 3′-UTR, including AREs are essential. In contrast, limited information is available regarding the nature of IL-6 mRNA stability and regulation by MAPK p38α. Using a p38α-deficient cell line, strict criteria regarding signaling intermediate requirements for IL-6 expression and mRNA stability could be applied. According to previous p38α inhibitor experiments, we predicted that IL-6 expression was down-regulated in p38α−/− MEFs at post-transcriptional level. Consistent with our expectation, IL-6 mRNA decay increased in p38α−/− MEFs compared with p38α+/+ cells.

In the present study, MEF cells treated with IL-1 resulted in phosphorylation of p38α to produce IL-6. Fibroblast IL-6 production was inhibited by SB203580 (26). To determine p38α is critical for the IL-1-induced IL-6 production by using p38α−/− cells, the normal expression of IL-1 receptor in p38α−/− cells is required. It has been reported that p38α−/− ES cells also gave rise to a population of IL-1R-positive cells after the in vitro differentiation process (32). The percentage of cells that express IL-1R was comparable in p38α+/+ and p38α−/− cultures. Our data show that reconstitution of p38α cDNA in p38α−/− cells restored the IL-1β-induced IL-6 production consistent with that the decrease of IL-6 production is due to the p38α deficiency rather than the defect of IL-1R. p38α is critical but not essential for the IL-6 production. Therefore, failure to increase IL-6 production by overexpression p38α in wide-type cells was not unexpected. It is possible that other proteins are limiting the involvement of p38α. For example, MAPK/extracellular signal-regulated kinase kinase kinase 3 (MEKK3), which is the potent activator of MAPKs, is essential for the IL-1-induced IL-6 production (33). However, it was somewhat surprising that p38AF decreased the IL-6 production in both p38α+/+ and
p38α−/− cells. Four members of the p38 MAPK family have been cloned: p38α, -β, -γ, and -δ (34,35). p38β shares ~74% sequence identity with p38α and contains the TGY dual phosphorylation motif observed in p38α (36). p38β is activated by IL-1 stimulation, but 4-folder lower than p38α (37). In addition, MK2 is the substrate of both p38α and p38β (37). Therefore, we predict that p38AF decreased the activation of MK2 by the co-repression of p38β.

In our present study, no significant effects of p38α deficiency on IL-6 proximal promoter reporter activity were demonstrated (data not shown). This result is consistent with previous reports from our group, where p38 inhibitors had minimal effects on IL-6 proximal promoter in MC3T3-E1 osteoblastic-like cells (38). Others have reported that SB203580 did not effect the activation of the IL-6 promoter by IL-1β in human fibroblast-like synoviocytes (26). Collectively, these results indicate that the effect of p38α deficiency on IL-6 gene expression results from the down-regulation of post-transcriptional level, however, we cannot rule out any role of transcriptional activation in these present studies.

SB203580 significantly reduced the mRNA stability of endogenous IL-6. The results were also confirmed in vivo by comparing half-life of IL-6 mRNA between p38α+/+ and p38α−/− MEFs. To analyze the regulation of IL-6 mRNA stability, the luciferase reporter gene system was employed. We found that p38α stabilized the mRNA reporter construct carrying the IL-6 3′-UTR. All these data supported the conclusion that p38α signaling increases IL-6 mRNA stability via 3′-UTR. The degradation of endogenous IL-6 mRNA and mRNA reporter construct containing the IL-6 3′-UTR is only marginally affected in p38α+/+ MEFs. This is probably due to the less classic ARE and perhaps weaker IL-6 ARE elements of the IL-6 3′-UTR compared with TNFα and other cytokines. TNFα, granulocyte macrophage-colony stimulating factor, and IL-3 AREs are typical class II AREs with a core AUUUA motif cluster, which mediated rapid mRNA decay (30). The AREs of c-myc and c-fos are prototypes of class I, containing one to three scattered copies of the AUUUA motif, whereas other cytokine transcripts, including IL-2, IL-4, and IL-6 contain class I-like AREs that are non-clustered (30). These non-clustered motifs are considered weaker AREs. The differences in cells types and stimulus may also account for this minimal degradation. In the present study, we observed only modest changes with IL-6 ARE reporter activity following stimulation with IL-1β (data not shown). These data are consistent with the concept that the IL-6 ARE is weaker compared with other AREs or alternatively there are differences in the MEF cells lines used in these studies.

A paradigm that has been helpful in understanding the regulation of post-transcriptional control was that RNA-binding protein binds to specific cis-acting elements in target transcript and activate 3′-to-5′-exonucleolytic decay or/and influence the translational control. Some RNA-binding proteins, like tristetraprolin, AU-binding factor 1, and K homology splicing-regulatory protein, promote mRNA decay, whereas others, like members of the Hu family, prevent mRNA degradation (15–17,19,20). Deletion or mutation of AUUUA motifs could alter the components of mRNA-trans factor complex and affect the luciferase activity of reporter gene containing the IL-6 3′-UTR with mutated AUUUA motifs. Using this approach, we identified ARE1, ARE2, and ARE5 as the cis-elements regulated by p38α in IL-6-3′-UTR. Interestingly, mutation of ARE1 and ARE2 resulted in the enhanced expression due to the deficiency of p38α, whereas mutation of ARE5 decreased the luciferase expression in the presence of p38α. These data are consistent with the idea that p38α is required for the ARE1 and ARE2 to repress expression and for ARE5 to enhance expression.

Our data indicated that distinct cis-elements of 3′-UTR exert opposing influence on the post-transcriptional fate of IL-6 mRNA. A similar bi-functional role for the 3′-UTR has been described for other mRNAs, including RNase-L (39). In that study, eight AREs were identified
in the RNase-L 3′-UTR, and deletion analysis identified AREs 7 and 8 served as a strong positive regulatory function, whereas AREs 2 and 4 are critical for the negative regulatory function. Also, HuR and AU-binding factor 1 were found to bind to p21 and cyclin D1 mRNAs in the nucleus, and independently operate opposite function in the cytoplasm depending on the physiological context (40).

RNA-binding proteins recognize RNA targets in a sequence-specific manner, and the secondary structure context of the binding site also affects the binding affinity (31). Binding sites are often located in single-stranded RNA regions. For example, HuR protein influences mRNA stability by binding to the motif NNTTNNTTT where HuR affinity correlates with the single-strandedness of this binding motif (41,42). IL-6 3′-UTR-(56–173) displays a typical “stem-loop” or hairpin structure. This structure might be critical for IL-6 3′-UTR-(56–173) inducing rapid mRNA decay.

Recently studies have shown that microRNA targeting of ARE appeared to be an essential step in ARE-mediated mRNA degradation (43). The ability of microRNA to target mRNA is directed by the paring of microRNA to mRNA. It is an intriguing possibility that microRNA is implicated for p38α to regulate the repressive elements in IL-6 3′-UTR.

CONCLUSION

Cell signaling through p38α MAPK is necessary for post-transcriptional regulation of many pro-inflammatory cytokines. In this study, the cis-acting elements of IL-6 3′-UTR mRNA that required p38α signaling for mRNA stability and translation were identified. AUUUA motif mutation analysis using gene reporter systems performed in p38α+/+ and p38α−/− MEF cells revealed that ARE1, ARE2, and ARE5 in IL-6 3′-UTR were targeted by p38α, and IL-6 3′-UTR-(56–173) is critical for p38 to promote mRNA stability. The predicted RNA secondary structure of these ARE reporter mutants was consistent with the ability to alter reporter expression data, suggesting that the molecular targets of p38 MAPK that interact with ARE mutants are dependent upon IL-6 3′-UTR secondary structure.

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FIGURE 1. IL-6 expression in MEFs requires p38α

A. MEFs were cultured in the presence of IL-1β (1 ng/ml), TNFα (5 ng/ml), and E. coli LPS (10 μg/ml) for 24 h. The culture supernatants were harvested, and IL-6 concentrations were measured by enzyme-linked immunosorbent assay. B. p38α+/+ and p38α−/− MEFs were seeded into 12-well plates in duplicate and transfected with empty vector pcDNA3, pcDNA3-p38α, and pcDNA3-p38AF. After 24 h, the cells were left untreated or were stimulated with IL-1β (1 ng/ml) for 24 h, and IL-6 from cultures supernatants was measured by enzyme-linked immunosorbent assay. Data represent the average of duplicate experiments repeated three times (n = 3; *, p < 0.05).
FIGURE 2. Endogenous IL-6 mRNA is stabilized by p38α signaling

A, p38α^{+/+} MEFs were seeded into 60-mm plates. After 24 h of IL-1β (1 ng/ml) stimulation with or without SB203580 (5 μM), actinomycin D (10 μg/ml) was added, and total RNA was harvested at indicated time points. Reverse transcription-PCR for IL-6 and GAPDH were performed as described under “Experimental Procedures.” B, graphical depiction of IL-6 mRNA decay from panel A normalized to GAPDH. C, p38α^{+/+} and p38α^{-/-} MEF cells were stimulated by IL-1β (1 ng/ml) for 24 h. Actinomycin D (10 μg/ml) was added, and the mRNA level of IL-6 was measured by quantitative real-time PCR. Data represent the average of three independent experiments.
FIGURE 3. IL-6 mRNA was stabilized by p38α via 3'-UTR

A, pGL3-Promoter and pGL3-IL6 3'-UTR were transfected into p38α+/+ and p38α−/− MEFs. After 24 h, the mRNA stability of ARE was assessed by actinomycin D chase experiments. The mRNA level of firefly luciferase was measured by Northern analysis. This experiment is representative of two independently performed experiments. B, quantification of the Northern blot shown in panel A (n = 2). The intensity of firefly luciferase mRNA bands was quantified using Quantity One software (Bio-Rad) and normalized to 28 S rRNA.
FIGURE 4. Reporter-gene assay identifies p38α-dependent elements in the 3′-UTR of IL-6

A, schematic representation of IL-6 3′-UTR deletion and ARE mutation reporter constructs. 

B and C, p38α+/+ and p38α−/− MEFs were transiently transfected with pGL3promoter, pGL3-IL-6-3′-UTR, or pGL3-IL-6 3′-UTR vectors containing the indicated deletion and ARE mutants of UTR shown in A. Renilla luciferase (pEF-RLuc) vector was co-transfected as transfection efficiency control. Significant differences in reporter expression between p38α+/+ and p38α−/− is indicated (*, p < 0.05; **, p < 0.01). The values represent from three independent experiments, and each experiment was performed in duplicate.
FIGURE 5. Proximal AREs were targeted by p38α to stabilize ARE reporter mRNA
A and B, pGL3-IL6 3′-UTR-(56–173) and (A) pGL3-IL6 3′-UTR-(172–403) (B) were
transfected into p38α+/+ and p38α−/− MEFs. After 24 h, the mRNA stability of ARE was
assessed by actinomycin D pulse-chase. The mRNA level of firefly luciferase was measured
by real-time reverse transcription-PCR and normalized to GAPDH mRNA levels. Results
represent three independent experiments measured in duplicate.
FIGURE 6. Major structural differences in IL-6 3′-UTR are observed following ARE mutations. Secondary structures of full-length (A) and mutant IL-6 3′-UTR (B–F) were predicted using M-fold software. Highlighted regions in B, C, and F indicate major loop change in RNA structure as predicted following ARE1, -2, and -5 mutations.