Regulation of Cyclooxygenase 2 mRNA Stability by the Mitogen-Activated Protein Kinase p38 Signaling Cascade

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Received 24 January 2000/Returned for modification 6 March 2000/Accepted 20 March 2000

A tetracycline-regulated reporter system was used to investigate the regulation of cyclooxygenase 2 (Cox-2) mRNA stability by the mitogen-activated protein kinase (MAPK) p38 signaling cascade. The stable β-globin mRNA was rendered unstable by insertion of the 2,500-nucleotide Cox-2 3′ untranslated region (3′ UTR). The chimeric transcript was stabilized by a constitutively active form of MAPK kinase 6, an activator of p38. This stabilization was blocked by SB203580, an inhibitor of p38, and by two different dominant negative forms of MAPK-activated protein kinase 2 (MAPKAPK-2), a kinase lying downstream of p38. Constitutively active MAPKAPK-2 was also able to stabilize chimeric β-globin–Cox-2 transcripts. The MAPKAPK-2 substrate hsp27 may be involved in stabilizing β-globin–Cox-2 transcripts were partially stabilized by phospho-mimetic mutant forms of hsp27. A short (123-nucleotide) fragment of the Cox-2 3′ UTR was necessary and sufficient for the regulation of mRNA stability by the p38 cascade and interacted with a HeLa protein immunologically related to AU-rich element/poly(U) binding factor 1.

Eicosanoids play a critical role in several physiological and pathophysiological processes, including blood clotting, wound healing, kidney function, acute inflammation, and cardiovascular disease (13, 19). The rate-limiting step in eicosanoid synthesis is catalyzed by cyclooxygenase (Cox) enzymes, which are encoded by two distinct genes. The Cox-1 gene is principally homeostatic in function and possesses a typical, GC-rich housekeeping promoter (55). In contrast the Cox-2 gene resembles an early response gene. It is strongly induced by mitogenic and proinflammatory stimuli, superinduced by inhibitors of protein synthesis, and acutely regulated at both transcriptional and posttranscriptional levels (17, 37–39, 46, 47). Posttranscriptional regulation is critical in determining the strength and duration of Cox-2 gene induction by external stimuli yet is poorly understood. Neither the sequences which regulate Cox-2 mRNA metabolism nor the trans-acting factors mediating this regulation have been identified.

Several proinflammatory treatments which induce Cox-2 gene expression also stimulate the mitogen-activated protein kinase (MAPK) p38. This kinase is activated by phosphorylation at threonine and tyrosine residues, catalyzed by the dual-specificity kinase MAPK kinase 6 (MKK6) (11, 23, 42). In turn, activated p38 phosphorylates numerous substrates which include kinases such as MAPK-activated protein kinase 2 (MAPKAPK-2) and -3 (18, 34, 49). Substrates of MAPKAPK-2 and -3 include the small heat shock protein hsp27, an abundant cytoplasmic protein of uncertain function (15, 18, 53). In a variety of cells treated with different inducing agents, specific inhibitors of p38 block the accumulation of Cox-2 mRNA (21, 29, 33, 41, 43, 45). In HeLa cells stimulated with interleukin 1 (IL-1) and in primary human monocytes stimulated with bacterial lipopolysaccharide inhibition of p38 results in a rapid and specific destabilization of Cox-2 mRNA but has little effect upon Cox-2 transcription (12, 44).

Regulation of mRNA stability is often mediated by sequences within the 3′ untranslated region (3′ UTR). Several Cox-2 transcripts differing in 3′ UTR length due to alternative polyadenylation site usage have been described, the two major transcripts being 4.6 and 2.8 kb long (38, 47). The most abundant (4.6-kb) transcript has a 3′ UTR of 2,515 nucleotides (nt), containing 22 copies of the pentamer sequence AUUUAA (see Fig. 1). This sequence is found in the 3′ UTRs of numerous unstable cytokine- and protooncogene-encoding mRNAs and is a prominent feature of AU-rich elements (AREs) which regulate mRNA stability (6–8, 50). Two conserved regions (CR) have been noted within the Cox-2 3′ UTR (38). CR1 lies immediately 3′ to the translation termination codon and contains six AUUUAA motifs, of which three are overlapping. CR2 lies 1,700 nt 3′ to the translation termination codon and contains three dispersed AUUUAA motifs (Fig. 1).

Several proteins have been shown to interact specifically with AU-rich RNA stability determinants, and have been implicated in positive or negative regulation of mRNA stability. For example ARE/poly(U) binding factor 1 (AUFI), a member of the hnRNP D family of RNA binding proteins, is present in a cytosolic fraction which accelerates c-myc mRNA decay in vitro (3, 60). Increased AUFI expression is associated with decreased mRNA stability in vivo (5, 40, 53), and AUFI overexpression can antagonize mRNA stabilization in vivo (31). Furthermore the immunodepletion of AUFI from cytoplasmic extracts increases the stability of an ARE-containing transcript in an in vitro degradation assay (4).

The tetracycline-responsive reporter system (20, 59) permits regulatory sequences and pathways to be mapped by performing RNA stability experiments in the absence of toxic transcriptional inhibitors. Reporter mRNAs are transcribed from a tetracycline-responsive promoter in a cell line expressing a chimeric, tetracycline-responsive transcription factor, IIA (20). In the presence of tetracycline, binding of tIA to the
promoter is blocked and transcription is inhibited. Using this system, referred to herein as the TET-off system, we demonstrate that the effects of p38 are mediated by its downstream kinase MAPKAPK-2 and may involve the phosphorylation of the MAPKAPK-2 substrate hsp27. A conserved, 123-nucleotide ARE lying immediately 3′ to the translation termination codon (CRI) is necessary and sufficient for stabilization by the p38 pathway and interacts with a HeLa cell protein which is immunologically related to AUFI.

**MATERIALS AND METHODS**

**Plasmids.** The M KK6E expression vector was a gift of J. Han. MAPKAPK-2 expression vectors were donated by C. Marshall. Human Cox-2 3′ UTR fragments were amplified by reverse transcription-PCR from IL-1-stimulated human gingival fibroblast RNA, using Moloney murine leukemia virus reverse transcriptase and Vent polymerase (New England Biolabs). Human tumor necrosis factor alpha (TNF-α) and c-myc 3′ UTR fragments were amplified from genomic DNA using TaqPlus polymerase (Stratagene). A mouse TNF-α 3′ UTR fragment was amplified from a mouse TNFα genomic clone (gift of D. Shakhov), using Vent polymerase. PCR products were cloned into pCR-Blunt (Invitrogen) and then excised with BglII or BamHI and cloned into the BglII site of pTetBEBB (gift of Ann-Bin Shyu).

RiboProbes vectors were constructed as follows. A 352-bp HincII-XbaI luciferase fragment was cloned from pGL3c reporter plasmid and fused to the human Cox-2 3′ UTR (gift of D. Fitzgerald) into pBluescript KS that had been digested with EcoRV and XbaI. A 269-bp SphI-BglII fragment was cloned from a human Cox-2 cDNA construct (gift of D. Fitzgerald) into luciferase plasmid that had been digested with BamHI and Clal. A 256-bp BstYI-ClaI fragment was cloned from a human Cox-2 3′ UTR fragment digested with BamHI and Crla. A 209-bp SphI-BglII fragment was cloned from pTetBEBB into pBluescript KS that had been digested with BamHI and EcoRV. To construct hsp27 expression vectors, the hsp27 open reading frame was excised from pGEX2T-hsp27 (gift of S. Lumb), using BamHI, and cloned in frame at the BamHI site of pFlagCMV2 (Sigma-Aldrich). Mutagenesis was performed with the QuikChange kit (Stratagene). Sequences of oligonucleotides are available upon request from the corresponding author. Novel DNA constructs were commercially sequenced by ACG (London, United Kingdom).

**Cell culture and transfection.** HeLa-TO cells (Clontech) were maintained in Dulbecco’s modified Eagle medium—10% fetal calf serum supplemented with 10% heat-inactivated foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 g/l of L-glutamine, 2.5 g/l of GlutaMax, 0.05 μg/ml of trypsin, 0.025% of gentamicin, 0.05% of non-essential amino acids, and 5% of 2-mercaptoethanol (Gibco). Cells were seeded in six-well plates at a density of 1.5 × 10^5 cells/well. The following day cells were transfected using SuperFect (Qiagen). The amount of total transfected DNA was kept constant within all experiments by addition of appropriate empty expression vectors and/or BlueScript plasmid (Stratagene). After 24 h, Tetracycline (Sigma) was added at a final concentration of 100 nm/g, and cells were harvested in guanidine thiocyanate lysis buffer (Ambion) at different intervals, as indicated in each figure. Lysates were passed through shredder columns (Qiagen) and stored at −20°C. In some experiments 1 μM SB203580 (Calbiochem) or vehicle control (dimethyl sulfoxide [0.05%]) was added to cells 30 min prior to the addition of tetracycline.

**Ribonuclease protection assay.** RiboProbes template constructs were linearized by appropriate restriction digestion and purified by phenol-chloroform extraction and ethanol precipitation. RiboProbes were synthesized using T7 polymerase (Boehringer Mannheim) in the presence of 50 Ci of [α-32P]UTP (800 Ci/mmol; American Radiolabeled Chemicals) and [γ-32P]ATP (600 Ci/mmol; American Radiolabeled Chemicals). The final concentration of unlabeled UTP in in vitro transcription reactions was 12 μM, except in the case of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), where it was 36 μM. Ribonuclease protection assays were carried out using the Direct Protect kit (Ambion). Under the conditions of hybridization DNA-RNA heteroduplexes are not detected. Protected RNA fragments were resolved by electrophoresis on denaturing 4% polyacrylamide gel. Gels were visualized and quantified by phosphorimaging (Fuji FLA 2000) and autoradiography. Autoradiographs are shown in all figures. Each experiment was performed at least three times, and results of experiments were used to check that quantitations were within the linear range of the assay.

**Western blotting.** HeLa-TO cells in six-well dishes were transfected with 780 ng of wild-type or mutant hsp27 expression vector and 220 ng of pBlueScript as described above. After 24 h proteins were harvested in radioimmunoprecipitation assay buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose (Sartorius). The membrane was probed with a monoclonal antibody to the flag epitope (anti-Flag M2 [Sigma-Aldrich]) and then with a peroxidase-coupled secondary antibody (Dako). Proteins were detected using the enhanced chemiluminescence system (Amersham).

**Preparation of HeLa extracts.** HeLa-TO cells were grown to confluence in 175-cm² flasks. The cells were cooled on ice for 5 min, rinsed once with ice-cold phosphate-buffered saline, and lysed by addition of a buffer containing 10 mM HEPES (pH 7.6), 3 mM MgCl₂, 40 mM KCl, 2 mM dithiothreitol, 5% glycerol, 0.5% NP-40, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 10 μM EDTA, and 4 μg of pepstatin and 4 μg of aprotinin in ml). After 15 min on ice, nuclei were removed by centrifugation at 600 × g for 10 min. The supernatant was aliquoted and snap frozen at −70°C.

**RESULTS**

The Cox-2 3′ UTR mediates regulation of mRNA stability by the p38 pathway. The tetracycline-responsive reporter construct pTetBEBB (59), contains a rabbit β-globin genomic fragment downstream of tetracycline operator sequences and a minimal promoter (Fig. 1). Transcriptional activity of this construct was rapidly switched off by addition of 100 ng/ml tetracycline to the culture medium (unpublished data).

A cDNA fragment encoding the Cox-2 3′ UTR was inserted at the BglII site of pTetBEBB (Fig. 1). The resulting construct (pTetBEBB-Cox-2.5) was transiently transfected into HeLa-TO cells with a luciferase expression vector and with or without a vector expressing constitutively active MKK6. After 24 h cells were treated with SB203580 or vehicle, and then tetracycline was added and cells were harvested at intervals as indicated in Fig. 2. A ribonuclease protection assay was used to quantify the β-globin—Cox-2.5 reporter transcript, Cox-2, luciferase, and GAPDH mRNAs (the last two as controls for transfection efficiency and gel loading). In all such experiments endogenous Cox-2 expression was up-regulated by MKK6, and this was
reversed by SB203580, confirming that the Cox-2 gene is a target for the p38 pathway in this particular HeLa cell line. Up-regulation of luciferase expression by MKK6 was not reproducible. The antisense β-globin probe spans an intron-exon boundary (Fig. 1), and detects an unspliced pre-mRNA species, which is indicated in Fig. 2A. Following the addition of tetracycline, this pre-mRNA rapidly disappeared, as transcription was inhibited and the pre-mRNA was processed.

Little or no decay of the β-globin transcript was detected over a 6-h tetracycline chase (see Fig. 4 for this and additional controls). In contrast the chimeric β-globin–Cox2.5 transcript decayed with a half-life of approximately 1 h. In the presence of constitutively active MKK6, the half-life of this transcript increased more than twofold. MKK6 coexpression increased the quantity of transcript detected at the start of the tetracycline chase, presumably because the increased transcript stability resulted in a greater mRNA accumulation during the 24 h prior to the addition of tetracycline. The addition of 1 µM SB203580 shortly before the addition of tetracycline reversed the MKK6-dependent stabilization of the chimeric transcript. In spite of very different starting levels, the transcripts decayed with identical half-lives in the absence of MKK6 or in the presence of both MKK6 and SB203580. Therefore, differences in rates of decay do not simply reflect limited capacity within the degradative machinery but are related to the activity of the p38 pathway. In HeLa cells phorbol myristate acetate is a potent activator of MAPKs p42 and p44 but not p38 and strongly induces expression of the Cox-2 gene. Treatment of HeLa-T0 cells with phorbol myristate acetate had no impact upon the stability of the β-globin–Cox2.5 chimeric mRNA (unpublished data). The Cox-2 3′ UTR therefore confers instability and p38-dependent stabilization upon a heterologous mRNA.

CR1 of the Cox-2 3′ UTR is necessary and sufficient for the regulation of mRNA stability by the p38 pathway. To determine what proportion of the 2.515-nt Cox-2 3′ UTR is required for p38-mediated regulation of stability, we initially cloned 1.4- and 0.6-kb 3′ UTR fragments into pTetBBB (Fig. 1). These fragments correspond to the 3′ UTRs present within two minor Cox-2 transcripts, which terminate at noncanonical ATT AAA polyadenylation signals (PA1 and PA2 in Fig. 1) (38, 47). The β-globin–Cox1.4 and β-globin–Cox0.6 transcripts behaved similarly to β-globin–Cox2.5 (Fig. 3). Therefore, only the first 600 nt of the Cox-2 3′ UTR is required for the regulation of mRNA stability by the p38 pathway and CR2 is dispensable. This is in agreement with our previous observation that endogenous 4.6- and 2.8-kb Cox-2 transcripts are identically destabilized by SB203580 in actinomycin D chase experiments (12, 44).

The 0.6-kb 3′ UTR fragment was further subdivided into a 0.1-kb fragment which contained six AUUUA repeats (equivalent to CR1) and a 0.5 kb fragment which contained only one AUUUA sequence (Fig. 1). The β-globin–Cox0.5 transcript was stable under all conditions (Fig. 3). In contrast the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3). We conclude that CR1 contains all of the sequence elements required to mediate the regulation of mRNA stability by the p38 pathway. Under all p38-activating conditions tested, the β-globin–Cox0.1 transcript was unstable and was very strongly stabilized by MKK6. This effect was significantly reversed in the presence of 1 µM SB203580 (Fig. 3).
not a general property of AREs. Neither synthesis nor degradation of the highly stable β-globin transcript from the tetracycline-regulated promoter was influenced by MKK6. The CR1 fragment did not confer instability or MKK6 responsiveness when placed in reverse orientation; therefore, it does not possess enhancer-like properties, and transcriptional effects of MKK6 coexpression can be discounted. Further, the regulation of mRNA stability by p38 is not simply a consequence of the high (78%) AU content of CR1, as this AU richness is preserved in β-globin–Cox0.1(as). Finally, all chimeric β-globin transcripts were assessed by Northern blotting and shown to be of the anticipated sizes (data not shown).

Regulation of mRNA stability is mediated by MAPKAPK-2. To further dissect the role of the p38 pathway in the regulation of Cox-2 mRNA stability, we used a series of mutants of MAPKAPK-2, a kinase which is phosphorylated and activated by p38 (18, 49). Wild-type MAPKAPK-2 had little or no impact upon the stability of β-globin–Cox0.1 transcripts; however, a constitutively active form (AspX3) (2) strongly stabilized the reporter transcript (Fig. 5A and 5C). Stabilization was also observed with β-globin–Cox2.5 and was not affected by 1 μM SB203580, consistent with the site of action of the drug in the p38 signal transduction cascade (unpublished data).

The stabilization of β-globin–Cox0.1 by MKK6 was blocked by two distinct dominant negative forms of MAPKAPK-2 (1), a nonphosphorylatable mutant (A222/334) and a kinase dead mutant (A207) (Fig. 5B and 5D). We observed a slight but reproducible additive stabilization of the reporter transcript by coexpression of MKK6 and wild-type MAPKAPK-2. Virtually identical results were obtained with β-globin–Cox2.5 (unpublished data). The results described here suggest that the regulation of mRNA stability by the p38 pathway is mediated largely or entirely by the p38 substrate MAPKAPK-2.

Chimeric β-globin–Cox transcripts are partially stabilized by hsp27 mutants. The only known substrates of the kinase MAPKAPK-2 are the transcription factors ATF1, CREB, and SRF (24, 26, 54) and the small heat shock protein hsp27 (15, 18, 53). hsp27 is an abundant cytoplasmic protein thought to play a role in cell survival following stress (14, 28, 35). MAPKAPK-2 phosphorylates three serine residues (15, 78, and 82) in human hsp27 and two serine residues (15 and 90) in the rodent homologue hsp25. Phosphorylation is thought to regulate the functional properties of the small heat shock proteins in part by controlling their association into dimers or homopolymers (27, 48). Self-association is primarily regulated by S90 phosphorylation in hamster hsp25. Phosphorylation of
S15 has little or no effect upon self-association but may regulate the interaction of hsp25 and/or hsp27 with other cellular proteins such as actin (27).

To investigate the potential role of hsp27 in the regulation of Cox-2 mRNA stability, we generated mutants in which serine 15, serines 78 and 82, or all three phospho-acceptor serines were mutated to alanine or glutamic acid. The triple glutamate substitution mutant hsp27EEE stabilized β-globin-Cox0.1 mRNA, while the single and double glutamate substitution mutants had less effect (Fig. 6A). Virtually identical results were obtained with β-globin–Cox2.5 (unpublished data). Equal expression of all mutants was confirmed by Western blotting (Fig. 6C).

The phosphorylation of hsp27 may contribute to the stabilization of mRNA by the MAPK p38 pathway, with a potential role for each of the sites in hsp27 phosphorylated by MAPKAPK-2. In this assay system the stabilizing effect of hsp27EEE was weak compared to that of MKK6 (Fig. 6) or MAPKAPK-2 itself (Fig. 5), and the alanine substitution mutants had no dominant negative effect on stabilization by MKK6 (unpublished data). However, the effects of phosphorylation site substitutions in a noncatalytic protein such as hsp27 are difficult to predict, especially within a cell which abundantly expresses the wild-type protein. In order to rule out or to prove more conclusively that hsp27 is involved in the regulation of mRNA stability, it may be necessary to use hsp27 knockout cells. It is possible that an unidentified substrate of MAPKAPK-2 plays a more significant role in this process.

**CR1 interacts with AUF1 or an immunologically related protein.** In electrophoretic mobility shift assays using a Cox0.1 probe and a HeLa-TO cytoplasmic extract three protein-RNA complexes were observed, with the lowest mobility complex (C3 in Fig. 7) being rather diffuse. No complexes were detected with the Cox0.5 or Cox0.1 antisense probes. All protein interactions with the Cox0.1 probe could be blocked by competition with an excess of unlabeled poly(U) but not with poly(A) (Fig. 7B), poly(C), or poly(G) (unpublished data). Complexes C3 and C4 were common to Cox0.1 and c-myc probes, while complex C5 was detected only with the Cox0.1 probe and complexes C1 and C2 were common to c-myc and TNF-α probes (Fig. 8B).

The ARE-binding proteins AUF1 and HuR are both present in HeLa cells and bind to the c-myc 3’ UTR and to poly(U) RNA (3, 32, 36, 60). Because of the similarities between Cox0.1 and c-myc RNA-protein complexes, we hypothesized that AUF1, HuR, or both proteins might interact with the CR1
sequence. Therefore, electrophoretic mobility shift assays were carried out using HeLa-TO cytoplasmic extract and Cox0.1 probe in the presence of antibodies raised against HuR, AUF1, or an irrelevant protein (JNK3) (Fig. 8A). A supershifted complex was detected in the presence of the AUF1 antibody, suggesting that a protein which interacts with the CR1 sequence is identical (or closely related) to AUF1. No supershifted complexes were detected with a nonimmune serum or with HuR or JNK3 antiserum. An anti-AUF1 supershifted complex was also detected using the c-myc but not the TNF-α probe (Fig. 8B). The formation of supershifted bands was not accompanied by substantial reduction of RNA-protein complexes C3 to C5. Immunodepletion of AUF1 from the HeLa-TO cytoplasmic extract did not significantly inhibit the formation of complexes C3 to C5 (unpublished data). Therefore, an AUF1-related protein is present in HeLa-TO cells and is able to bind to the CR1 probe but represents only a small proportion of the CR1-binding activity detected in electrophoretic mobility shift assays.

**DISCUSSION**

We previously reported that Cox-2 mRNA stability is regulated by p38 in human monocytes and in HeLa cells (12, 44). Those studies employed pharmacological inhibitors of transcription and of p38, each of which is a potential source of artifacts. The transcriptional inhibitor actinomycin D is cytotoxic, induces nucleocytoplasmic shuttling of several RNA binding proteins (16, 22), and may artificially stabilize some mRNAs (9, 51, 58). At high concentrations SB203580 is able to inhibit some JNK isoforms (10, 56), although at the 1 mM concentration we have used there is little or no effect upon HeLa cell JNK activity (44). At the concentration used, tetracycline has no discernible effects upon HeLa-TO cells other than the regulation of the tetracycline-responsive promoter. The present study, using a tetracycline chase procedure, provides proof of p38-dependent Cox-2 mRNA stabilization, operating through the p38 substrate MAPKAPK-2, and possibly
mediated in part by the phosphorylation of the small heat shock protein hsp27.

The p38-dependent stabilization of mRNA is sequence-specific, since 3' UTR sequences derived from c-myc or TNFα destabilise the β-globin reporter transcript, but do not confer responsiveness to the p38 pathway. Using a similar system, it has recently been demonstrated that the p38 pathway regulates the stability of reporter transcripts containing IL-6, IL-8, c-fos, and granulocyte-macrophage colony-stimulating factor (GM-CSF) AREs (57). Thus, p38 is able to regulate the stability of a subset of mRNAs containing class I AREs and a subset of mRNAs containing class II AREs. For comparison, several of the relevant ARE sequences are illustrated in Fig. 9. The TNF-α ARE is strikingly similar to CR1, and we find endogenous TNF-α mRNA stability to be regulated by p38 in RAW264.7 mouse macrophage cells (unpublished data).

The basis of selectivity of p38 action is not clear, but such selectivity most likely arises from specific protein-RNA interactions. We have shown that CR1 interacts with AUF1, a member of the hnRNP D family which may be involved in the degradation of labile mRNAs (4, 5, 30, 40, 52, 60). AUF1 is a relatively small proportion of the CR1-binding activity detected in electrophoretic mobility shift assays. However, the cell contains multiple ARE-binding factors which may not function in the control of degradation; therefore, this finding does not rule out an involvement of AUF1 in the regulation of Cox-2 mRNA stability by p38 in RAW264.7 mouse macrophage cells (unpublished data).

The p38-responsive region of the Cox-2 3' UTR forms several complexes with HeLa-TO cytoplasmic proteins. Complexes C3 to C5 are discussed in the text. FP, free probe. (A) Electrophoretic mobility shift assays were performed using Cox0.1, Cox0.1 antisense, and Cox0.5 RNA probes and 10 μg of HeLa-TO cytoplasmic extract. (B) Electrophoretic mobility shift assays were performed using a Cox0.1 RNA probe and 10 μg of HeLa-TO cytoplasmic extract in the presence of a 0- to 1,000-fold excess (by mass) of homopolyribonucleotides poly(A) or poly(U), as indicated.
define determinants of p38 responsiveness by extending the comparison of p38-sensitive and -insensitive ARE-containing transcripts using the system described here. It will also be interesting to extend the comparison of RNA-protein complexes involving p38-sensitive and p38-insensitive AREs and to determine whether the formation of complex C5 is specific to p38-sensitive AREs.

The most-abundant (4.6-kb) Cox-2 transcript contains a 2,515-nt 3'-UTR, while the second-most-abundant (2.8-kb) transcript contains a 603-nt 3'-UTR (38, 47). Only 123 nt, immediately 3' to the translation termination codon, are required for the regulation of mRNA stability by p38. Evolutionary conservation of p38-mediated stability regulation is suggested by the high degree of conservation of this region (between mouse and human transcripts: 77% for CR1, 100% for the AUUUA motifs within CR1, and 64% for the entire 3'-UTR). The 4.6- and 2.8-kb transcripts are similarly destabilized by inhibition of p38 (12, 44) but are differentially destabilized by the anti-inflammatory glucocorticoid dexamethasone (47), suggesting an involvement of distal sequences (possibly CR2). Regulation of stability by dexamethasone requires glucocorticoid receptor-mediated gene expression and could not be reconstituted using posttranscriptional reporter constructs (37, 46, 47). The p38 and glucocorticoid pathways therefore seem to employ distinct mechanisms and distinct cis-acting sequences to regulate Cox-2 mRNA stability. Thus, both transcriptional and posttranscriptional regulatory elements of the Cox-2 gene have evolved a capacity to respond to diverse extracellular signals.

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