The Proximal Region of the 3'-Untranslated Region of Cyclooxygenase-2 is Recognized by a Multimeric Protein Complex Containing HuR, TIA-1, TIAR, and the Heterogeneous Nuclear Ribonucleoprotein U*

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Cyclooxygenase-2 (COX-2) is an early response gene induced in renal mesangial cells by interleukin-1β (IL-1β). The 3'-untranslated region (3'-UTR) of COX-2 mRNA plays an important role in IL-1β induction by regulating message stability and translational efficiency. The first 60 nucleotides of the 3'-UTR of COX-2 are highly conserved and contain multiple copies of the regulatory sequence AUUUA. Introduction of the 60-nucleotide sequence into the 3'-UTR of a heterologous reporter gene resulted in a 70% decrease in reporter gene expression. Electrophoretic mobility shift assays (EMSAs) demonstrated that mesangial cell nuclear fractions contain a multimeric protein complex that bound this region of COX-2 mRNA in a sequence-specific manner. We identified four members of the protein-RNA complex as HuR, TIA-1, TIAR, and the heterogeneous nuclear ribonucleoprotein U (hnRNP U). Treatment of mesangial cells with IL-1β caused an increase in cytosolic HuR, which was accompanied by an increase in COX-2 mRNA that co-immunoprecipitated with cytosolic HuR. Therefore, we propose that HuR binds to the proximal region of the 3'-UTR of COX-2 following stimulation by IL-1β and increases the expression of COX-2 mRNA by facilitating its transport out of the nucleus.

Many early response genes encoding cytokines, lymphokines, and proto-oncogenes are transiently expressed in response to extra cellular stimuli. Although the gene can be transcriptionally regulated, the level of expression is determined in large part by changes in the half-life and/or translational efficiency of the mRNA. These changes are regulated through interactions between specific RNA sequences and trans-acting RNA-binding proteins that influence nuclear export, targeting to the ribosome, and translation of the mRNA into protein.

Among the best studied regulatory sequences are the adenosine- and uridine-rich elements (AREs),¹ found in the 3'-UTR of many short lived mRNAs (1–5). Most AREs contain multiple copies of the sequence AUUUA, which are sufficient to confer message instability when placed in the 3'-UTR of a normally stable reporter message. Additionally, AREs can play a role in regulating translational efficiency of target mRNAs (6–8). Once cells are activated by specific extracellular signals, these same ARE sequences are required for message stabilization and/or increased translational efficiency.

Several RNA-binding proteins have been isolated based on their ability to regulate mRNA expression through ARE sequences. Some of these proteins promote message expression, e.g. hnRNP A1 (9) and HuR (10, 11), whereas others primarily decrease message expression, e.g. AUF1 (hnRNP D) (12–14), tristetraprolin (TTP) (15–17), TIAR and TIA-1, (6, 8) and CUGBP2 (18). All of these proteins are believed to function in the cytoplasm but appear to be regulated by shuttling in and out of the nucleus. The mechanism whereby AREs regulate message expression in the presence of multiple and sometimes diametrically opposed signals is not fully understood.

Cyclooxygenase (COX) catalyzes the rate-limiting step in the biosynthesis of prostaglandins (19). Three isoforms of COX have been identified as follows: 1) COX-1, a constitutively expressed isoform; 2) COX-2, an early response gene that can be induced by a variety of mitogenic and pro-inflammatory stimuli; and 3) COX-3, an alternatively spliced variant of COX-1 (20). In rat mesangial cells, interleukin-1β (IL-1β) caused a significant increase in COX-2 expression due, in part, to altered mRNA stability and translational efficiency (21–23). The 3'-UTR of COX-2 is greater than 2 kilobases in length and, depending on the species, contains 11–22 copies of the ARE core sequence AUUUA. It has been shown previously that the 3'-UTR of COX-2 contains multiple regulatory elements, some of which co-localized with several of the AREs (18, 21, 24–26). One such element identified in the first 60 nucleotides of the 3'-UTR of murine COX-2 contained seven AUUUA repeats and was able to decrease message stability and translational efficiency of a heterologous reporter gene (21). This region of the murine 3'-UTR is highly conserved across species and aligns within the region of human COX-2 mRNA that was shown to bind HuR (27), AUF1 (25), and CUGBP2 (18). Furthermore, dysregulated expression of HuR promoted increased COX-2 expression in colon cancer cells (27). Thus, the binding of HuR and other yet to be determined proteins to the 3'-UTR

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EXPERIMENTAL PROCEDURES

Mesangial Cell Culture—Primary rat mesangial cell cultures were prepared from male Sprague-Dawley rats as described previously (28). Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 15 mM HEPES. Where indicated, mesangial cells were stimulated with IL-1β (100 units/ml) for 60 min.

Plasmids—Reporter gene constructs were generated as described previously (21). Briefly, various regions of the 3′-UTR of COX-2 were amplified by PCR using primers terminating in XhoI recognition sequences. PCR products were ligated in the unique XhoI site of the pGL3 control vector (Promega) located in the 3′-UTR of the firefly luciferase gene. Vectors used for synthesis of RNA probes for electrophoretic mobility shift assays (EMSA) were constructed by amplifying the same 3′-UTR sequences, except that the primers encoded a HindIII recognition site at the 5′ end and a BamHI site at the 3′ end. The PCR products were ligated into the HindIII and BamHI sites of pGEM7ZFD (Stratagene).

Transient Transfections—Mesangial cells were transiently transfected using SuperFect Transfection Reagent (Qiagen). Cells were plated into 60-mm dishes, grown to 50–75% confluency, and transfected with HuR, TIA-1, and TIAR expression plasmids at a ratio of 1:1:1. The day after transfection, transfected cells were harvested by scraping into hypotonic buffer containing 10 mM HEPES, pH 7.5, 1.5 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTG, 1 mM MgCl₂, and 25% glycerol (with protease inhibitors). Nuclei were incubated on ice for 15 min with vortexing every 2–3 min and centrifuged for 5 min at 15,000 × g. Supernatant was saved as the nuclear fraction and stored at −70 °C.

EMSA and Antibody Supershifts—Typically, 5–10 μg of cell fraction protein was incubated for 30 min at 37 °C in binding buffer (10 mM HEPES, pH 7.6, 5 μM MgCl₂, 40 mM KCl, 1 mM DTG, 5% glycerol, and 5 μg/ml heparin) with 1–5 pmol of labeled RNA probe in a final volume of 20 μl. For supershift experiments, binding mixtures included 0.2 μg of affinity-purified IgG raised against HuR, TIA-1, TIA-2, or TTP (Santa Cruz Biotechnology), 1 μg of anti-TIA-1 antibody (Upstate Biotechnology), 1 μl of anti-hnRNP A1 and anti-hnRNP U (generous gift of Gideon Dreyfuss), or 1 μl of normal goat serum (Sigma). RNase T1 (10 units) was added to EMSA or supershift samples and incubated for 15 min at 37 °C. Loading buffer (5 μl containing 80% glycerol and 0.1% bromphenol blue in 50 mM Tris-Cl, pH 7.5, was added, and samples were electrophoresed (250 V) on 4% polyacrylamide gels (for pre-run for 1 h at 250 V) containing 44 mM Tris-Cl, pH 8.3, 44 mM boric acid, 1 mM EDTA, 4% acrylamide-bisacrylamide (29:1), and 2.5% glycerol. EMSAs were visualized by autoradiography.

Immunoprecipitation—Antibodies were immobilized onto an agarose gel using a Seize Primary immunoprecipitation kit (Pierce Biotechnology). An equivalent of 5 μg of immobilized antibody was added to 5 μg of nuclear protein and incubated at room temperature for 2 h. Immunoprecipitates were isolated by centrifugation (1 min at 12,000 × g) and washed twice with immunoprecipitation buffer (25 mM Tris, 150 mM NaCl, pH 7.2). Total RNA was extracted from immunoprecipitates using an RNasy RNA isolation kit (Qiagen). Western blots were probed with monoclonal anti-HuR antibody (Santa Cruz Biotechnology) at a 1:1000 dilution. Signal detection was carried out using an enhanced chemiluminescence system (ECL, Amersham Biosciences). Western analyses of cell fractionations was carried out using the polyclonal anti-hnRNP A1 and anti-hnRNP U (Santa Cruz Biotechnology) at a 1:1000 dilution and a monoclonal anti-GAPDH antibody (Ambion) at a 1:4000 dilution. Cytosolic HuR was quantitated on the Discovery Series densitometer, and the band intensities were measured using Quantity One software (Bio-Rad).

RESULTS

AREs from the 3′-UTR of COX-2 Regulate Message Stability—The first 60 nucleotides of the 3′-UTR of COX-2 message contains multiple copies of the sequence AUUUAG (Fig. 1, Truncation #1). We have reported previously that this region of the 3′-UTR of COX-2 had a significant effect on post-transcriptional regulation of COX-2 expression (21). These 60 nucleotides contain a major translational control element and caused a decrease in message stability when placed in a chimeric reporter gene expressed in an immortalized mouse mesangial cell system. In this study, we used primary rat mesangial cells to further define key regulatory sequences and trans-acting protein factors that may play important roles in regulating the expression of COX-2 in response to stimulation with IL-1β.

Insertion of the first 60 nucleotides of the 3′-UTR of COX-2 into the 3′-UTR of a luciferase reporter message resulted in a 70% decrease in luciferase activity and a similar decrease in steady-state mRNA levels when expressed in primary rat mesangial cells (Fig. 2). This region contains seven copies of the sequence AUUUAG, i.e. three that are overlapping and located within the proximal 25 nucleotides and four copies clustered within the distal 28 nucleotide region (Fig. 1, Truncation #1). Removal of the first 19 nucleotides (Fig. 1, Truncation #2) disrupted the proximal overlapping AUUUAG repeats but had no effect on luciferase reporter gene activity or mRNA levels (Fig. 2). Truncation from the proximal 5′-end to nucleotide 24 (Fig. 1, Truncation #3) resulted in an additional 75% decrease (93% overall) in luciferase activity and mRNA levels (Fig. 2). This deletion coincides with the removal of all of the first three AUUUAG consensus sequences (Fig. 1, Truncation #3), suggest-
Nuclear Protein Complexes Bind to COX-2 AREs—To determine whether changes in luciferase activity following truncation of the 3'-UTR of COX-2 were reflected by altered binding of protein(s) to this region of the message, we performed EMSAs using radiolabeled RNA probes corresponding to the regions of the three truncations shown in Fig. 1 and protein fractions isolated from rat mesangial cells. Incubation of the RNA probe encoded by the first 60 nucleotides of the 3'-UTR of COX-2 with mesangial cell nuclear protein fractions resulted in the formation of three prominent RNA-protein complexes (Fig. 3). The RNA-protein interaction was sequence-specific, because the addition of increasing concentrations of non-labeled RNA of the same nucleotide sequence effectively competed for all three complexes, whereas there was no effect when including equal amounts of a non-related RNA of the same size derived from the TOPO TA cloning vector. All of the three complexes were present at similar levels using each of the various truncated RNA probes (Fig. 4; non-immune controls), suggesting that removal of the proximal portion of the RNA had no significant effect on the formation of the RNA-protein complexes. Incubating probes with cytosolic fractions also resulted in formation of three RNA-protein complexes that had a similar electrophoretic mobility but at a consistently lower intensity compared with complexes formed using an equal mass of nuclear proteins (results not shown). Stimulation of rat mesangial cells with IL-1β for 30–120 min had no effect on the pattern of shift and little or no change in the intensity of the shifted RNA probe (results not shown).

TIA-1, TIAR, HuR, and hnRNP U Bind the ARE Region of the 3'-UTR of COX-2—As a first step toward identifying which proteins were present in the protein-RNA complexes, we included in the binding incubation antibodies to known RNA-binding proteins, as well as non-immune serum as a control, and assessed either the formation of a higher molecular weight RNA-protein complex (supershift) or disruption of one or more of the RNA-protein complexes. Both results would suggest that the epitope recognized by the antibody was present in the complex. The three major RNA-protein complexes were still present when non-immune serum was included in the binding reaction (Fig. 4, lanes labeled NI). In contrast, inclusion of both anti-TIA-1 and anti-TIAR antibodies produced a supershift (Fig. 4A, S1) that was accompanied by a significant reduction in RNA-protein complex C2. The intensity of the supershift increased as the RNA probe was truncated, suggesting that a more stable complex forms between TIA-1 and TIAR and the shortened RNA probes. Addition of HuR antibody also resulted in a supershift (Fig. 4A, S2) and a loss of the C3 complex. The supershift was most prominent with the 60-nucleotide probe and decreased as the probes were truncated. However, the disappearance of the C3 complex occurred to the same extent for all three RNA probes. A less prominent complex migrating below complex C3 also disappeared from the 1–60 probe when HuR antibodies were added, suggesting that HuR may form more than one complex with COX-2 RNA. Thus, it appears that HuR binds all the RNA probes, but only in the presence of the 1–60 probe was a...
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Fig. 3. Detection of RNA protein complexes that form with the first 60 nucleotides of the 3'-UTR of COX-2. Radiolabeled RNA probes corresponding to nucleotides 1–60 were incubated with nuclear protein fractions for 30 min at 37 °C, digested with RNase T1 (10 units), and subjected to electrophoresis on non-denaturing polyacrylamide gels. Complexes were detected by a change in electrophoretic mobility compared with free probes (FP). Increasing amounts of non-labeled RNA probes were added to radiolabeled RNA prior to the addition of cellular fractions. Radiolabeled RNA-protein complexes (C1, C2, and C3) were inhibited by increasing concentrations of non-labeled RNA of the same sequence but not by non-related RNA species of the same nucleotide length.

Complex formed that was stable enough to result in a supershift. A supershift was also observed when using the anti-hnRNP U antibody (Fig. 4B, S1). In this case, the supershift was only seen in the presence of the truncated probes and failed to occur with the full-length probe. Inclusion of antibodies directed against TTP or hnRNP A1 failed to show any observable supershift but did result in a decreased intensity of some of the complexes, raising the possibility that these proteins may also be directly or indirectly associated with the RNA probes. In our hands, EMSA experiments using mesangial cellular fractions failed to detect formation of a protein-RNA complex between AUF1 and this region of the 3'-UTR of murine COX-2.

Stimulation of Rat Mesangial Cells with IL-1β Caused a Transient Increase in Cytosolic HuR—It has been reported that HuR is shuttled from the nucleus to the cytoplasm in response to various cellular stresses (29–34). To determine whether cytoplasmic HuR increased in response to IL-1β stimulation, we isolated cytoplasmic protein fractions from mesangial cells at various times after treatment with IL-1β and determined the HuR protein content. The purity of the cell fractions was assessed by Western analysis using an antibody directed against the splicing factor U1 snRNP 70 as a marker of nuclear fractions and an antibody against GAPDH as a cytosolic protein marker. The Western results indicate that we have isolated nuclear and cytoplasmic cell fractions that are free of any detectable cross-contamination and that the vast majority of HuR resides in the nuclear fraction (Fig. 5A). When we probed for cytosolic HuR using increasing amounts of protein, we found that HuR was detectable in the cytosol and that cells treated with IL-1β for 60 min had appreciably more cytosolic HuR than non-stimulated control cells (Fig. 5B). Time course experiments indicate that cytosolic HuR increased as early as 15 min following administration of IL-1β and peaked at 30 min (Fig. 6A). Cytosolic levels of HuR remained elevated for at least 4 h and returned to control levels within 24 h. There was no appreciable decrease in nuclear HuR levels, presumably due to the fact that only a small portion of total HuR exited the nucleus. Cytosolic GAPDH remained constant over this time period (Fig. 6A).

Because HuR binds the 3'-UTR of COX-2 in the EMSA experiments and IL-1β stimulation increased cytosolic HuR, we next tested whether there were corresponding changes in cytosolic HuR-COX-2 mRNA complexes. Cytosolic fractions were immunoprecipitated with anti-HuR antibodies, and the amount of co-immunoprecipitated COX-2 mRNA was measured by quantitative RT-PCR. The DNA transcribed by reverse transcriptase was quantitated using a cDNA clone of COX-2 as standard. The amount of COX-2 mRNA present in immunoprecipitates of cytosolic fraction from non-stimulated cells was below the detectable limit of the assay. Cells treated with IL-1β contained detectable amounts of cytosolic HuR-COX-2 mRNA complexes that increased from 30 to 60 min (Fig. 6B), suggesting that increased transport of HuR into the cytosol facilitated the export of COX-2 mRNA.

DISCUSSION

Post-transcriptional regulation of gene expression is tightly orchestrated through complex interactions of various RNA-binding proteins with target mRNAs. The best characterized target sequence is the ARE. Identification of different ARE-binding proteins and the elucidation of their functions has been the subject of many recent investigations. The AREs located in the proximal region of the 3'-UTR of COX-2 play an important role in regulating gene expression (21, 24–26). This region also has been shown to be recognized by the RNA-binding proteins HuR (27), AUF1 (25), and CUBP2 (18). Here we report that the first 60 nucleotides of the 3'-UTR of murine COX-2 were recognized by TIA-1, TIAR, and hnRNP U, in addition to HuR.

Based on EMSAs using truncated RNA probes we have shown that TIA-1 and TIAR both bind to a 37-nucleotide region of the 3'-UTR of COX-2 mRNA. This corresponds to the region reported to affect message translation (21). Both TIAR and TIA-1 have been shown to bind an ARE-containing region of
the 3′-UTR of tumor necrosis factor (TNF-α), resulting in translational silencing of the TNF-α message (6, 8). Furthermore, overexpression of TIA-1 in COS cells resulted in decreased expression of a reporter gene harboring the ARE region of TNF-α (35). Thus, it seems probable that binding of TIA-1 and/or TIAR to the 3′-UTR of COX-2 promotes translational silencing of the message. We also found that hnRNP U binds to this region of the 3′-UTR of COX-2. It is not clear at this moment what the functional significance of this binding is; however, hnRNP U has been shown to bind RNA (36, 37) as well as other proteins such as the glucocorticoid receptor (38). The ability to bind both RNA and proteins raises the possibility that hnRNP U may act as a scaffolding protein and mediate interactions between target mRNAs and proteins regulating mRNA expression. Based on the report by Lasa et al. (25), we predicted that AUF1 and the 3′-UTR of COX-2 could form a protein-RNA complex stable to EMSA and supershift assays. The inability to detect a stable complex may represent differences in cell-type and/or species-specific interactions or be due to differences in experimental conditions.

Another well characterized ARE-binding protein is HuR. Binding of mRNA by HuR is associated with increased message stability (10, 11, 39). A number of HuR target mRNAs have been reported, including the message encoding COX-2 protein (27). We have determined that one of the HuR binding sites is within the first 60 nucleotides of the 3′-UTR of murine COX-2, as evidenced by the HuR antibody-dependent supershift (Fig. 4A, S2). The supershift was accompanied by a significant decrease of a major RNA-protein complex, C3, and a minor lower molecular weight complex. When truncated RNA probes were incubated with the anti-HuR antibody, the decrease in C3 was still evident, but there was no supershift (Fig. 4A), suggesting that more than one HuR-RNA complex was present and that the more stable supershifted complex required the full 60-nucleotide probe.

Translocation of HuR from the nucleus to the cytoplasm has been shown in response to a variety of stimuli, including heat shock (29–31), serum stimulation (32), UV light (33), and nutrient limitation (34). In response to heat shock, HuR binds the proteins pp32 and APRIL, which modulates the ability to transport HuR out of the nucleus through interactions with nuclear export factor CRM1 (31). The CRM1-dependent transport can be inhibited by leptomycin B. Recently, it has been reported that leptomycin B inhibited COX-2 mRNA stabilization by blocking nuclear export of the COX-2 message (40). Although it was not determined whether HuR played a direct role in the transport of COX-2 mRNA, it does represent a reasonable conclusion. Here, we show that IL-1β stimulation caused an increase in cytosolic HuR protein levels and HuR-COX-2 mRNA complexes. Thus, we propose that in response to IL-1β stimulation there was increased binding of HuR to COX-2 mRNA, which facilitates export of the message out of the nucleus.

Taken together, our results suggest that multiple proteins can bind to a relatively short portion of the 3′-UTR of COX-2.
The types of proteins we have identified have profound effects on message expression by regulating mRNA export from the nucleus and influencing the ability of the message to be either transported to polysomes for translation or targeted for degradation. Some of these proteins, such as HuR and TIA-1, apparently have contradictory effects on the fate of the target message. Therefore, the challenge still remains to determine the mechanism whereby cells decide whether to increase or decrease gene expression when presented with RNA-protein complexes containing potentially opposing regulatory proteins.
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