Structural and functional dissection of a conserved destabilizing element of cyclo-oxygenase-2 mRNA: evidence against the involvement of AUF-1 [AU-rich element/poly(U)-binding/degradation factor-1], AUF-2, tristetraprolin, HuR (Hu antigen R) or FBP1 (far-upstream-sequence-element-binding protein 1)

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COX-2 (cyclo-oxygenase-2) mRNA is degraded rapidly in resting cells, but is stabilized by the mitogen-activated protein kinase p38 signalling pathway in response to pro-inflammatory stimuli. A conserved ARE (AU-rich element) of the COX-2 3' untranslated region, CR1 (conserved region 1), acts as a potent instability determinant, and mediates stabilization in response to p38 activation. A detailed structural and functional analysis of this element was performed in an attempt to identify RNA-binding proteins involved in the regulation of COX-2 mRNA stability. Destabilization of a β -globin reporter mRNA was dependent upon two distinct AREs within CR1, each containing three copies of the sequence AUUUA. CR1 was shown to bind AUF-1 [ARE/poly(U)-binding/degradation factor-1] and/or AUF-2, HuR (Hu antigen R), TTP (tristetraprolin) and FBP1 (far-upstream-se-

quence-element-binding protein 1), yet these factors did not appear to account for the effects of CR1 upon mRNA stability. Mutant sequences were identified that were incapable of destabilizing a reporter mRNA, yet showed unimpaired binding of FBP1 and AUF-1 and/or -2. TTP was absent from the HeLa cell line used in this analysis. Finally, RNA interference experiments argued against a prominent role for HuR in the CR1-mediated regulation of mRNA stability. We conclude that at least one critical regulator of COX-2 mRNA stability is likely to remain unidentified at present.

Key words: AU-rich element (ARE), cyclo-oxygenase-2, mitogen-activated protein kinase p38, mRNA stability, post-transcriptional regulation.

INTRODUCTION

The COX (cyclo-oxygenase) enzymes COX-1 and COX-2 perform a rate-limiting step in the synthesis of eicosanoids (prostaglandins, leukotrienes, thromboxanes, etc.) from arachidonic acid. Whereas COX-1 is expressed constitutively in some tissues and plays a largely homeostatic role, COX-2 expression is increased by a wide variety of stimuli, including growth factors and pro-inflammatory agonists. The inducible synthesis of eicosanoids by COX-2 is implicated in the control of inflammation, ovulation, kidney function, wound healing and other physiological processes [1]. Increased COX-2 gene expression and elevated levels of eicosanoids are often observed in both chronically inflamed tissues and solid tumours [1], perhaps reflecting the existence of regulatory mechanisms that are common to inflammation and tumorigenesis [2]. Both pharmacologically and genetically, COX-2 is clearly defined as a major therapeutic target in both cancers and chronic inflammatory diseases [1]. Therefore it is clearly important to understand the regulation or dysregulation of COX-2 gene expression in health or disease.

The COX-2 gene is tightly regulated at both the transcriptional and post-transcriptional levels, and is often expressed in a transient manner. In unstimulated cells, COX-2 mRNA is typically

transcribed at a low rate and degraded rapidly, resulting in low basal levels of expression. The inherent instability of COX-2 mRNA is determined by its 3' UTR (untranslated region). Alternative usage of polyadenylation signals gives rise to two major COX-2 transcripts of 4.6 and 2.8 kb. The 3' UTR of the shorter transcript is 0.6 kb long and contains seven copies of the mRNA-destabilizing motif AUUUA. Six of these are clustered within a highly conserved region, CR1 (conserved region 1), lying immediately 3' of the translation termination site. The longer transcript has a 2.5 kb 3' UTR that contains an additional 15 AUUUA motifs, three of them within a second evolutionarily conserved region (CR2). Such AREs (AU-rich elements) regulate mRNA stability via interactions with sequence-specific RNA-binding proteins, which influence either (or both) of two critical steps in eukaryotic mRNA decay: deadenylation and/or the subsequent 3' to 5' degradation of the mRNA body [3,4]. A number of ARE-binding proteins have been shown to regulate these processes positively or negatively. The competition between stabilizing and destabilizing ARE-binding proteins is thought to determine mRNA decay rates.

AREs may also play a role in the modulation of COX-2 mRNA stability in response to external agonists, contributing to dramatic changes in steady-state mRNA levels in stimulated

Abbreviations used: ARE, AU-rich element; AUF, ARE/poly(U)-binding/degradation factor; COX, cyclo-oxygenase; COX76 (etc.), 76-nucleotide fragment of the COX-2 3' UTR (etc.); CR, conserved region; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBP, far-upstream-sequence-element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hnRNP, heterogeneous nuclear ribonucleoprotein; HuR, Hu antigen R; IL, interleukin; LPS, lipopolysaccharide; MALDI, matrix-assisted laser-desorption ionization; MAPK, mitogen-activated protein kinase; MKK6, MAPK kinase 6; NP40, Nonidet P40; RPA, ribonuclease protection assay; siRNA, small interfering RNA; TIA-1, T-cell-restricted intracellular antigen-1; TIAR, TIA-1-related protein; TNF α , tumour necrosis factor α ; TTP, tristetraprolin; UTR, untranslated region.

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cells [5,6]. For example CR1 is a potent mRNA-destabilizing element, whose function is blocked by the MAPK (mitogen-activated protein kinase) p38 signal transduction pathway [7]. Hence the activation of MAPK p38 by various pro-inflammatory stimuli results in COX-2 mRNA stabilization and increased inflammatory eicosanoid synthesis [8–10]. The COX-2 3' UTR has also been implicated in the regulation of mRNA by diverse signalling pathways, including the ERK (extracellular-signal-regulated kinase) [11,12] and Akt/protein kinase B pathways [13]. Discrepancies between COX-2 mRNA and protein expression levels have been observed under certain conditions, suggesting a level of translational regulation, which may also involve the 3' UTR [14]. In summary, the COX-2 3' UTR appears to function as a complex post-transcriptional regulatory element, integrating a variety of signals to modulate the stability or translatability of the transcript, and hence the production of eicosanoids.

It follows that the overexpression of COX-2 in chronic inflammation or cancer could arise from misregulation at the post-transcriptional level, and the failure of mechanisms that normally serve to restrain COX-2 biosynthesis. At least three distinct processes might result in such post-transcriptional up-regulation, and potential examples of all three of these processes have been described in the context of chronic inflammation or cancer. Because the two major COX-2 transcripts differ in their 3' UTR sequences and can be regulated differentially at a post-transcriptional level, altered usage of polyadenylation sites could modulate COX-2 gene expression [15]. Secondly, COX-2 mRNA stabilization might be a consequence of changes in the expression of stabilizing or destabilizing ARE-binding proteins [16,17]. Thirdly, the constitutive activation of signal transduction pathways, such as the Akt/protein kinase B pathway, may stabilize COX-2 mRNA and lead to overexpression of COX-2 protein [13].

In order to understand the regulation of COX-2 gene expression in normal physiology and in pathophysiological states, it is critical to identify RNA-binding proteins involved in post-transcriptional regulation. Many proteins have already been demonstrated to interact with the COX-2 3' UTR. These include: AUF-1 [ARE/poly(U)-binding/degradation factor-1] and AUF-2 [7,18], members of the hnRNP (heterogeneous nuclear ribonucleoprotein) D family that display mRNA-stabilizing or -destabilizing functions according to context and isoform [3]; hnRNPA₀, an ARE-binding protein whose phosphorylation is regulated by the MAPK p38 pathway [19]; HuR (Hu antigen R), previously identified as an mRNA-stabilizing factor [16,17,20]; the translational repressors TIA-1 (T-cell-restricted intracellular antigen-1) and TIAR (TIA-1-related protein) [21]; and CUG-binding protein 2, which is reported to both stabilize COX-2 mRNA and inhibit its translation [22].

In HeLa cells stimulated with IL-1 (interleukin-1), the 4.5 and 2.8 kb COX-2 transcripts decayed with similar kinetics [9]. CR1 is present in both of these mRNAs, whereas CR2 is present only in the longer transcript. In these cells CR1 destabilized a reporter mRNA as efficiently as the full-length 3' UTR, and its response to MAPK p38 activation was indistinguishable from that of the full-length 3' UTR [7]. Therefore at least some aspects of the post-transcriptional regulation of COX-2 can be ascribed to CR1. We performed a detailed structural and functional analysis of this element in order to identify proteins with which it interacts, and determine their roles in the regulation of mRNA stability. We report that CR1 is a target for AUF-1 and/or AUF-2, HuR, TTP (tristetraprolin) and FBP1 (far-upstream-sequence-element-binding protein 1). However, CR1-mediated mRNA degradation does not correlate with binding of any of these factors, and therefore COX-2 mRNA decay may be regulated by proteins that remain to be identified.

EXPERIMENTAL

Materials

To generate β -globin reporter constructs, oligonucleotides (Life Technologies) with *Bam*HI/*Bgl*II-compatible cohesive ends were annealed and cloned into the *Bgl*II site of pTetBBB, a gift from Ann-Bin Shyu (Department of Biochemistry and Molecular Biology, University of Texas, Houston, TX, U.S.A.). *In vitro* transcription constructs were generated by cloning the same double-stranded oligonucleotides at the *Bam*HI site of pBluescript KS+ (Stratagene). All constructs were confirmed by sequencing (ABC; Imperial College London). Vectors expressing a constitutively active form of the MAPK p38 activator MKK6 (MAPK kinase 6) or FLAG-tagged HuR were described previously [23]. Rabbit polyclonal antisera against AUF-1/AUF-2 [18] and TTP [24] were described previously. A mouse monoclonal antibody against HuR was from Molecular Probes Inc. A mouse monoclonal antibody against α -tubulin was from Sigma-Aldrich. A human antibody against topoisomerase I was from Immunovision. Antibodies against hnRNP A₀, TIA-1 and TIAR were generously provided by Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, U.K.) and Paul Anderson (Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, MA, U.S.A.). A rabbit polyclonal antiserum against the FBP1 N-terminal peptide GGVNDAFK-DALQR was raised by Harlan SeraLabs. General chemical reagents, including *Salmonella* LPS (lipopolysaccharide), were from Sigma-Aldrich unless otherwise stated. Radiochemicals were from Amersham Biosciences.

Preparation of labelled RNA probes and RNA competitors

Constructs for the generation of β -globin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) riboprobes were described previously [7]. Template plasmids were linearized by appropriate restriction digestion, and purified using a QIAquick gel extraction kit (Qiagen). *In vitro* transcription in the presence of [α -³²P]UTP was performed as described [7]. Competitor RNAs were synthesized using the Ampliscribe kit (Epicentre Technologies). A trace quantity (0.1 μ Ci) of [α -³²P]UTP was included in the *in vitro* transcription reaction in order to accurately quantify the yield of the reaction.

Transfection, RPA (ribonuclease protection assay) and RNA interference

HeLa Tet Off cells (BD Biosciences) and RAW 264.7 cells were maintained at 37 °C under 5% CO₂ in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum. The HeLa Tet Off medium additionally contained 100 μ g/ml G418. Transfections were performed using Superfect (Qiagen) as described previously [23]. Briefly, HeLa Tet Off cells at approx. 50% confluence in six-well dishes were transfected with 100 ng of reporter construct (pTetBBB derivative), 100 ng of pCMV-MKK6E or empty vector and 800 ng of pBluescript carrier DNA. After 24 h, tetracycline was added to a final concentration of 100 ng/ml, and at the time points indicated cells were harvested by scraping into guanidine thiocyanate lysis buffer (Ambion). RPAs to quantify β -globin (reporter) and GAPDH (loading control) mRNAs were performed using a Direct Protect RPA kit (Ambion) according to the manufacturer's instructions. For RNA interference experiments, cells were transfected with siRNAs (small interfering RNAs) 24 h before transfection with plasmid constructs as described above. siRNA duplexes were purchased from Dharmacon

(Lafayette, CO, U.S.A.), and were directed against the target sequences AAGCGCGCUUUGUAGGAUUCG (Scramble) or CUACCUCUCCUCAGAACAUUG (HuR). Duplexes were prepared and transfected according to the manufacturers' instructions, using Oligofectamine transfection reagent (Invitrogen).

Preparation of cell extracts

Cells were chilled on ice for 5 min, washed once with ice-cold PBS, then harvested by scraping and pelleted by centrifugation at 600 *g* for 10 min. The cell pellet was washed again with ice-cold PBS and subjected to hypotonic lysis on ice in buffer A [10 mM Hepes, pH 7.6, 3 mM MgCl₂, 40 mM KCl, 2 mM DTT (dithiothreitol), 5 % glycerol, 0.5 % NP40 (Nonidet P40), 0.5 mM PMSF, 10 μ M E64, 4 μ g/ml aprotinin, 4 μ g/ml pepstatin, 100 μ M sodium orthovanadate and 1 μ M microcystin]. Nuclei were pelleted by centrifugation at 600 *g* for 10 min. The post-nuclear supernatant was fractionated by centrifugation at 100 000 *g* for 1 h at 4 °C to yield a pellet and soluble supernatant (S100). The pellet was solubilized in buffer A by three 10 s bursts of sonication, then clarified by centrifugation at 10 000 *g* for 10 min at 4 °C, and the supernatant (P100) collected. Nuclei were washed three times with nuclear wash buffer (10 mM Hepes, pH 7.6, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 10 μ M E64, 4 μ g/ml aprotinin, 4 μ g/ml pepstatin, 100 μ M sodium orthovanadate and 1 μ M microcystin). Washed nuclei were resuspended in buffer A and lysed by three 10 s bursts of sonication. The extract was clarified by centrifugation at 12 000 *g* for 20 min at 4 °C, and the supernatant collected. Protein concentrations of extracts were determined by the Bradford assay [24a].

EMSAs (electrophoretic mobility shift assays) and UV cross-linking

³²P-labelled RNA probes (approx. 50 fmol) were incubated for 20 min on ice with 0.5–20 μ g of nuclear, S100 or P100 protein extract in 20 μ l of bandshift buffer (10 mM Hepes, pH 7.6, 3 mM MgCl₂, 20 mM KCl, 1 mM DTT, 5 % glycerol). RNase T₁ (Roche) and heparin sulphate (Sigma) were added to final concentrations of 50 units/ml and 5 mg/ml respectively, followed by incubation on ice for a further 5 min. Then 5 μ l of loading buffer (80 % glycerol, 0.1 % Bromophenol Blue) was added and RNA–protein complexes were resolved by electrophoresis on a 4 % (w/v) native acrylamide gel containing 0.5 $\times$ Tris/borate/EDTA (1 $\times$ TBE = 45 mM Tris/borate/1 mM EDTA) and 2.5 % glycerol. Supershift and competition experiments were similar, except that protein was preincubated with antibody, antiserum, control IgG, pre-immune serum or unlabelled competitor RNA in bandshift buffer for 10 min prior to the addition of probe. For UV cross-linking, binding reactions were set up as described above, and after 20 min they were exposed to 120 mJ of UV using a Stratilinker (Stratagene). RNase T₁ and heparin sulphate were then added as above. After a further 5 min incubation on ice, SDS/PAGE loading buffer was added and samples were subjected to SDS/PAGE (10 % gels). Gels were dried down on 3MM blotting paper (Whatman) and visualized using a phosphorimager (FLA2000; Fuji).

Chromatography and MS

Throughout chromatographic purification, RNA-binding activities were monitored by EMSA as above. Starting with approx. 2 $\times$ 10⁸ HeLa Tet Off cells, an S100 fraction was prepared as above, except that Hepes, pH 7.6, was replaced by Tris/HCl, pH 9.5, in

buffer A. The S100 extract was loaded on to a 3 ml FFQ column (Amersham Biosciences), which had been pre-equilibrated with 10 column volumes of buffer A (minus protease inhibitors). The run-through was collected and the column was eluted with a linear 0–1 M NaCl gradient over 20 fractions. The run-through was adjusted to pH 7.4 using 1 M HCl, and then applied to a 1 ml heparin column (Amersham Biosciences) that had been pre-equilibrated with 10 vol. of 20 mM Tris/HCl (pH 7.4)/0.1 % NP40. The column was eluted with a linear 0–1 M NaCl gradient over 20 fractions. Fraction 10 was concentrated from 1 ml to 50 μ l using a Microcon 10 column (Amicon), then applied to a SMART Superose 12 column (Amersham Biosciences), which had been pre-equilibrated in buffer B (20 mM Tris/HCl, 300 mM NaCl, 0.1 % NP40). Material was eluted over 20 column volumes of buffer B. Each fraction was divided in two. One aliquot was used directly in EMSA with a COXC76 probe (see the Results section), and the other was diluted by addition of 80 μ l of buffer C (25 mM Hepes, pH 7.6, 1 mM DTT, 1 M KCl, 0.1 % Brij 35), then combined with poly(U)–Sephacrose 4B beads (Amersham Biosciences) and incubated at 4 °C for 1 h with continuous agitation. Beads were washed with buffer C containing only 0.5 M KCl, then with buffer C containing no KCl. Proteins were eluted by boiling in SDS/PAGE loading buffer. Eluates were electrophoresed on a SDS/polyacrylamide (10 %) gel and silver stained as described [23]. In-gel trypsinolysis was performed using an Investigator Progest (Genomic Solutions, Huntingdon, U.K.) robotic digestion system, as described previously [23]. The resulting mixtures of peptides were characterized by MALDI (matrix-assisted laser-desorption ionization) MS, using a ToFSpec 2E instrument (Micromass, Manchester, U.K.), from an α -cyano-4-hydroxycinnamic acid matrix, or by tandem electrospray MS on a Micromass Q-ToF spectrometer. Mass spectra were searched against SwissProt/TREMBL as described [25].

Western blotting

Protein extracts were electrophoresed on SDS/polyacrylamide (10 %) gels and transferred to PVDF membranes (NEN). Filters were blocked with 5 % (w/v) dried milk in PBS containing 0.1 % Tween 20, then probed with primary antibodies (1:1000) and appropriate horseradish peroxidase-coupled secondary antibodies (1:2000). Proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences).

RESULTS

Definition of a minimal COX-2 mRNA-destabilizing element

A 123-nucleotide fragment of the COX-2 3' UTR destabilized an otherwise stable β -globin reporter mRNA [7]. To identify proteins involved in this effect, we initially performed further deletion analysis using a tetracycline-regulated mRNA stability assay as described [7]. Briefly, 3' UTR fragments are cloned downstream of a tetracycline-responsive promoter and rabbit β -globin open reading frame in the plasmid pTetBBB. Following transient transfection of a cell line that expresses a chimaeric tetracycline-regulated transcription factor, transcription of the reporter mRNA is halted by addition of tetracycline, and reporter mRNA is quantified by RPA. The COX-2 3' UTR fragments used in this study are detailed in Figure 1. A 76-nucleotide fragment of the COX-2 3' UTR (COX76) was identical in function to the longer 123-nucleotide fragment. It destabilized the reporter mRNA and mediated stabilization upon co-expression of MKK6E, a constitutively active form of the p38 activator MKK6

Figure 3 Binding of nuclear proteins requires both proximal and distal AUUUA clusters of CR1

(A) EMSAs were performed with TNF75, COX76 and COXC76 probes, and 0, 0.625, 1.25, 2.5, 5, 10 or 20 μ g of nuclear extract. (B) EMSAs were performed with COXC76 probe and 10 μ g of nuclear extract, in the presence of 0-, 1-, 2-, 5-, 10-, 20- or 40-fold excess of unlabelled competitor RNA as indicated.

fragment was observed (Figure 3B). The G $\rightarrow$ C mutated fragment was indistinguishable from the unaltered COX76 fragment in function (Figure 2 and Table 1), whereas neither the COX33 nor the COX43 fragment destabilized the β -globin reporter mRNA detectably (Table 1). Hence both p38-sensitive mRNA decay *in vivo* and protein–RNA complex formation *in vitro* require an extended ARE.

CR1 interacts with HuR and members of the hnRNP D family

In order to identify proteins interacting with the minimal destabilizing element, the COXC76 probe was used in EMSAs with various HeLa cell fractions. In IL-1-stimulated HeLa cells, a relatively small proportion of endogenous COX-2 mRNA was associated with free polysomes in the cytoplasm. The majority was associated with the endoplasmic reticulum (results not shown), which co-purified with the nucleus under the conditions described (see the Experimental section). We attempted a more rigorous purification of an endoplasmic reticulum fraction as a source of COX-2 mRNA-binding proteins, but could not recover active RNA-binding proteins from this extract, which contained high concentrations of salts and detergents. Therefore EMSAs were performed using a 'nuclear' fraction, the soluble supernatant remaining after high-speed centrifugation of the cytoplasm (S100) or the solubilized pellet from this centrifugation step (P100). The nuclear fraction also contained endoplasmic reticulum components. The P100 fraction contained free cytosolic polysomes. The S100 fraction is known to contain ARE-binding proteins, deadenylating and 3' to 5' exonucleolytic activities [26,27].

The P100 fraction and the COXC76 probe formed a single retarded complex of high mobility (P5 in Figure 4, left panel; complexes are numbered in order of increasing mobility, and

Figure 4 CR1 is recognized by HuR and AUF-1/AUF-2

EMSA were performed with probe COXC76 and 10 μ g of P100 extract (left panel), 10 μ g of nuclear extract (middle panel) or 2 μ g of nuclear extract (right panel). A monoclonal antibody against HuR, control IgG, an antiserum against AUF-1/AUF-2 or control pre-immune serum (PI) was incubated with the protein extract in binding buffer for 10 min prior to the addition of probe. The positions of RNA–protein complexes P5, N2, N4 and N5 are indicated. Supershifted complexes are indicated by asterisks.

the protein source is indicated by the letter P, S or N for P100, S100 or nuclear fraction respectively). In the presence of a monoclonal antibody against the RNA-binding protein HuR, P5 was strongly depleted and a novel supershifted complex was observed. In EMSAs the nuclear fraction formed three retarded bands, labelled N2, N4 and N5 (Figure 4, middle panel). N2 was poorly resolved, and may consist of more than one RNA–protein complex. N5 was identical in mobility to P5, and was also supershifted by an anti-HuR antibody. This is consistent with reports that HuR is a nuclear–cytoplasmic shuttling protein [20]. N4 was similarly depleted and supershifted in the presence of the anti-HuR antibody; therefore complexes P5, N5 and N4 contain HuR.

Supershifts were also attempted using a previously described peptide antiserum that recognizes both AUF-1 and the closely related RNA-binding protein AUF-2/CBF-A (C A-rich G box-binding factor A) [18]. AUF-1 and AUF-2 are both present in HeLa cells, and appear very similar in function [18]. Our AUF-1-specific antisera do not supershift RNA–protein complexes; therefore we were unable to distinguish between AUF-1 and AUF-2 in the present study. The anti-AUF-1/AUF-2 antiserum supershifted the nuclear complexes N2 and N4 (Figure 4, right panel). Because N4 is supershifted by antibodies or antisera against HuR or AUF-1/AUF-2, this complex appears to contain both proteins. It is not yet clear whether the formation of such a mixed complex involves interaction between HuR and AUF-1/AUF-2 proteins, or independent interactions of proteins with two distinct binding sites contained by the COXC76 probe.

RNA–protein binding reactions were set up using probe COXC76 and a nuclear extract. RNA–protein complexes were UV cross-linked, treated with RNaseT₁, separated by SDS/PAGE and detected by autoradiography. Under these conditions two cross-linked RNA–protein complexes were detected (Figure 5A). Both of these could be immunoprecipitated using an anti-HuR antibody, but not by protein G or an isotype-matched control antibody. The anti-AUF-1/AUF-2 serum did not immunoprecipitate any labelled RNA–protein complexes under identical conditions (results not shown). The P100 extract formed cross-linked complexes similar to those observed with the nuclear extract, and again these could be immunoprecipitated by an anti-HuR monoclonal antibody (results not shown). Under identical conditions, proteins in the S100 extract did not cross-link with the COXC76 probe (results not shown).

Figure 5 Cross-linking of HuR to CR1

(A) Binding reactions were set up with probe COXC76 and 10 μ g of nuclear extract. After 20 min, reaction mixtures were UV cross-linked and treated with RNase T₁. RNA–protein complexes were immunoprecipitated (IP) with a monoclonal antibody against HuR, with control IgG, or with protein G (PrG) alone. Pellets (P) and supernatants (S) from the immunoprecipitation procedure were subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel, and cross-linked complexes were detected by phosphorimaging. Starting material (i.e. cross-linked RNA–protein complexes not subjected to immunoprecipitation) was run in the first lane. Positions of molecular mass markers are indicated to the left. (B) Nuclear extracts were prepared from HeLa cells transiently transfected with an empty vector (CMV) or a vector expressing FLAG-tagged HuR (CMV–HuR). Binding reactions were set up with probe COXC76 and 10 μ g of nuclear extract. After 20 min, reaction mixtures were UV cross-linked and treated with RNase T₁ or RNase A, then subjected to SDS/PAGE on a 10% (w/v) polyacrylamide gel. Cross-linked complexes were detected by phosphorimaging.

The HuR-containing cross-linked complexes were of estimated masses 58 and 82 kDa. In Western blots of HeLa cells with an anti-HuR antibody, a single protein band of approx. 34 kDa was detected (results not shown), and the predicted mass of the RNase T₁ limit digest of the COXC76 probe is approx. 24 kDa. The most likely explanation is that the cross-linked complexes each contain one molecule of HuR, but differ in the number of RNA molecules to which the protein is cross-linked, i.e. two in the upper and one in the lower complex. Treatment of RNA–protein

complexes with the more promiscuous nuclease RNase A prior to immunoprecipitation resulted in the recovery of a single, high-mobility HuR–RNA complex similar in molecular mass to HuR itself. These observations are consistent with an ability of HuR to make two simultaneous contacts with discrete AU-rich binding sites. Finally, the overexpression of epitope-tagged HuR gave rise to cross-linked complexes of slightly greater mass than those involving endogenous HuR, whether RNA–protein complexes were treated with RNase A or RNase T₁.

CR1 is a target for FBP1

The soluble cytoplasmic extract (S100) formed a complex with the COXC76 probe (labelled S1 in Figure 6A). This was clearly distinct in mobility from the complexes observed with the nuclear or P100 extracts, and was not supershifted by any of the antisera or antibodies tested, including those against AUF-1/AUF-2 or HuR (results not shown). In some experiments an additional complex, S3, was observed (e.g. see Figure 6C). The proteins present in complex S3 are antigenically related to those in S1 (see below), and may arise from proteolytic cleavage of an RNA-binding protein during the preparation of the extract. We attempted to identify proteins present in S1 and S3 by a chromatographic approach. An S100 extract was fractionated by anion-exchange chromatography. Binding activity was recovered in the flow-through (results not shown), which was then applied to a heparin column and eluted with a 0–1 M salt gradient. Binding activity eluted in three fractions, over approx. 350–550 mM NaCl (results not shown). These fractions were concentrated and fractionated by gel filtration. Eluted fractions were split into two, and either used in EMSA or subjected to affinity purification using poly(U)–Sepharose. No binding activity was detected in the poly(U)–Sepharose supernatants, indicating efficient adsorption of CR1-binding proteins by this matrix. Poly(U) beads were eluted in Laemmli loading buffer, and the eluates inspected by SDS/PAGE and silver staining.

COXC76 probe binding activity was recovered from the gel filtration column in three fractions, corresponding to molecular

Figure 6 CR1 is recognized by FBP1

(A) CR1-binding proteins in the S100 fraction were chromatographically purified as described in the Experimental section. Fractions 9–16 eluted from the gel filtration column were used in EMSAs with probe COXC76 (upper panel). The same fractions were adsorbed on to poly(U)–Sepharose 4B, and eluates were subjected to SDS/PAGE on a 10% gel, followed by silver staining (lower panel). Positions of molecular mass markers are indicated to the left. (B) Tryptic peptides from the 70 kDa band in (A) were analysed by MS. The sequence of human FBP1 protein is shown. Peptides identified by MALDI MS are underlined, and four peptides sequenced by tandem electrospray MS are indicated in bold. (C) EMSAs were performed with probe COXC76 and 10 μ g of HeLa cell S100 extract. Pre-immune or anti-FBP1 serum was incubated with the protein extract prior to the addition of probe in the second and third lanes respectively. The positions of the S100 complexes S1 and S3 are shown, and supershifted complexes are indicated by asterisks.

Figure 7 Subcellular localization and RNA-binding specificity of FBP1

(A) EMSAs were performed with probe COXC76 and 10 μ g of HeLa cell S100 extract in the presence of increasing amounts of unlabelled COXC76 competitor RNA or unrelated GAPDH 3' UTR competitor RNA. The position of the major S100 complex S1 is indicated. (B) Nuclear (N), S100 (S) and P100 (P) fractions were prepared from HeLa cells following lysis by Dounce homogenization or using an NP40-containing lysis buffer. Fractions were Western blotted for topoisomerase (Topo), tubulin or FBP1.

masses of approx. 65–75 kDa (Figure 6A). Binding activity was precisely co-distributed with a highly purified poly(U)-binding protein of approx. 70 kDa. MALDI MS and quadrupole time-of-flight tandem MS (Figure 6B) identified this 70 kDa protein as FBP1, a member of a family of three closely related nucleic acid-binding proteins that are related to hnRNP K [28]. Four tryptic peptides sequenced by quadrupole time-of-flight MS were unique to FBP1 (Figure 6B). Tryptic peptides covering approx. 54 % of the FBP1 sequence were identified by MALDI MS, and all but a few of these were unique to FBP1 (Figure 6B). No peptides specific to FBP2 or FBP3 were recovered from the 70 kDa band. Finally, a rabbit polyclonal antiserum was raised against residues 25–37 of FBP1, a region that is relatively poorly conserved in FBP2 and FBP3 (8/13 and 6/13 identical residues respectively). This antiserum strongly supershifted the cytoplasmic complexes S1 and S3 (Figure 6C), suggesting that both of these complexes contained FBP1. FBP2 and FBP3 may not be expressed in HeLa cells, or may fail to bind to CR1 under the conditions described.

FBP1 was identified initially as a nuclear single-stranded DNA-binding protein that regulated the expression of *c-myc* via binding to the far upstream sequence element of the *c-myc* locus [29]. Members of the FBP family have been implicated in mRNA processing, localization or degradation. They are principally nuclear, but also localize to discrete cytoplasmic loci [30,31]. An affinity purification method was recently used to identify cytoplasmic proteins interacting with the TNF α ARE, and three of the proteins identified in that study were FBP1–FBP3 [19]. FBP2, otherwise known as KSRP, can stimulate the decay of ARE-containing transcripts via recruitment of the exosome [32]. The binding specificity and localization of FBP1 were therefore examined in further detail. In competition assays, FBP1 displayed approx. 4-fold higher affinity for the CR1 sequence than for a control sequence lacking ARE motifs (Figure 7A). This result suggests a modest selectivity of FBP1 for the COX-2 ARE. The

Figure 8 CR1 is recognized by TTP

RAW264.7 cells were left untreated or were stimulated with 10 ng/ml LPS for 1 h. Unfractionated cytoplasmic extracts were prepared and 10 μ g portions were used in EMSAs with probe COXC76 in the presence of a pre-immune serum or an antiserum raised against the C-terminus of TTP. The position of the major TTP-containing complex is shown at the left, and a supershifted complex is indicated by an asterisk.

localization of FBP1 was assessed by Western blotting of nuclear and cytoplasmic S100 fractions (Figure 7B). The integrity of these fractions was confirmed using topoisomerase I and tubulin as respective markers. Following lysis in the presence of NP-40 according to our standard protocol for cell fractionation (see the Experimental section), FBP1 was readily detected in the S100 fraction. However, following lysis by Dounce homogenization, FBP1 was mostly present in the nucleus, as reported previously [33], with a small quantity in the cytosol. Topoisomerase I was consistently recovered in the nuclear fraction, and tubulin in the cytosolic fraction, under both conditions of extract preparation.

CR1 is a target for TTP

The RNA-destabilizing factor TTP (also known as Nup475 or TIS11) may be phosphorylated either by p38 itself or by the p38-activated kinase MAPKAPK-2 (MAPK-activated protein kinase 2) [24,34]. It has been suggested that the regulation of mRNA stability by p38 may be mediated by changes in TTP function; for example, TTP might function as an mRNA destabilizer, which is inactivated by the p38 pathway [34]. TTP was shown to bind to a distal region of the COX-2 3' UTR; however, its ability to interact with CR1 was not addressed specifically in that study [15]. The optimal TTP-binding site has been identified as the nonameric sequence UUAUUUAUU [35], which is present only once in the COX-2 3' UTR, within CR1. To investigate the interaction of TTP with CR1, we used extracts of LPS-stimulated RAW264.7 cells (Figure 8). The RAW264.7 extract formed an LPS-inducible complex with the COXC76 probe that could be supershifted by an anti-TTP antiserum; hence CR1 is able to bind to TTP. However, we were not able to detect the expression of endogenous TTP in HeLa cells by Northern blotting, Western blotting or EMSA supershift (results not shown).

Mutational analysis of CR1

As described above, the principal CR1-binding factors detected by EMSA using HeLa cell extracts were HuR, FBP1 and AUF-1/AUF-2. To investigate the roles of these proteins in the post-transcriptional regulation of COX-2 gene expression, we performed a mutational analysis of CR1, attempting to correlate changes in function with changes in affinity for RNA-binding

Figure 9 Inactivating mutations of CR1 impair binding of HuR, but not FBP1 or AUF-1/AUF-2

EMSA were performed using an S100 extract (**A**) or a nuclear extract (**B**) with probe COXC76, COXC76m2 or COXC76m4. The positions of major S100 complexes S1 and S3 and nuclear complexes N2, N4 and N5 are indicated. (**C**) Binding reactions were set up using 10 µg of nuclear extract and probe COXC76, COXC76m2 or COXC76m4. After 20 min, UV cross-linking was performed, and complexes were treated with RNase T1 and subjected to SDS/PAGE. Cross-linked RNA–protein complexes were detected by phosphorimaging. Numbers denote molecular mass (kDa).

proteins. In order to conserve the size and approximate AU content of RNA fragments, the point mutation AUUUA to AUCUA was made within individual pentamer motifs in the context of the COXC76 fragment. Mutation of two proximal pentamers (COXC76m1) or two distal pentamers (COXC76m3) resulted in a partial loss of destabilizing function. The moderately unstable β -globin/COXC76m1 and β -globin/COXC76m3 transcripts could still be stabilized by co-expression of the MAPK p38 activator MKK6E (Table 1). Mutation of three proximal pentamers (COXC76m2) or three distal pentamers (COXC76m4) resulted in complete loss or severe impairment of destabilizing function (Table 1 and Figure 2), consistent with the previous observation that both regions of CR1 contribute to mRNA destabilization. The COXC76m4 sequence retained a very weak destabilizing

function, and was responsive to p38 activation, whereas the p38-responsiveness of COXC76m2 could not be determined.

The abilities of mutated CR1 sequences to interact with RNA-binding proteins were then examined. In the cytosolic S100 fraction (Figure 9A), the COXC76, COXC76m2 and COXC76m4 probes formed identical complexes (S1 and S3); therefore the loss of destabilizing function is not correlated with the loss of binding of FBP1. In the nuclear fraction (Figure 9B), probes COXC76m2 and COXC76m4 displayed unimpaired formation of complex N2, which contains hnRNP D protein(s); therefore the loss of destabilizing function is not correlated with loss of hnRNP D binding.

Probes COXC76m2 and COXC76m4 failed to form the HuR-containing complexes N4 and N5 (Figure 9B), and also failed to cross-link to HuR *in vitro* (Figure 9C). The failure to cross-link could be due to the mutation of residues that are critical for RNA–protein cross-linking rather than RNA–protein complex formation. However, unlabelled COXC76m2 and COXC76m4 also failed to compete with COXC76 in cross-linking reactions (results not shown). Taken together with the EMSA result, this suggests that COXC76m2 and COXC76m4 bind HuR with very low affinity. The functionally intermediate mutants COXC76m1 and COXC76m3 were also unable to cross-link to HuR *in vitro*, although a consistent defect in binding to HuR was not observed in EMSAs (results not shown). In summary, partial impairment of the destabilization function of CR1 was correlated with a subtle change in HuR binding, whereas complete loss of destabilizing function was correlated with complete loss of the ability to bind to HuR.

HuR does not play a critical role in the regulation of COX-2 mRNA stability

HuR is generally thought to function as an mRNA-stabilizing factor [20], and thus the surprising correlation between reporter mRNA instability and HuR binding was investigated in more detail. First, overexpression of HuR resulted in stabilization of a β -globin/COXC76 reporter mRNA (Figure 10A). Similar results have been described using other ARE-containing reporter

Figure 10 HuR does not play a major role in the CR1-mediated regulation of reporter mRNA stability

(**A**) HeLa Tet Off cells were transiently transfected with 100 ng of pTetBBB-COXC76 and 100 ng of empty vector or vector expressing epitope-tagged HuR. Total DNA was made up to 1 µg using pBluescript as a carrier. After 24 h, tetracycline was added to a final concentration of 100 ng/ml and cells were harvested after 0, 1, 2 or 4 h. β -Globin reporter mRNA and GAPDH loading control mRNA were quantified by RPA, and the normalized ratio of β -globin to GAPDH was plotted semi-logarithmically against time. This experiment was performed twice with similar results. HeLa cells were transfected with siRNA directed against HuR, or with a control siRNA (Scramble or Scr.) that has no known eukaryotic target. After 24 h, cells were transfected with 100 ng of pTetBBB-COXC76, 100 ng of a vector expressing constitutively active MKK6 (MKK6E) or the corresponding empty vector, and 800 ng of pBluescript. After a further 24 h the cells were harvested for Western blotting of HuR (**B**), or tetracycline chases were performed (**C**). β -Globin and GAPDH mRNAs were quantified by RPA. This experiment was performed twice with similar results.

mRNAs, and are consistent with a stabilizing function for HuR [20]. However, protein overexpression can give rise to artefactual results, and therefore RNA interference was also used to inhibit endogenous HuR expression (Figure 10B). In the absence of any stimulus, an estimated 95% reduction in endogenous HuR protein expression did not alter the stability of β -globin/COXC76 reporter mRNA (Figure 10C, upper panel). The stabilization of β -globin/COXC76 mRNA by MKK6E was not blocked by RNA interference-mediated knock-down of HuR expression (Figure 10C, lower panel). Therefore HuR appears not to be required for the rapid degradation of β -globin/COXC76 reporter mRNA in the absence of MAPK p38 activity, or for stabilization of the reporter in response to MAPK p38 activation.

DISCUSSION

CR1, a short ARE from the COX-2 3' UTR, acts as a potent mRNA-destabilizing element, and mediates mRNA stabilization in response to activation of the MAPK p38 signalling pathway in HeLa cells [7]. We performed a detailed structural and functional analysis of CR1, with three aims: (1) to characterize a minimal p38-responsive element as a means of gaining insight into selective mRNA stabilization by the p38 pathway; (2) to identify proteins interacting with this element in HeLa cell extracts; and (3) to identify RNA-binding proteins involved in the rapid degradation of CR1-containing transcripts and/or the inhibition of this decay by the MAPK p38 pathway.

Previous studies identified a minimal mRNA-stabilizing element as the nonameric motif UUAUUUA[U/A][U/A] [36,37]. CR1 consists of two discrete AU-rich domains, each of which contains a copy of this nonameric sequence, yet neither domain in isolation was capable of destabilizing a β -globin reporter mRNA. Instead, reporter mRNA destabilization and p38 responsiveness required a relatively extended ARE containing both domains of CR1. This is reminiscent of observations described by Holtmann and colleagues [38]. In similar assays, a 49-nucleotide IL-8 ARE containing four AUUUA motifs, and a granulocyte/macrophage colony-stimulating factor ARE containing five overlapping AUUUA motifs, were insufficient for p38-sensitive reporter mRNA decay, and each required additional flanking AU-rich sequences to function. Another recent publication described the identification of 45 transcripts stabilized by MAPK p38 in THP-1 cells [39]. These differed widely in the length and structure of their 3' UTRs, with some containing as few as one copy of the AUUUA motif [6]. The basis of selective mRNA stabilization by MAPK p38 is not clear from the inspection of primary structure. However, several of the p38-responsive elements that we have examined appear, like CR1, to contain at least two discrete AU-rich domains (G. Sully, C. Tchen, A. Mavropoulos and A. R. Clark, unpublished work). It is possible that secondary structures formed by these domains play an important role in the post-transcriptional actions of the MAPK p38 pathway.

The present study confirms previous observations that the COX-2 3' UTR is a target of the RNA-binding protein HuR. The CR1 probe and the P100 fraction formed a single RNA-protein complex that contained HuR. Other known RNA-binding proteins did not appear to be present in this complex. An apparently identical complex was formed by the nuclear extract, consistent with the shuttling of HuR between the nucleus and the cytoplasm [20]. Prominent nuclear complexes contained AUF-1 and/or AUF-2. Although these proteins were also present in the cytoplasm, we were not able to detect interactions between CR1 and AUF-1/2 other than in the nuclear fraction. Interestingly, the nuclear complex N4 was supershifted by antibodies against either HuR or AUF-1/2. To our knowledge this is the first demonstration

of a mixed complex involving HuR and members of the hnRNP D family; however, its significance remains unclear.

In the cytoplasmic fraction the most prominent RNA-protein interaction involved FBP1, a member of a family of three proteins that are related to hnRNP K [28]. Members of the FBP family interact with diverse RNA elements, including AREs [19,32], a splicing regulatory sequence of c-Src mRNA [40], a stability determinant of GAP-43 (growth-associated protein 43) mRNA [41] and sequences involved in the cytoplasmic localization of microtubule-associated protein 2 and β -actin mRNAs [30,31,42]. There is little obvious similarity between these putative RNA targets, and FBP1 has additionally been shown to interact with single-stranded DNA in the context of the c-myc far upstream sequence element [29]. The present study does not suggest a highly specific interaction of FBP1 with CR1, but further studies are required to determine the nucleic acid-binding specificities of these proteins. Members of the FBP family are principally nuclear, although there is evidence for cytoplasmic expression and function, for example in the localization of specific mRNAs [30,31,33,42]. In the present study, FBP1 was relatively easily released from a HeLa cell nuclear fraction in the presence of NP40. An involvement in the cytoplasmic localization of COX-2 mRNA remains possible; therefore the association of FBP1 and COX-2 mRNA *in vivo* is under further investigation.

In the course of these studies, we used antibodies against TIA-1, TIAR and hnRNP A₀ to investigate the binding of these proteins to CR1. Under the conditions employed, no interactions of these proteins with a CR1 probe were detected in RNA EMSAs (results not shown). We were unable to detect TTP in HeLa cells. However, using extracts from an LPS-treated mouse macrophage cell line as a source of TTP, we demonstrated that this RNA-binding protein recognizes CR1, consistent with the presence of a putative high-affinity binding site within this region [35]. Although TTP clearly does not play a role in the regulation of COX-2 mRNA stability in HeLa cells, it may do so in other cell types. It would be interesting to determine whether the expression of COX-2 is dysregulated in a TTP knockout mouse that overexpresses TNF α protein as a consequence of TNF α mRNA stabilization [43].

Current models of ARE-mediated regulation of mRNA decay invoke competition between stabilizing and destabilizing factors. It is suggested that changes in mRNA stability are brought about by changes in the expression, subcellular localization or RNA affinity of such factors. Of the CR1-binding proteins we detected, HuR, AUF-1 and AUF-2 are known regulators of mRNA stability. Although regulation of mRNA decay by FBP1 has not been demonstrated, it is closely related to KSRP, a protein thought to destabilize ARE-containing mRNAs by means of recruitment of the exosome [32]. We therefore investigated the effects of overexpression of the p38 activator MKK6E, treatment with IL-1 or UV light, all of which result in powerful activation of MAPK p38. No changes in RNA-protein interactions were observed in any HeLa cell fraction following these treatments (results not shown). Furthermore, we were not able to uncouple mRNA destabilization and MAPK p38-mediated stabilization. All of the CR1 mutants studied were either devoid of destabilizing function or destabilized a reporter mRNA to some extent, and remained responsive to MAPK p38 activation. Hence it was not possible directly to identify mediators of post-transcriptional regulation by MAPK p38. The RNA-binding factor responsible for the rapid degradation of CR1-containing mRNAs in the absence of p38 activity was similarly elusive. Mutations that resulted in complete loss of destabilizing function had no impact on the binding of AUF-1/2, suggesting that these proteins are not sufficient to direct mRNA decay. The binding of FBP1 was likewise unaffected by CR1-inactivating mutations.

On the other hand, a close correlation existed between the destabilizing function of CR1 mutants and their ability to bind HuR. Previous studies have described similar correlations between the destabilizing function of AREs and their affinities for HuR [44–47], which was originally identified on this basis as a putative destabilizing factor. This conclusion was later challenged by the observation that HuR overexpression caused stabilization of ARE-containing reporter mRNAs [20]. We likewise observed HuR-dependent stabilization of a reporter mRNA containing CR1. Because overexpression experiments can be prone to artefacts, we also used RNA interference to investigate the role of HuR in CR1-directed mRNA degradation. Surprisingly, an almost complete knockdown of endogenous HuR protein had no detectable effect on CR1-mediated mRNA decay in either the absence or the presence of a p38 activator. siRNA-mediated knockdown of endogenous HuR also did not influence the stability of endogenous COX-2 mRNA in the absence or presence of IL-1 (J. L. E. Dean, K. Alford and J. Saklatvala, unpublished work). We conclude that HuR is unlikely to play a prominent role in the regulation of COX-2 mRNA stability by MAPK p38 in HeLa cells. Consistent with this conclusion, we have shown that MAPK p38 regulates mRNA deadenylation [48], whereas HuR is thought to regulate decay of the mRNA body [26,49].

A major conclusion is that the regulation of COX-2 mRNA stability by MAPK p38 cannot be explained in terms of the known RNA-binding proteins HuR, AUF-1 and -2, TTP or members of the FBP family. It is likely that at least one important post-transcriptional regulator of COX-2 remains to be identified. Presumably this is similar to HuR in its RNA-binding specificity, but is not readily detected in EMSAs. Possible reasons include: (1) failure of short RNA probes to form secondary structures that contribute to RNA–protein interactions; (2) instability of RNA–protein interactions during electrophoresis; (3) release of highly abundant non-specific RNA-binding proteins during cell fractionation; and/or (4) extremely low abundance of functionally significant RNA-binding proteins. To overcome these difficulties, we are presently attempting to identify proteins that are associated with the full-length COX-2 mRNA *in vivo*, and to determine how these interactions are influenced by mutation of the CR1 element.

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REFERENCES

- Turini, M. E. and DuBois, R. N. (2002) Cyclooxygenase-2: a therapeutic target. *Annu. Rev. Med.* **53**, 35–57.
- Coussens, L. M. and Werb, Z. (2002) Inflammation and cancer. *Nature (London)* **420**, 860–867.
- Guhaniyogi, J. and Brewer, G. (2001) Regulation of mRNA stability in mammalian cells. *Gene* **265**, 11–23.
- Wilusz, C. J., Wormington, M. and Peltz, S. W. (2001) The cap-to-tail guide to mRNA turnover. *Nat. Rev. Mol. Cell Biol.* **2**, 237–246.
- Shim, J. and Karin, M. (2002) The control of mRNA stability in response to extracellular stimuli. *Mol. Cell* **14**, 323–331.
- Clark, A. R., Dean, J. L. and Saklatvala, J. (2003) Post-transcriptional regulation of gene expression by mitogen-activated protein kinase p38. *FEBS Lett.* **546**, 37–44.
- Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J. and Clark, A. R. (2000) Regulation of cyclooxygenase 2 mRNA stability by the mitogen-activated protein kinase p38 signaling cascade. *Mol. Cell. Biol.* **20**, 4265–4274.
- Dean, J. L., Brook, M., Clark, A. R. and Saklatvala, J. (1999) p38 mitogen-activated protein kinase regulates cyclooxygenase-2 mRNA stability and transcription in lipopolysaccharide-treated human monocytes. *J. Biol. Chem.* **274**, 264–269.
- Ridley, S. H., Dean, J. L., Sarsfield, S. J., Brook, M., Clark, A. R. and Saklatvala, J. (1998) A p38 MAP kinase inhibitor regulates stability of interleukin-1-induced cyclooxygenase-2 mRNA. *FEBS Lett.* **439**, 75–80.
- Bachelor, M. A., Silvers, A. L. and Bowden, G. T. (2002) The role of p38 in UVA-induced cyclooxygenase-2 expression in the human keratinocyte cell line, HaCaT. *Oncogene* **21**, 7092–7099.
- Zhang, Z., Sheng, H., Shao, J., Beauchamp, R. D. and DuBois, R. N. (2000) Posttranscriptional regulation of cyclooxygenase-2 in rat intestinal epithelial cells. *Neoplasia* **2**, 523–530.
- Xu, K., Robida, A. M. and Murphy, T. J. (2000) Immediate-early MEK-1 dependent stabilization of rat smooth muscle cell cyclooxygenase-2 mRNA by Gq-coupled receptor signaling. *J. Biol. Chem.* **275**, 23012–23019.
- Sheng, H., Shao, J. and DuBois, R. N. (2001) K-Ras-mediated increase in cyclooxygenase 2 mRNA stability involves activation of the protein kinase B1. *Cancer Res.* **61**, 2670–2675.
- Cok, S. J. and Morrison, A. R. (2001) The 3'-untranslated region of murine cyclooxygenase-2 contains multiple regulatory elements that alter message stability and translational efficiency. *J. Biol. Chem.* **276**, 23179–23185.
- Sawaoka, H., Dixon, D. A., Oates, J. A. and Boutaud, O. (2003) Tristetraprolin binds to the 3'-untranslated region of cyclooxygenase-2 mRNA. A polyadenylation variant in a cancer cell line lacks the binding site. *J. Biol. Chem.* **278**, 13928–13935.
- Nabors, L. B., Gillespie, G. Y., Harkins, L. and King, P. H. (2001) HuR, a RNA stability factor, is expressed in malignant brain tumors and binds to adenine- and uridine-rich elements within the 3' untranslated regions of cytokine and angiogenic factor mRNAs. *Cancer Res.* **61**, 2154–2161.
- Dixon, D. A., Tolley, N. D., King, P. H., Nabors, L. B., McIntyre, T. M., Zimmerman, G. A. and Prescott, S. M. (2001) Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. *J. Clin. Invest.* **108**, 1657–1665.
- Dean, J. L., Sully, G., Wait, R., Rawlinson, L., Clark, A. R. and Saklatvala, J. (2002) Identification of a novel AU-rich-element-binding protein which is related to AUF1. *Biochem. J.* **366**, 709–719.
- Rousseau, S., Morrice, N., Pegg, M., Campbell, D. G., Gaestel, M. and Cohen, P. (2002) Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. *EMBO J.* **21**, 6505–6514.
- Brennan, C. M. and Steitz, J. A. (2001) HuR and mRNA stability. *Cell. Mol. Life Sci.* **58**, 266–277.
- Dixon, D. A., Balch, G. C., Kedeshera, N., Anderson, P., Zimmerman, G. A., Beauchamp, R. D. and Prescott, S. M. (2003) Regulation of cyclooxygenase-2 expression by the translational silencer TIA-1. *J. Exp. Med.* **198**, 475–481.
- Mukhopadhyay, D., Houchen, C. W., Kennedy, S., Dieckgraefe, B. K. and Anant, S. (2003) Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2. *Mol. Cell* **11**, 113–126.
- Dean, J. L., Wait, R., Mahtani, K. R., Sully, G., Clark, A. R. and Saklatvala, J. (2001) The 3' untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. *Mol. Cell. Biol.* **21**, 721–730.
- Mahtani, K. R., Brook, M., Dean, J. L., Sully, G., Saklatvala, J. and Clark, A. R. (2001) Mitogen-activated protein kinase p38 controls the expression and posttranslational modification of tristetraprolin, a regulator of tumor necrosis factor alpha mRNA stability. *Mol. Cell. Biol.* **21**, 6461–6469.
- Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Wait, R., Miller, I., Eberini, I., Cairoli, F., Veronesi, C., Battocchio, M., Gemeiner, M. and Gianazza, E. (2002) Strategies for proteomics with incompletely characterized genomes: the proteome of *Bos taurus* serum. *Electrophoresis* **23**, 3418–3427.
- Ford, L. P., Watson, J., Keene, J. D. and Wilusz, J. (1999) ELAV proteins stabilize deadenylated intermediates in a novel *in vitro* mRNA deadenylation/degradation system. *Genes Dev.* **13**, 188–201.
- Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L. and Brewer, G. (1993) Purification, characterization, and cDNA cloning of an AU-rich element RNA-binding protein, AUF1. *Mol. Cell. Biol.* **13**, 7652–7665.
- Davis-Smyth, T., Duncan, R. C., Zheng, T., Michelotti, G. and Levens, D. (1996) The far upstream element-binding proteins comprise an ancient family of single-strand DNA-binding transactivators. *J. Biol. Chem.* **271**, 31679–31687.
- Duncan, R., Bazar, L., Michelotti, G., Tomonaga, T., Kruttsch, H., Avigan, M. and Levens, D. (1994) A sequence-specific, single-strand binding protein activates the far upstream element of c-myc and defines a new DNA-binding motif. *Genes Dev.* **8**, 465–480.
- Rehbein, M., Wege, K., Buck, F., Schweizer, M., Richter, D. and Kindler, S. (2002) Molecular characterization of MARTA1, a protein interacting with the dendritic targeting element of MAP2 mRNAs. *J. Neurochem.* **82**, 1039–1046.
- Gu, W., Pan, F., Zhang, H., Bassell, G. J. and Singer, R. H. (2002) A predominantly nuclear protein affecting cytoplasmic localization of beta-actin mRNA in fibroblasts and neurons. *J. Cell Biol.* **156**, 41–51.

- 32 Chen, C. Y., Gherzi, R., Ong, S. E., Chan, E. L., Raijmakers, R., Puij, G. J., Stoecklin, G., Moroni, C., Mann, M. and Karin, M. (2001) AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**, 451–464
- 33 He, L., Weber, A. and Levens, D. (2000) Nuclear targeting determinants of the far upstream element binding protein, a c-myc transcription factor. *Nucleic Acids Res.* **28**, 4558–4565
- 34 Carballo, E., Cao, H., Lai, W. S., Kennington, E. A., Campbell, D. and Blakeshear, P. J. (2001) Decreased sensitivity of tristetraprolin-deficient cells to p38 inhibitors suggests the involvement of tristetraprolin in the p38 signaling pathway. *J. Biol. Chem.* **276**, 42580–42587
- 35 Worthington, M. T., Pelo, J. W., Sachedina, M. A., Applegate, J. L., Arseneau, K. O. and Pizarro, T. T. (2002) RNA binding properties of the AU-rich element-binding recombinant Nup475/TIS11/tristetraprolin protein. *J. Biol. Chem.* **277**, 48558–48564
- 36 Zubiaga, A. M., Belasco, J. G. and Greenberg, M. E. (1995) The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol. Cell. Biol.* **15**, 2219–2230
- 37 Lagnado, C. A., Brown, C. Y. and Goodall, G. J. (1994) AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: the functional sequence within AU-rich elements may be UUAUUUA(U/A)(U/A). *Mol. Cell. Biol.* **14**, 7984–7995
- 38 Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C. Y., Shyu, A. B., Muller, M., Gaestel, M., Resch, K. and Holtmann, H. (1999) The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. *EMBO J.* **18**, 4969–4980
- 39 Frevel, M. A., Bakheet, T., Silva, A. M., Hissong, J. G., Khabar, K. S. and Williams, B. R. (2003) p38 Mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. *Mol. Cell. Biol.* **23**, 425–436
- 40 Min, H., Turck, C. W., Nikolic, J. M. and Black, D. L. (1997) A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. *Genes Dev.* **11**, 1023–1036
- 41 Irwin, N., Baekelandt, V., Goritchenko, L. and Benowitz, L. I. (1997) Identification of two proteins that bind to a pyrimidine-rich sequence in the 3'-untranslated region of GAP-43 mRNA. *Nucleic Acids Res.* **25**, 1281–1288
- 42 Snee, M., Kidd, G. J., Munro, T. P. and Smith, R. (2002) RNA trafficking and stabilization elements associate with multiple brain proteins. *J. Cell Sci.* **115**, 4661–4669
- 43 Carballo, E., Lai, W. S. and Blakeshear, P. J. (1998) Feedback inhibition of macrophage tumor necrosis factor- α production by tristetraprolin. *Science* **281**, 1001–1005
- 44 Myer, V. E., Fan, X. C. and Steitz, J. A. (1997) Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. *EMBO J.* **16**, 2130–2139
- 45 Sokolowski, M., Furneaux, H. and Schwartz, S. (1999) The inhibitory activity of the AU-rich RNA element in the human papillomavirus type 1 late 3' untranslated region correlates with its affinity for the elav-like HuR protein. *J. Virol.* **73**, 1080–1091
- 46 Vakalopoulou, E., Schaack, J. and Shenk, T. (1991) A 32-kilodalton protein binds to AU-rich domains in the 3' untranslated regions of rapidly degraded mRNAs. *Mol. Cell. Biol.* **11**, 3355–3364
- 47 Blaxall, B. C., Pende, A., Wu, S. C. and Port, J. D. (2002) Correlation between intrinsic mRNA stability and the affinity of AUF1 (hnRNP D) and HuR for A + U-rich mRNAs. *Mol. Cell. Biochem.* **232**, 1–11
- 48 Dean, J. L., Sarsfield, S. J., Tsounakou, E. and Saklatvala, J. (2003) p38 mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumour necrosis factor AU-rich elements by inhibiting deadenylation. *J. Biol. Chem.* **278**, 39470–39476
- 49 Peng, S. S., Chen, C. Y., Xu, N. and Shyu, A. B. (1998) RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J.* **17**, 3461–3470

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