

Functional Analysis of KSRP Interaction with the AU-Rich Element of Interleukin-8 and Identification of Inflammatory mRNA Targets^{∇†}

Reinhard Winzen,^{1‡} Basant Kumar Thakur,^{1‡} Oliver Dittrich-Breiholz,² Meera Shah,¹ Natalie Redich,¹ Sonam Dhamija,¹ Michael Kracht,² and Helmut Holtmann^{1*}

Institute of Biochemistry¹ and Institute of Pharmacology,² Medical School Hannover, D-30623 Hannover, Germany

Received 17 August 2007/Returned for modification 13 September 2007/Accepted 19 September 2007

mRNA stability is a major determinant of inflammatory gene expression. Rapid degradation of interleukin-8 (IL-8) mRNA is imposed by a bipartite AU-rich element (ARE) in the 3' untranslated region (R. Winzen et al., Mol. Cell. Biol. 24:4835–4847, 2004). Small interfering RNA-mediated knockdown of the ARE-binding protein KSRP resulted in stabilization of IL-8 mRNA or of a β -globin reporter mRNA containing the IL-8 ARE. Rapid deadenylation was impaired, indicating a crucial role for KSRP in this step of mRNA degradation. The two IL-8 ARE domains both contribute to interaction with KSRP, corresponding to the importance of both domains for rapid degradation. Exposure to the inflammatory cytokine IL-1 has been shown to stabilize IL-8 mRNA through p38 mitogen-activated protein (MAP) kinase and MK2. IL-1 treatment impaired the interaction of KSRP with the IL-8 ARE in a manner dependent on p38 MAP kinase but apparently independent of MK2. Instead, evidence that TTP, a target of MK2, can also destabilize the IL-8 ARE reporter mRNA is presented. In a comprehensive approach to identify mRNAs controlled by KSRP, two criteria were evaluated by microarray analysis of (i) association of mRNAs with KSRP in pulldown assays and (ii) increased amounts in KSRP knockdown cells. According to both criteria, a group of 100 mRNAs is controlled by KSRP, many of which are unstable and encode proteins involved in inflammation. These results indicate that KSRP functions as a limiting factor in inflammatory gene expression.

Rapid degradation controls the levels of many mRNAs that are translated into transiently expressed proteins. These include cytokines, growth factors, proto-oncogene products, and other proteins participating in acute reactions. The short half-lives of their mRNAs depend on regulatory RNA sequences, the most widely distributed being AU-rich elements (AREs) located in their 3' untranslated regions (UTRs) (8, 39). AREs have been divided into three classes, differing in the sequences and modes of degradation imposed by them (8). Class I AREs contain one to three scattered AUUUA motifs, and class II AREs contain multiple overlapping AUUUA motifs. Class III AREs are less well defined and lack an AUUUA motif. With the search pattern WWWU(AUUUA)UUUW, 4,000 human mRNAs have been reported to contain AREs and grouped into the ARED database (1), where the class II AREs are further subdivided into different groups, depending on the number of AUUUA motifs present in an ARE.

Interleukin-8 (IL-8) is a member of the CXC chemokine family, released from different types of cells in response to direct cell stress, pathogens, or the proinflammatory cytokines tumor necrosis factor (TNF) and IL-1 (reference 25 and references therein). It attracts and activates leukocytes and also

plays a role in angiogenesis. Studying its induction in response to IL-1, we previously observed that in addition to transcriptional activation of the IL-8 gene, its mRNA is stabilized (26, 46). The latter response involves the activation of p38 mitogen-activated protein (MAP) kinase and its substrate kinase MK2. Stabilization of IL-8 mRNA can contribute to enhanced IL-8 expression, e.g., in viral infection (22). Our recent studies showed that the IL-8 mRNA contains an ARE which consists of two functionally distinct domains. They cooperate for maximal destabilization and interaction with cytoplasmic proteins *in vitro* (44).

Control of mRNA degradation by AREs involves the function of proteins binding to them. ARE-binding proteins include destabilizing factors such as TTP, BRF1, or KSRP, which recruit RNA degrading enzymes, as well as stabilizing factors like HuR (12, 17). AUF1/hnRNP D has been shown to function in both ways.

The KH-type splicing regulatory protein (KSRP) was originally identified as a factor involved in regulated splicing of *c-src* (35). It contains four hnRNP K homology domains and is a member of the family of far upstream sequence binding proteins (FUBP) (11), also named FUBP2 accordingly. KSRP has been shown to play a role in rapid degradation of ARE-containing transcripts (3, 7, 9, 15, 18, 19, 32, 38). Other functions which depend on interactions with mRNA sequences distinct from AREs have been ascribed to KSRP or its homologs. A chicken homolog interacts with the zipcode sequence that controls β -actin mRNA localization (23). In rat, KSRP also binds to the β -actin zipcode sequence (40) and to a region determining localization of microtubule-associated protein 2 mRNA (36). In *Xenopus* oocytes, a member of the FUBP family stim-

* Corresponding author. Mailing address: Institute of Biochemistry, Medical School Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany. Phone: (49) 511-5323384. Fax: (49) 511-5322827. E-mail: holtmann.helmut@mh-hannover.de.

† Supplemental material for this article may be found at <http://mc.asm.org/>.

‡ R.W. and B.K.T. contributed equally to this work.

∇ Published ahead of print on 1 October 2007.

ulates removal of a translational repressor element from the Vg1 mRNA (30). Despite these observations, more comprehensive information on target mRNAs directly regulated by KSRP is largely missing.

We now report that in HeLa cells suppression of KSRP expression by small interfering RNA (siRNA) indicates an essential role for rapid degradation of IL-8 mRNA and its deadenylation as the initiating step in it. The two domains of the IL-8 ARE are involved in interaction with KSRP in vitro and in intact cells. Interaction with the IL-8 ARE is impaired by IL-1 in a manner dependent on p38 MAP kinase but not on MK2, explaining in part the stabilization induced by the p38/MK2 pathway. Microarray experiments indicate that KSRP interacts with numerous ARE-containing mRNAs but also with transcripts that lack AREs, including its own mRNA. By comparison of mRNAs associated with KSRP and upregulated by KSRP knockdown, using IL-1 stimulation as in vitro model of an inflammatory response, a group of bona fide targets of KSRP is defined. Their regulation of expression by KSRP is expected to have significant impact on inflammatory gene expression in vivo.

MATERIALS AND METHODS

Plasmids. The expression vectors for β -globin (ptetBBB) carrying insertions of the IL-8 3' UTR, for constitutively active MAP kinase kinase 6 (MKK6_{2E}) and MK2 (MK2_{EE}), and for negative interfering MK2 (MK2_{K76R}) have been described in detail previously (44, 46). ptetBBB-TNF₁₃₁₅₋₁₃₅₀ expresses an mRNA that contains the ARE of human TNF- α in its 3' UTR. To express KSRP with an N-terminal Strep tag (stKSRP), a sequence encoding the Strep-tactin target peptide Strep tag III (29) was inserted into pcDNA3.1-HisC-KSRP (a kind gift from Ching-Yi Chen). The KSRP sequence in the resulting plasmid was replaced by the coding region of green fluorescent protein (GFP) to express Strep-tagged GFP (stGFP) or of human TTP to express Strep-tagged TTP (stTTP).

Transfection and determination of mRNA degradation. HeLa cells constitutively expressing the tetracycline-controlled transactivator protein were transfected by the calcium phosphate method, and the degradation kinetics of plasmid-expressed mRNA was determined using the tet-off system as detailed earlier (44). Cells were stimulated with human recombinant IL-1 α (a gift from A. Stern and P. T. Lomedico, Hoffman-La Roche) where indicated. The degradation kinetics for endogenous mRNA was determined by inhibiting transcription with actinomycin D (5 μ g/ml). Total RNA was isolated and Northern blot analysis performed using digoxigenin-labeled antisense RNA probes. RNA half-lives were determined as described in reference 46, using a video imaging system and the Molecular Analyst program (Bio-Rad). siRNA duplexes (Qiagen) specifically interfering with expression of KSRP (18) or GFP (47) were transfected by the calcium phosphate method. Transfection was repeated 2 days later with the respective siRNA together with plasmids for reporter mRNA expression. Samples for determining KSRP protein levels and mRNA degradation were prepared the following day. Essentially similar increases in mRNA stability in KSRP knockdown cells were observed with a second siRNA duplex (modified from reference 3).

Analysis of poly(A) tail length. To resolve heterogeneity in poly(A) tail length, RNA samples were separated on denaturing polyacrylamide gels (4% [wt/vol] acrylamide, 7 M urea) as described previously (44). Running in 1 \times Tris-borate-EDTA was performed at about 10 V/cm for variable times, depending on the size of the mRNA monitored. Deadenylated reference RNAs were prepared by RNase H digestion in the presence of oligo(dT) (44).

Strep tag affinity chromatography. All steps were carried out in the cold. Cells were pelleted, resuspended at 10⁷ cells per 125 μ l in lysis buffer (20 mM β -glycerophosphate [pH 7.4], 150 mM NaCl, 7.5% [vol/vol] glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 0.5% [vol/vol] Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 200 units/ml RNase inhibitor [MBI Fermentas]) and kept on ice for 15 min. After centrifugation at 16,000 \times g, the supernatant was collected (cytoplasmic extract). Aliquots were frozen for determination of stKSRP protein and input mRNAs. Strep-tactin agarose beads (IBA) preincubated for 30 min with tRNA from *Escherichia coli* (50 μ g/ml) in 500 μ l wash buffer (20 mM β -glycerophosphate [pH 7.4], 150 mM NaCl, 1 mM EDTA) were washed twice in the same buffer and added to the cytoplasmic

extract. After 4 h with constant mixing, the beads were spun down and washed three times with wash buffer. Aliquots of the beads were heated to 95°C with sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for subsequent analysis of stKSRP amounts or were supplied with 10 μ g of bacterial rRNA as carrier RNA and subjected to total RNA isolation (RNA isolation kit; Macherey & Nagel).

Reverse transcription-PCR (RT-PCR). Total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and amplified with *Taq* polymerase (both from MBI Fermentas) and specific primers (MWG Biotech) for CXCL3 (sense, 5'TCTCGCACAGCTTCCCGA; antisense, 5'CTAGAAAGCTGCTGTTCTC), 14-3-3 ζ (sense, 5'AGGTCATCTTGGAGGGTC; antisense, 5'CTCC TTGGGTATCCGATG), I κ B ζ (sense, 5'CTCCTGTTGCACATCCGA; antisense, 5'CAGCTAACTTGAAGTGTGT), IL-8 (sense, 5'CTGGGCACAGAACTTAT GTTG; antisense, 5'GGTAAGCCTACACTTCCA), IL-8 (sense, ATCAATAT TTGTGCAAGAATTGG; antisense, TTTCAGATAAACAATAATGT); KSRP (sense, 5'GAGAAGATAGCGAGATCTAA; antisense, 5'GAATGTTCCACCTC TAACTA); FUBP3 (sense, 5'AATCAAGCAGTTGCAGGAG; antisense, 5'CCG TGTATGTTATCTCCTG), and TTP (sense, 5'GATCTGACTGCCATCTACG; antisense, TCACTCAGAAACAGAGATGC).

Western blot analysis. Proteins were separated by SDS-PAGE (10% acrylamide) and blotted onto polyvinylidene difluoride membranes (Millipore). Equal loading and protein transfer were controlled by staining with Coomassie brilliant blue. KSRP was detected by chemiluminescence using a specific monoclonal antibody (kindly provided by C.-Y. Chen) and alkaline-phosphatase-labeled secondary antibody.

Microarray analysis. RNA from pulldown or siRNA-mediated knockdown experiments was purified and Cy3-labeled cRNA prepared as described previously (21). For analysis of KSRP-associated mRNAs on high-density arrays, RNA was isolated from Strep-tactin pulldown in four independent experiments which yielded essentially similar sets of mRNAs enriched by stKSRP compared to stGFP. Global mRNA expression of input samples was analyzed in parallel. Table S1 in the supplemental material shows results from one experiment where stringency was increased by raising the NaCl concentration to 700 mM in the first two washes of Strep-tactin agarose beads after incubation with cytoplasmic extract (see above). Cy3-labeled cRNA was generated using an Amino Allyl MessageAmp II with Cy3 kit according to the manufacturer's recommendations (AM1795; Applied Biosystems). Even amplification efficiency was controlled by spiking reverse transcription reactions with a mixture of 10 different E1A mRNA species (5188-5977; Agilent Technologies) that were each detected by 32 probes on the microarray. cRNA quality, yield, and labeling efficiency were analyzed by an Agilent 2100 bioanalyzer. To minimize technically caused variations, cRNA for each RNA sample was generated in duplicate and pooled prior to labeling and hybridizing. Labeled cRNAs were hybridized to the Whole Human Genome Microarray (G4112F, ID 014850; Agilent Technologies) containing 45,015 probes for 15,713 annotated genes and 15,269 uncharacterized transcripts. cRNA fragmentation, hybridization, and washing steps were performed exactly as directed by the manufacturer's One-Color Microarray-Based Gene Expression Analysis Protocol V5.0.1 manual. Slides were scanned on an Agilent G2505 B microarray scanner at high (100%) and low (5%) photomultiplier tube settings. Data extraction and intra-array normalization were performed with Feature Extraction V9.1.3.1 software (Agilent protocol file GE1-v5_91_0806.xml). Flagged spots were excluded from further analysis. Interarray normalization was performed according to E1A signals. Intensity values close to background levels were replaced by surrogate values, calculated from E1A signals, to compensate for elevated bias at the low end of the dynamic intensity range. Ratio data of probes directed against the same RNA were averaged by calculating the geometric mean.

In vitro transcription and electrophoretic mobility shift assays. Radiolabeled RNA fragments corresponding to the IL-8 ARE and mutants thereof were synthesized with T7 RNA polymerase from linearized plasmids as described recently (44). The labeled RNA probes were purified using mini Quick Spin RNA columns (Roche Diagnostics) and incubated with cytoplasmic extract (prepared by Nonidet P-40 lysis as in reference 45) or with affinity-purified stKSRP eluted from the Strep-tactin agarose with desthiobiotin (10 mM; IBA). After digestion with RNase T1, samples were separated on nondenaturing polyacrylamide gels (44) or UV cross-linked with 900 mJ/cm² and separated by SDS-PAGE (45).

RESULTS

KSRP is essential for rapid degradation of IL-8 mRNA. Rapid turnover of IL-8 mRNA is largely dependent on an

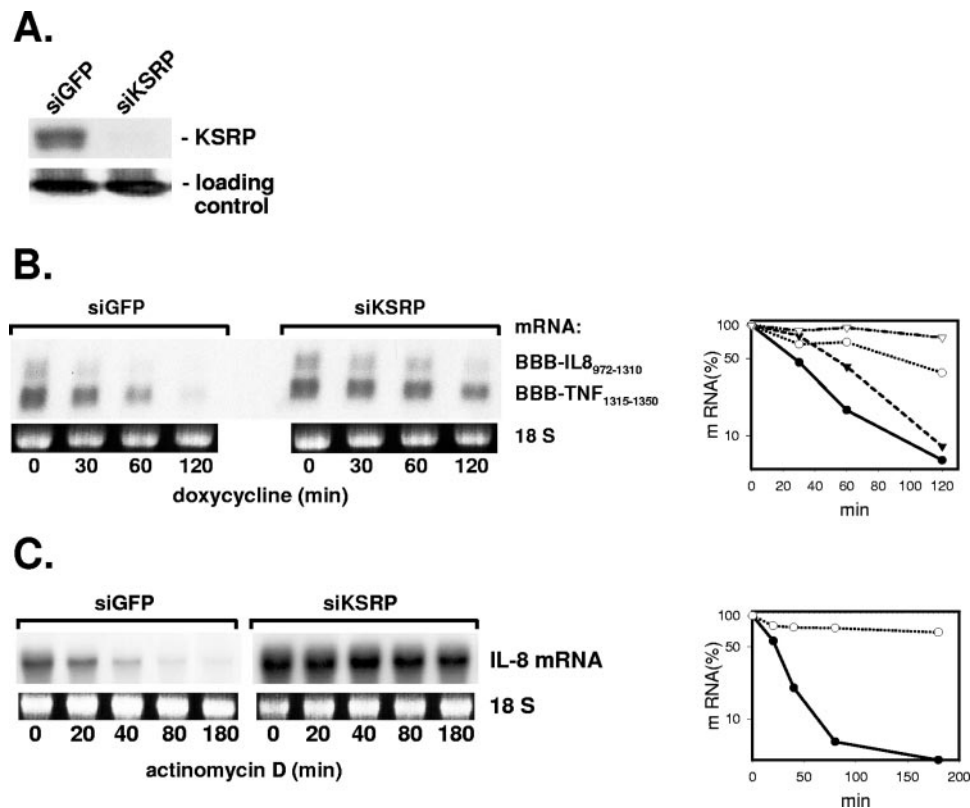


FIG. 1. Evidence for a crucial role for KSRP in the control of IL-8 mRNA decay. HeLa cells were transfected with siRNA specific for KSRP or for GFP as a control (siKSRP or siGFP, respectively). (A) Western blot with antibodies against KSRP to control knockdown efficiency. Coomassie brilliant blue staining of an irrelevant protein is shown as a loading control. (B) Cells were cotransfected with plasmids for β -globin mRNAs containing destabilizing elements of IL-8 or of TNF- α mRNA. At the indicated times after stopping transcription with doxycycline (3 μ g/ml), total RNA was isolated and analyzed by Northern blotting with a β -globin antisense probe. Ethidium bromide staining of the 18S rRNA is shown to allow comparison of the RNA amounts loaded. Results were quantified by a video analyzer system (amount of mRNA at the time of doxycycline addition [0 min] = 100%; circles, BBB-IL8₉₇₂₋₁₃₁₀; triangles, BBB-TNF₁₃₁₅₋₁₃₅₀; closed symbols, siGFP; open symbols, siKSRP). (C) Endogenous IL-8 mRNA was induced by incubating the cells for 2 h with IL-1 α (2 ng/ml). mRNA half-life was determined after stopping transcription with actinomycin D (5 μ g/ml). Northern blots were hybridized to an IL-8 antisense probe and results quantified as described for panel B.

ARE characterized in detail recently (44). To find out if the ARE-binding protein KSRP is involved in the function of the IL-8 ARE, HeLa cells were transfected with siRNAs specific for KSRP mRNA. Amounts of KSRP were strongly diminished compared to those in cells transfected with siRNA against GFP as a control (Fig. 1A). Degradation of β -globin reporter mRNAs carrying the IL-8 ARE as well as the TNF ARE was slowed down in the KSRP knockdown cells compared to that in the control cells (Fig. 1B). Endogenous IL-8 mRNA is barely detectable in unstimulated HeLa cells but strongly and rapidly induced by the proinflammatory cytokine IL-1. Two hours after addition of IL-1, the stability of endogenous IL-8 mRNA was strongly increased when KSRP expression was suppressed by siRNA (Fig. 1C).

Suppression of KSRP impairs deadenylation of IL-8 mRNA.

Evidence has been presented for recruitment of the deadenylating enzyme PARN by KSRP (18). To compare the poly(A) tail lengths of IL-8 mRNA in control and KSRP knockdown cells, the RNA samples were separated by PAGE. This revealed primarily short poly(A) tails in control cells after 2 h of stimulation with IL-1, as expected from previous work in which we showed that IL-1 induces rapid and transient IL-8 mRNA

expression with long poly(A) tails at the 1-h maximum, with rapid poly(A) tail shortening and decrease in RNA amounts ensuing thereafter (20). In the cells treated with siRNA against KSRP, IL-8 transcripts with intermediate and long poly(A) tails were prominent (Fig. 2A). The weighted arithmetic mean length of poly(A) tails increased from 58 nucleotides (nt) in control cells to 120 nt in KSRP knockdown cells. Furthermore, in the latter cells amounts and poly(A) tail lengths remained fairly constant for 1 hour after inhibiting transcription. Similarly, steady-state levels and poly(A) tail lengths of CCL20 mRNA were also increased in the KSRP knockdown cells (Fig. 2B) (mean length 136 nt versus 96 nt in the control cells). The mRNA of I κ B α is short lived but does not contain a typical ARE with overlapping AUUUA motifs in its 3' UTR. Amounts and poly(A) tail lengths are found to be hardly affected when cells treated with control or KSRP-specific siRNA are compared (Fig. 2B) (mean lengths of 97 and 110 nt, respectively), indicating that KSRP knockdown selectively affects only certain mRNAs. A stable mRNA for a housekeeping enzyme, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), also remains unaffected by suppressing KSRP levels (mean lengths of 107 and 104 nt, respectively, in control and

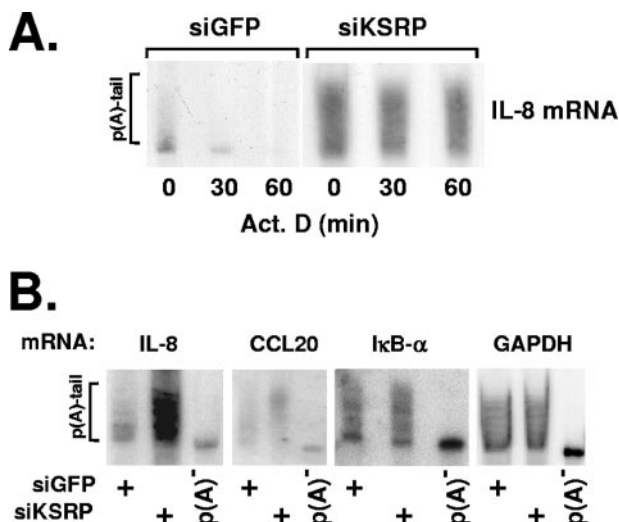


FIG. 2. Effect of KSRP knockdown on the poly(A) [p(A)] lengths of mRNAs. IL-1 α was added to HeLa cells transfected with siRNA specific for KSRP or for GFP (siKSRP or siGFP, respectively). (A) After 2 h, transcription was inhibited by addition of actinomycin D (Act. D; 5 μ g/ml). Total RNA was isolated at the indicated times thereafter, separated on a polyacrylamide gel, and analyzed by Northern blotting with an IL-8 antisense probe. (B) Polyacrylamide gel analysis of the indicated mRNAs from cells transfected with siRNAs and stimulated with IL-1 α as described for panel A. Aliquots of the RNAs from cells transfected with siRNA against KSRP were subjected to in vitro deadenylation [p(A)⁻].

KSRP knockdown). These results provide evidence for a role for KSRP in the rapid and specific deadenylation and degradation of endogenous ARE-containing mRNAs.

Both domains of the IL-8 ARE are involved in its interaction with KSRP. In UV cross-linking experiments, radiolabeled RNA corresponding to the IL-8 ARE forms protein-RNA complexes with cytoplasmic extract (Fig. 3A). Formation of the highest-molecular-weight complex is increased with cytoplasmic extract from cells expressing stKSRP. Furthermore, IL-8 ARE RNA can be UV cross-linked to purified stKSRP, resulting in a product of the expected molecular weight in SDS-PAGE. Complex formation of stKSRP and the IL-8 ARE is also detected in nondenaturing gels (Fig. 3A, right).

Interaction of KSRP with the IL-8 ARE was confirmed in pull-down assays with stKSRP. First, stKSRP plasmid was transfected in different amounts and stKSRP and total KSRP were detected by Western blot analysis (transfected and endogenous KSRP could not be separated in SDS-PAGE). In the range of plasmid amounts tested, strong increases in total KSRP were not observed (Fig. 3B). Amounts that corresponded to 7 μ g in the setting of Fig. 3B were chosen for further experiments. In Northern analysis of mRNAs associated with stKSRP after pull-down from cytoplasmic extract with Strep-tactin agarose beads, β -globin mRNA without an ARE was not detected (Fig. 3C). β -Globin mRNA with the ARE-containing region of IL-8 mRNA was selectively detected in pull-down from cells expressing stKSRP but not in pull-down from control cells. Importantly, endogenous IL-8 mRNA was also found to associate with stKSRP. GAPDH mRNA, which exhibits very high signal intensity in total RNA, was detected in

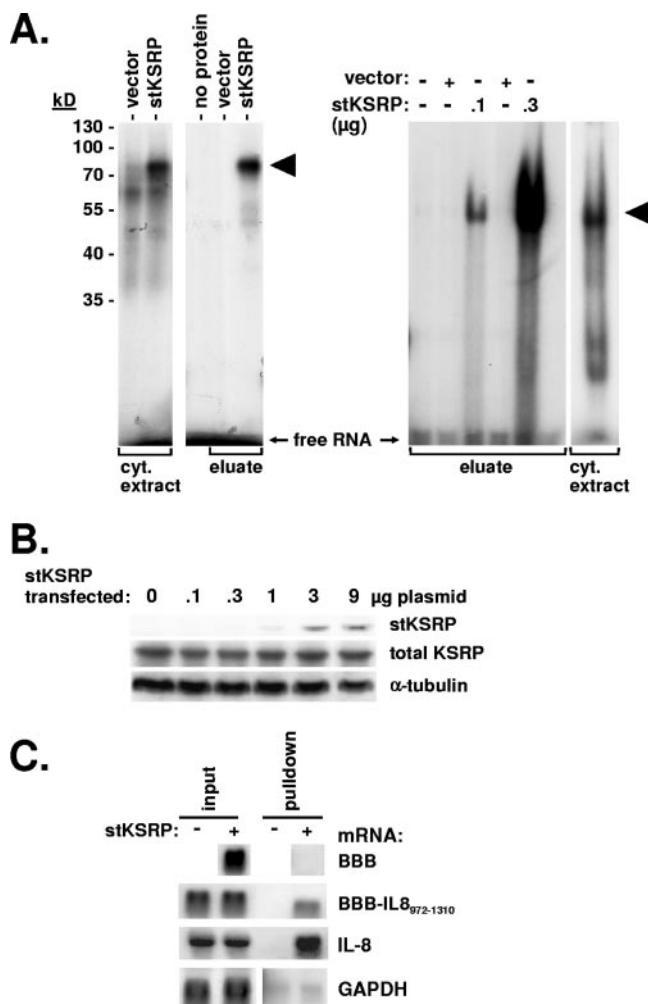


FIG. 3. Interaction of the IL-8 ARE with KSRP. (A) Radiolabeled in vitro-transcribed RNA corresponding to the IL-8 ARE was incubated alone (no protein), with cytoplasmic (cyt.) extracts from cells transfected with empty vector or with an stKSRP expression plasmid, or with eluate from Strep-tactin-agarose beads after incubation with the cytoplasmic extracts. Complexes were either separated by SDS-PAGE after UV cross-linking in vitro (left) or separated by nondenaturing gel electrophoresis (right). KSRP-RNA complexes are marked by arrowheads. (B) stKSRP expression plasmid was transfected in increasing amounts. Western blot detection of stKSRP was done with horseradish peroxidase-labeled Strep-tactin, and total KSRP and α -tubulin were detected with specific antibodies. (C) β -Globin reporter mRNAs without insertion (BBB) or with an IL-8 RNA fragment containing the ARE (BBB-IL-8₉₇₂₋₁₃₁₀) were coexpressed with stKSRP where indicated and the cells stimulated with IL-1 α . Total RNA was prepared from cytoplasmic extract (input) and from eluate of Strep-tactin beads (pull-down). β -Globin and endogenous IL-8 and GAPDH mRNAs were detected by Northern blot analysis.

spurious and not significantly differing amounts in both the specific and the control pull-down preparations.

We further studied the ARE-KSRP interaction by investigating the importance of the four AUUUA motifs and of the two functionally distinct domains in the ARE that we had previously defined (44). β -Globin reporter RNAs containing the ARE or derivatives thereof in the 3' UTR were expressed (Fig. 4A). Compared to the wild-type ARE with an intact

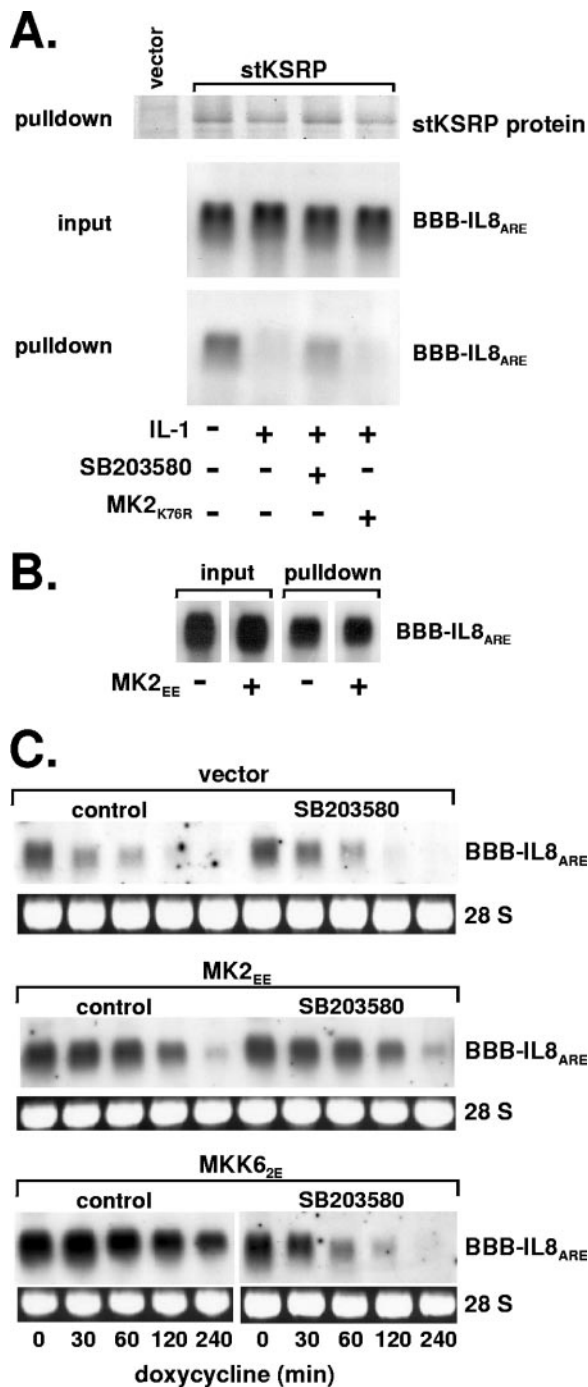


FIG. 5. Effect of the p38 MAP kinase cascade on the interaction of KSRP with the IL-8 ARE. (A) β -globin reporter mRNA containing the IL-8 ARE was coexpressed with stKSRP and a negative interfering mutant of MK2 (MK2_{K76R}) where indicated. The cells were incubated without or with IL-1 α (2 ng/ml) for 30 min in the absence or presence of SB203580 (2 μ M) added 15 min earlier. After pull-down of stKSRP, the protein was detected by SDS-PAGE and Coomassie brilliant blue staining, and associated β -globin-IL-8 ARE mRNA was detected by Northern blot analysis. (B) Pull-down of stKSRP and mRNA detection were performed as described for panel A for cells cotransfected with an expression vector for MK2_{EE} as indicated. (C) Degradation kinetics of β -globin-IL-8 ARE mRNA in cells transfected with empty vector or plasmids for expression of constitutively active mutants of MK2 (MK2_{EE}) or MKK6 (MKK6_{2E}) in the absence or presence of SB203580 (2 μ M).

involving p38 MAP kinase and its substrate MK2 (46). In cells expressing stKSRP and β -globin mRNA containing the IL-8 ARE, significantly less of the mRNA was associated with stKSRP after treatment of the cells with IL-1 than in untreated cells (Fig. 5A). Inhibition of p38 MAP kinase using the specific inhibitor SB203580 largely reversed this effect of IL-1. Selective activation of the p38/MK2 pathway by expressing a constitutively active form of the p38-selective MAP kinase kinase MKK6 also impaired KSRP-RNA interaction (not shown). These results are in accordance with a decreased interaction of phosphorylated KSRP with ARE-containing RNA demonstrated in vitro (3). On the other hand, a kinase-inactive mutant of MK2 which interferes with IL-1-induced mRNA stabilization (46) could not reverse impairment of KSRP-mRNA interaction. Furthermore, expression of a constitutively active mutant of MK2 (MK2_{EE}), which induced stabilization (46) (Fig. 5C), did not impair interaction (Fig. 5B). This and the observation by Briata et al. that MK2 does not phosphorylate KSRP indicates that the contribution of MK2 to stabilization of ARE-containing mRNAs is not reflected on this level.

We also tested whether MK2 has an indirect role in mRNA stabilization, e.g., facilitating export of active p38 MAP kinase from the nucleus to the cytoplasm (2, 14), where p38 MAP kinase might phosphorylate its target KSRP. This is unlikely, however: as shown in Fig. 5C, constitutively active MK2 induces mRNA stabilization (compare "control" in upper panel [vector] with "control" in middle panel [MK2_{EE}], which is independent of p38 MAP kinase activity (middle, compare "control" and SB203580). Stabilization induced by the upstream kinase MKK6 was inhibited by SB203580 as expected.

The IL-8 ARE is a target of TTP. MK2 can phosphorylate the ARE-binding protein TTP, which can impair its destabilizing activity toward its target mRNAs (16). To test whether TTP can contribute to regulation of IL-8 mRNA stability, stTTP was expressed in HeLa cells and pull-down performed after induction of IL-8 mRNA by IL-1. IL-8 mRNA was enriched in the stTTP pull-down fraction (Fig. 6A). Furthermore, purified TTP interacted with the IL-8 ARE in vitro (not shown). Since detection of a destabilizing effect of TTP might be obscured by the predominant destabilization of KSRP, the effect of TTP on the degradation of the IL-8 ARE-containing reporter RNA in KSRP knockdown cells was determined. Coexpression of stTTP resulted in a marked decrease in the steady-state level and the stability of the reporter RNA (Fig. 6B). Coexpression of constitutively active MK2 (MK2_{EE}) partly reverted this effect. These results indicate that TTP can participate in the control of IL-8 mRNA stability and may represent the MK2-sensitive component of rapid IL-8 mRNA degradation.

Microarray-based identification of mRNAs regulated by KSRP. To obtain information on the population of mRNAs potentially regulated by KSRP, HeLa cells were stimulated with IL-1. A stimulation period of 2 hours was chosen because at this time IL-1-induced mRNA stabilization has ceased (20, 46) but expression of transiently induced mRNAs is still high enough for analysis. Two parameters were determined for RNAs from these cells, (i) enrichment in pull-down with KSRP and (ii) increase in amount upon knockdown of KSRP (Fig. 7A). RNA was analyzed on an oligonucleotide array covering 30,982 human genes. After pull-down of stKSRP, 1,734 mRNAs

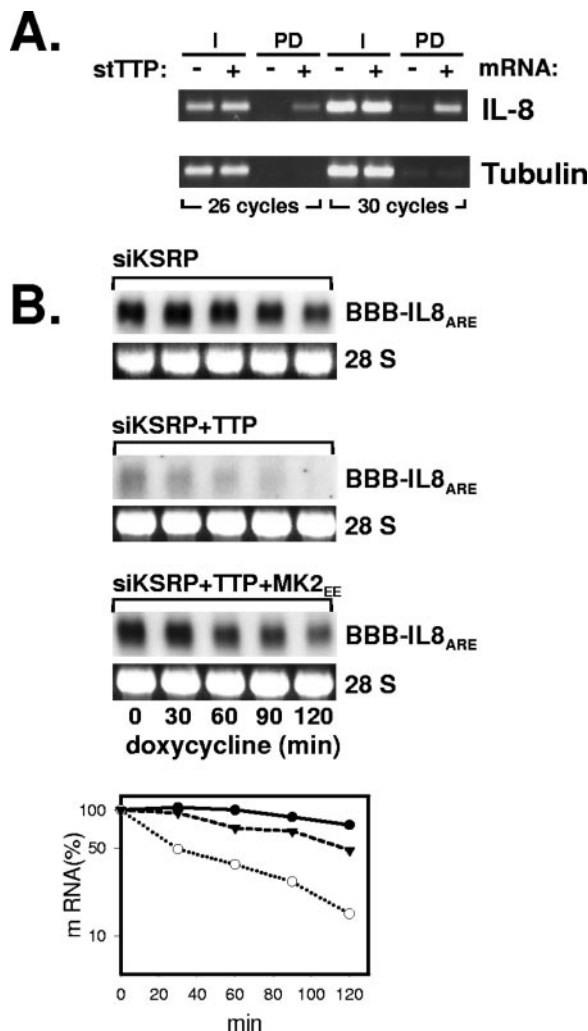


FIG. 6. Interaction of TTP with the IL-8 ARE. (A) HeLa cells were transfected with a plasmid carrying stTTP or empty vector. Total RNA from cytoplasmic extract (I) or Strep-tactin pull-down (PD) was analyzed by RT-PCR, with primers specific for IL-8 mRNA or β -tubulin mRNA as a control. (B) HeLa cells were transfected with siRNA against KSRP, a plasmid carrying the β -globin-IL-8 ARE reporter RNA, and expression vectors for stTTP and MK2_{EE} as indicated. The degradation kinetics of the reporter RNA was determined by Northern blot analysis and quantified as described for Fig. 1B. Closed circles, siKSRP; open circles, siKSRP plus TTP; triangles, siKSRP plus TTP plus MK2_{EE}.

(i.e., 11% of all functionally annotated genes that were detected by the Agilent array) were enriched more than twofold in the stKSRP fraction (see Table S1 in the supplemental material). Determination of amounts in the cytoplasmic extract (input) excluded the possibility that this was due to different starting levels (not shown). Enrichment in the stKSRP fraction was verified by RT-PCR for several mRNAs (Fig. 7B).

mRNA from two independent KSRP knockdown experiments was analyzed to estimate the impact of KSRP on gene expression. The cells were stimulated with IL-1 for 2 hours, and RNA was isolated immediately or after additional incubation for 3 hours with actinomycin D to obtain information on changes in mRNA degradation. RNAs of 427 annotated genes

showed a mean increase of twofold or higher in the KSRP knockdown cells compared to the level for GFP knockdown cells (see Table S2 in the supplemental material). For 1,674 of the 1,734 transcripts enriched in pull-down, complete data sets from both knockdown experiments were available. One hundred mRNAs were enriched in the stKSRP pull-down, and their levels also increased upon KSRP knockdown (Table 1). Forty-eight of them had short half-lives. Of these, 10 mRNAs, including that of IL-8, decayed more slowly in the KSRP knockdown cells (Table 1). This indicates that these mRNAs represent target transcripts for the destabilizing function of KSRP. Of note, 80% of them and 36% of all mRNAs in Table 1 were found in the ARED database (1), which lists mRNAs with AU-rich sequences in their 3' UTRs as defined by a bioinformatic approach.

Possible autoregulation of KSRP. Interestingly, among the transcripts associated with stKSRP was that of its homolog FUBP3 and also the KSRP mRNA itself (see Table S1 in the supplemental material). To exclude the possibility that the latter result merely reflects a higher input level (due to the additional mRNAs derived from the stKSRP vector), RT-PCR was performed with primers that amplify 3' UTRs not contained in the vector (Fig. 7B). The result clearly demonstrates an association of stKSRP with endogenous KSRP mRNA. The plasmid-derived KSRP mRNA contains part of the 3' UTR. Since that mRNA was also enriched, while a KSRP mRNA without 3' UTR sequences was not (not shown), a binding site for KSRP protein was further localized by expressing the β -globin mRNA with 3' UTR sequences from KSRP mRNA. Association with stKSRP in pull-down assays was found to be mediated by a non-ARE region of 104 nt 3' of the stop codon in the KSRP mRNA (Fig. 7C).

DISCUSSION

Rapid degradation of IL-8 mRNA and its inhibition by the p38/MK2 pathway are largely dependent on an ARE with a bipartite structure (44, 46). In this report, we define KSRP as a major factor involved in the function of this ARE, based on three lines of evidence obtained by (i) siRNA-mediated knockdown, (ii) *in vitro* protein-ARE interaction, and (iii) copull-down of RNA and protein. (i) siRNA-mediated knockdown of KSRP slowed down degradation of endogenous IL-8 mRNA and of a reporter mRNA containing the IL-8 ARE (Fig. 1). Consistent with prior reports that KSRP can recruit the deadenylating enzyme PARN to the mRNA (3, 9, 18), the IL-8 mRNA has long poly(A) tails in the KSRP knockdown cells. Evidence that, in addition to PARN, exosome components and, more recently, the decapping enzyme Dcp2 are recruited by KSRP has been presented (9, 18). Decapping and 5'-to-3' degradation are involved in ARE-mediated decay according to other studies (33, 41). Whether KSRP is essential for deadenylation of all mRNAs that it destabilizes or only for some of them, as shown for IL-8 and CCL20 in this study, is not known. Perhaps recruiting the exosome or enzymes of 5'-to-3' degradation is the major function for degradation of other mRNA targets. Of interest, it has been reported that KSRP interacts with the ARE-binding protein TTP (32), which may contribute to determining the subsequent events in degradation. (ii) KSRP directly interacts with the IL-8 ARE *in vitro* (Fig. 3A).

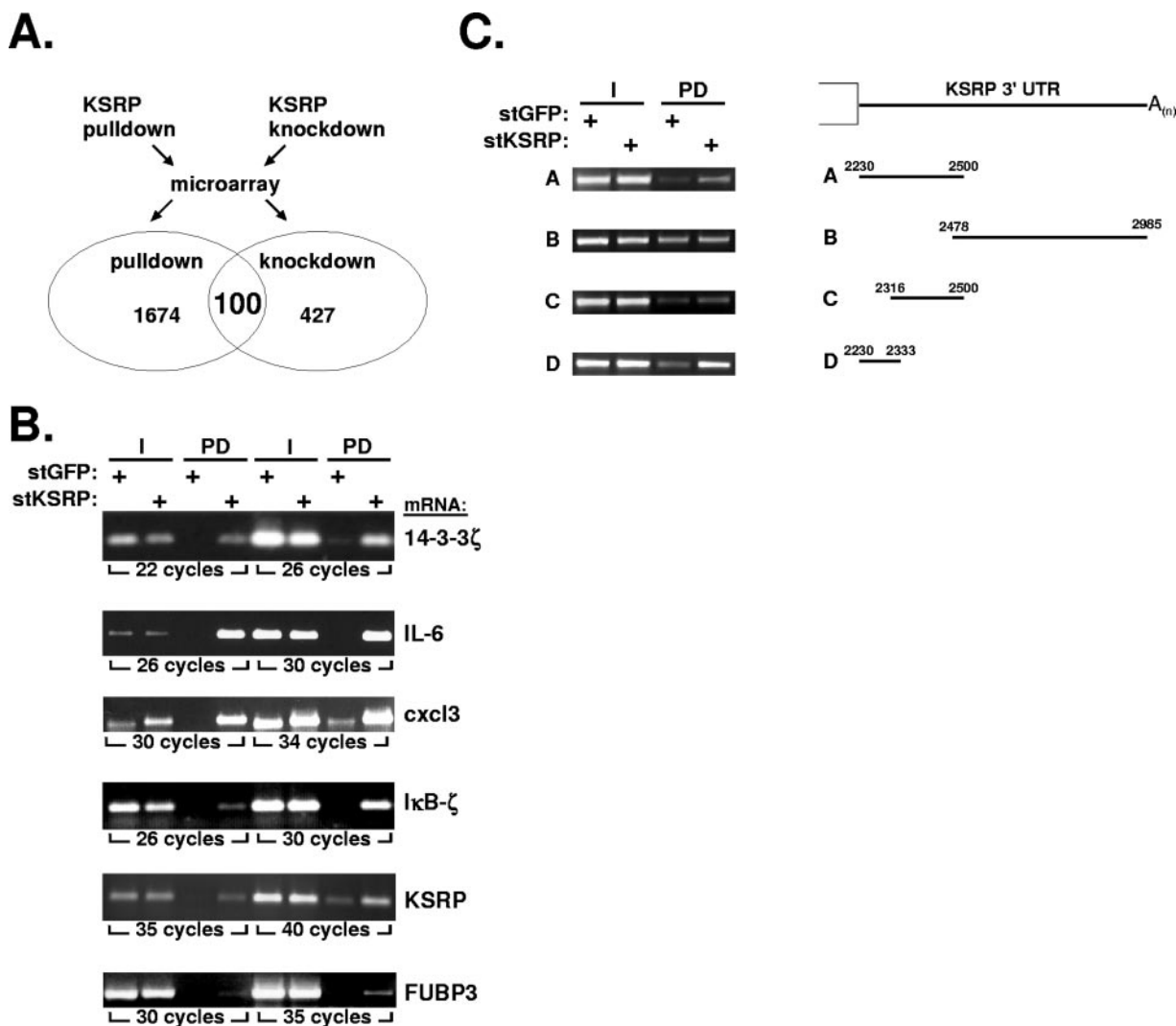


FIG. 7. Interaction of KSRP with endogenous mRNAs. (A) HeLa cells were transfected with stKSRP or stGFP as a control for pull-down assays and with siRNA against KSRP or against GFP as a control for knockdown assays. After stimulation for 2 h with IL-1 α , total RNA was isolated and subjected to microarray analysis as described in Materials and Methods. mRNAs associated with stKSRP in pull-down and increased in KSRP knockdown cells were identified. Details on mRNAs positive for both parameters (with ratios of >2 for signal intensities of stKSRP/stGFP and siRNA against KSRP/GFP) are presented in Table 1. (B) Enrichment of the indicated mRNAs in the stKSRP pull-down was confirmed by RT-PCR. (C) Input (I) and stKSRP pull-down (PD) samples from cells expressing β -globin mRNA with the indicated insertions of the KSRP 3' UTR were analyzed for the respective mRNA by RT-PCR with β -globin mRNA-specific primers.

One of the complexes formed with cytoplasmic extract from HeLa cells could be ascribed to KSRP interaction. We have reported a bipartite structure for the IL-8 ARE with a core domain containing the four AUUUA motifs that mediates moderate destabilization and an auxiliary domain which does not destabilize on its own but enhances the destabilizing effect of the core domain (44). The data in Fig. 4 indicate that this behavior reflects the weak interaction of KSRP with the individual domains compared to that with the complete ARE. (iii) Endogenous IL-8 mRNA as well as a reporter mRNA containing the IL-8 ARE is found associated with KSRP pulled down from cytoplasmic extracts. The extent of interaction assayed in this way for the complete ARE and its domains parallels that observed in gel shift assays and, most importantly, their destabilizing activity (Fig. 4). Clearly, the interaction of the IL-8

ARE with KSRP as assayed by gel shift as well as by copull-down from cell lysates shows that both the four AUUUA motifs and the auxiliary domain are as essential for maximal interaction with KSRP as they are for maximal mRNA destabilization in intact cells (44) (Fig. 4). This suggests that the degree of destabilization is directly dependent on the interaction with KSRP.

Rapid ARE-dependent degradation of IL-8 mRNA is impaired upon activation of the p38 MAP kinase/MK2 signaling pathway (46). Recently, p38 MAP kinase has been shown to phosphorylate KSRP at threonine 692, which inhibits its binding and destabilizing function for AREs of myogenin and p21 in a muscle cell differentiation model (3). In agreement with this report, IL-1 α impairs association of KSRP with mRNA containing the IL-8 ARE in pull-down assays (Fig. 5A). This

TABLE 1. mRNAs enriched in pulldown and increased in knockdown of KSRP^a

RefSeq accession no.	Description	Gene name	mRNA status		ARE class ^d
			Unstable ^b	Stabilized in KSRP knockdown ^c	
NM_001200	Bone morphogenetic protein 2 (BMP2), mRNA	<i>BMP2</i>	+	+	
NM_000758	Colony stimulating factor 2 (granulocyte-macrophage) (CSF2), mRNA	<i>CSF2</i>	+	+	II
NM_002089	Chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA	<i>CXCL2</i>	+	+	II
NM_002090	Chemokine (C-X-C motif) ligand 3 (CXCL3), mRNA	<i>CXCL3</i>	+	+	II
NM_018948	ERBB receptor feedback inhibitor 1 (ERRFI1), mRNA	<i>ERRFI1</i>	+	+	
NM_004864	Growth differentiation factor 15 (GDF15), mRNA	<i>GDF15</i>	+	+	II
NM_002166	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2), mRNA	<i>ID2</i>	+	+	I
NM_000600	Interleukin 6 (beta 2 interferon, beta 2) (IL6), mRNA	<i>IL6</i>	+	+	I
NM_000584	Interleukin 8 (IL8), mRNA	<i>IL8</i>	+	+	II
NM_000963	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA	<i>PTGS2</i>	+	+	II
NM_000024	Adrenergic beta-2-, receptor, surface (ADRB2), mRNA	<i>ADRB2</i>	+		
NM_016201	Angiotensin-like 2 (AMOTL2), mRNA	<i>AMOTL2</i>	+		
NM_025047	ADP-ribosylation factor-like 14 (ARL14), mRNA	<i>ARL14</i>	+		I
NM_006577	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2 (B3GNT2), mRNA	<i>B3GNT2</i>	+		I
NM_170735	Brain-derived neurotrophic factor (BDNF), transcript variant 1, mRNA	<i>BDNF</i>	+		I
NM_001901	Connective tissue growth factor (CTGF), mRNA	<i>CTGF</i>	+		
NM_001554	Cysteine-rich angiogenic inducer 61 (CYR61), mRNA	<i>CYR61</i>	+		
NM_012242	Dickkopf homolog 1 (<i>Xenopus laevis</i>) (DKK1), mRNA	<i>DKK1</i>	+		
NM_012266	DnaJ (Hsp40) homolog, subfamily B, member 5 (DNAJB5), mRNA	<i>DNAJB5</i>	+		
NM_004419	Dual specificity phosphatase 5 (DUSP5), mRNA	<i>DUSP5</i>	+		
NM_001946	Dual specificity phosphatase 6 (DUSP6), transcript variant 1, mRNA	<i>DUSP6</i>	+		
NM_004951	Epstein-Barr virus-induced gene 2 (lymphocyte-specific G protein-coupled receptor) (EBI2), mRNA	<i>EBI2</i>	+		
NM_001955	Endothelin 1 (EDN1), mRNA	<i>EDN1</i>	+		
NM_001956	Endothelin 2 (EDN2), mRNA	<i>EDN2</i>	+		I
NM_023037	Furry homolog (<i>Drosophila</i>) (FRY), mRNA	<i>FRY</i>	+		
NM_005261	GTP binding protein overexpressed in skeletal muscle (GEM), transcript variant 1, mRNA	<i>GEM</i>	+		
NM_005279	G protein-coupled receptor 1 (GPR1), mRNA	<i>GPR1</i>	+		
NM_005328	Hyaluronan synthase 2 (HAS2), mRNA	<i>HAS2</i>	+		
NM_001945	Heparin-binding EGF-like growth factor (HBEGF), mRNA	<i>HBEGF</i>	+		II
NM_182757	IBR domain-containing 2 (IBRDC2), mRNA	<i>IBRDC2</i>	+		
NM_001547	Interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA	<i>IFIT2</i>	+		
NM_005544	Insulin receptor substrate 1 (IRS1), mRNA	<i>IRS1</i>	+		
NM_000214	Jagged 1 (Alagille syndrome) (JAG1), mRNA	<i>JAG1</i>	+		II
NM_031419	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta (NFKBIZ), transcript variant 1, mRNA	<i>NFKBIZ</i>	+		
NM_006186	Nuclear receptor subfamily 4, group A, member 2 (NR4A2), transcript variant 1, mRNA	<i>NR4A2</i>	+		I
NM_021127	Phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA	<i>PMAIP1</i>	+		I
NM_005398	Protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C), mRNA	<i>PPP1R3C</i>	+		
NM_000958	Prostaglandin E receptor 4 (subtype EP4) (PTGER4), mRNA	<i>PTGER4</i>	+		I
NM_198965	Parathyroid hormone-like hormone (PTH1LH), transcript variant 1, mRNA	<i>PTH1LH</i>	+		I
NM_144593	Ras homolog enriched in brain-like 1 (RHEBL1), mRNA	<i>RHEBL1</i>	+		
NM_014575	Schwannomin interacting protein 1 (SCHIP1), mRNA	<i>SCHIP1</i>	+		
NM_000450	Selectin E (endothelial adhesion molecule 1) (SELE), mRNA	<i>SELE</i>	+		II
NM_005842	Sprouty homolog 2 (<i>Drosophila</i>) (SPRY2), mRNA	<i>SPRY2</i>	+		I
NM_003222	Transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma) (TFAP2C), mRNA	<i>TFAP2C</i>	+		
NM_000594	Tumor necrosis factor (TNF, superfamily, member 2) (TNF), mRNA	<i>TNF</i>	+		II
NM_001003818	Tripartite motif-containing 6 (TRIM6), transcript variant 1, mRNA	<i>TRIM6</i>	+		

Continued on facing page

TABLE 1—Continued

RefSeq accession no.	Description	Gene name	mRNA status		ARE class ^d
			Unstable ^b	Stabilized in KSRP knockdown ^c	
NM_033035	Thymic stromal lymphopoietin (TSLP), transcript variant 1, mRNA	<i>TSLP</i>	+		I
NM_024640	<i>yrdC</i> domain-containing (<i>E. coli</i>) (YRDC), mRNA	<i>YRDC</i>	+		
NM_023039	Ankyrin repeat, family A (RFXANK-like), 2 (ANKRA2), mRNA	<i>ANKRA2</i>			
NM_001233	Caveolin 2 (CAV2), transcript variant 1, mRNA	<i>CAV2</i>			I
NM_025214	Coiled-coil domain-containing 68 (CCDC68), mRNA	<i>CCDC68</i>			I
NM_004591	Chemokine (C-C motif) ligand 20 (CCL20), mRNA	<i>CCL20</i>			
NM_012129	Claudin 12 (CLDN12), mRNA	<i>CLDN12</i>			
NM_030627	Cytoplasmic polyadenylation element binding protein 4 (CPEB4), mRNA	<i>CPEB4</i>			II
NM_001912	Cathepsin L (CTSL), transcript variant 1, mRNA	<i>CTSL</i>			
NM_001565	Chemokine (C-X-C motif) ligand 10 (CXCL10), mRNA	<i>CXCL10</i>			
NM_005409	Chemokine (C-X-C motif) ligand 11 (CXCL11), mRNA	<i>CXCL11</i>			
NM_000782	Cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1), nuclear gene encoding mitochondrial protein, mRNA	<i>CYP24A1</i>			
NM_019885	Cytochrome P450, family 26, subfamily B, polypeptide 1 (CYP26B1), mRNA	<i>CYP26B1</i>			
AK026768	cDNA: FLJ23115 fis, clone LNG07933	<i>DPY19L1P1</i>			
NM_022726	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4 (ELOVL4), mRNA	<i>ELOVL4</i>			I
NM_001432	Epiregulin (EREG), mRNA	<i>EREG</i>			I
NM_144503	F11 receptor (F11R), transcript variant 4, mRNA	<i>F11R</i>			
NM_001993	Coagulation factor III (thromboplastin, tissue factor) (F3), mRNA	<i>F3</i>			II
NM_020066	Formin 2 (FMN2), mRNA	<i>FMN2</i>			
NM_013409	Follistatin (FST), transcript variant FST344, mRNA	<i>FST</i>			
NM_013372	Gremlin 1, cysteine knot superfamily, homolog (<i>Xenopus laevis</i>) (GREM1), mRNA	<i>GREM1</i>			
NM_001548	Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), transcript variant 2, mRNA	<i>IFIT1</i>			
NM_016584	Interleukin 23, alpha subunit p19 (IL23A), mRNA	<i>IL23A</i>			
NM_002192	Inhibin, beta A (activin A, activin AB alpha polypeptide) (INHBA), mRNA	<i>INHBA</i>			I
NM_000210	Integrin, alpha 6 (ITGA6), mRNA	<i>ITGA6</i>			
NM_005574	LIM domain only 2 (rhombotin-like 1) (LMO2), mRNA	<i>LMO2</i>			
NM_033049	Mucin 13, cell surface-associated (MUC13), mRNA	<i>MUC13</i>			
NM_021229	Netrin 4 (NTN4), mRNA	<i>NTN4</i>			
NM_002581	Pregnancy-associated plasma protein A, pappalysin 1 (PAPP), mRNA	<i>PAPP</i>			
NM_002607	Platelet-derived growth factor alpha polypeptide (PDGFA), transcript variant 1, mRNA	<i>PDGFA</i>			
NM_016205	Platelet-derived growth factor C (PDGFC), mRNA	<i>PDGFC</i>			
NM_006823	Protein kinase (cyclic AMP-dependent, catalytic) inhibitor alpha (PKIA), transcript variant 6, mRNA	<i>PKIA</i>			
NM_001005377	Plasminogen activator, urokinase receptor (PLAUR), transcript variant 3, mRNA	<i>PLAUR</i>			I
NM_000959	Prostaglandin F receptor (FP) (PTGFR), transcript variant 1, mRNA	<i>PTGFR</i>			
NM_003020	Secretogranin V (7B2 protein) (SCG5), mRNA	<i>SCG5</i>			
NM_002998	Syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan) (SDC2), mRNA	<i>SDC2</i>			
NM_002999	Syndecan 4 (amphiglycan, ryudocan) (SDC4), mRNA	<i>SDC4</i>			
NM_006216	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2 (SERPINE2), mRNA	<i>SERPINE2</i>			
NM_005025	Serpin peptidase inhibitor, clade I (neuroserpin), member 1 (SERPINI1), mRNA	<i>SERPINI1</i>			
NM_203349	SHC (Src homology 2 domain-containing) family, member 4 (SHC4), mRNA	<i>SHC4</i>			
NM_003759	Solute carrier family 4, sodium bicarbonate cotransporter, member 4 (SLC4A4), mRNA	<i>SLC4A4</i>			
NM_032229	SLIT and NTRK-like family, member 6 (SLITRK6), mRNA	<i>SLITRK6</i>			I
NM_006714	Sphingomyelin phosphodiesterase, acid-like 3A (SMPDL3A), mRNA	<i>SMPDL3A</i>			

Continued on following page

TABLE 1—Continued

RefSeq accession no.	Description	Gene name	mRNA status		ARE class ^d
			Unstable ^b	Stabilized in KSRP knockdown ^c	
NM_003122	Serine peptidase inhibitor, Kazal type 1 (SPINK1), mRNA	<i>SPINK1</i>			
NM_030964	Sprouty homolog 4 (<i>Drosophila</i>) (SPRY4), mRNA	<i>SPRY4</i>			I
NM_015000	Serine/threonine kinase 38-like (STK38L), mRNA	<i>STK38L</i>			
NM_018423	Serine/threonine/tyrosine kinase 1 (STYK1), mRNA	<i>STYK1</i>			I
NM_003236	Transforming growth factor alpha (TGFA), mRNA	<i>TGFA</i>			I
NM_003243	Transforming growth factor beta receptor III (betaglycan, 300 kDa) (TGFB3), mRNA	<i>TGFB3</i>			
NM_024847	Transmembrane channel-like 7 (TMC7), mRNA	<i>TMC7</i>			
NM_003304	Transient receptor potential cation channel, subfamily C, member 1 (TRPC1), mRNA	<i>TRPC1</i>			I
NM_016061	Yippee-like 5 (<i>Drosophila</i>) (YPEL5), mRNA	<i>YPEL5</i>			
NM_019006	Zinc finger, AN1-type domain 6 (ZFAND6), mRNA	<i>ZFAND6</i>			
NM_003407	Zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA	<i>ZFP36</i>			II

^a Listed are mRNAs with >2-fold signal intensities in stKSRP pulldown compared to those in stGFP pulldown and >2-fold signal intensities in KSRP knockdown cells compared to those in GFP knockdown cells in two independent assays (for details, see Materials and Methods). Boldface indicates mRNAs destabilized by KSRP.

^b mRNAs labeled “unstable” (+) decreased to 50% or less at 3 h after actinomycin D addition in GFP knockdown cells in both assays.

^c For mRNAs labeled “stabilized” (+), the ratio of signal intensities for 3 h of actinomycin D treatment to those at 0 h is >1.33-fold higher for KSRP knockdown than for GFP knockdown cells.

^d ARE classification was done using the ARED3.0 database search tool (<http://rc.kfshrc.edu.sa/ared>). ARE-containing as well as unstable mRNAs are highly significantly overrepresented ($P < 0.0001$, Fisher's exact test).

effect of IL-1, like its mRNA stabilization, is sensitive to inhibition of p38 MAP kinase. Interestingly, an inhibitory mutant of MK2 which interferes with mRNA stabilization (46) did not affect IL-1-induced impairment of KSRP-ARE interaction (Fig. 5A), and an active mutant of MK2 which induces stabilization did not mimic the effect of IL-1 (Fig. 5B). KSRP is not phosphorylated by MK2 (3). However, it cannot be ruled out that MK2 modifies KSRP function indirectly. The stabilization of the reporter-IL-8 ARE mRNA by an active form of MK2 in the presence of a p38 inhibitor (Fig. 5C) argues against a mere supportive role for MK2 for the effect of p38 MAP kinase, e.g., by facilitating its export from the nucleus (2, 14). MK2 may also affect other proteins involved in degradation. A known substrate of MK2 is the ARE-binding protein TTP, and activation of the p38/MK2 pathway has been associated with increased stability of TTP target mRNAs (6, 10, 24, 34, 42). We therefore investigated a possible role for TTP in degradation of IL-8 mRNA and its modulation by MK2. TTP, expressed in small amounts as suggested to detect its destabilizing activity (31) and to limit artifacts (4), accelerated degradation of an IL-8 ARE containing reporter RNA under conditions where basal degradation was slow due to KSRP knockdown. Acceleration of degradation was partly reversed by coexpressing active MK2 (Fig. 6). This, together with the copulldown of TTP and endogenous IL-8 mRNA, suggests that MK2 contributes to stabilization of IL-8 mRNA in part by impairing TTP function. It has to be noted, however, that TTP is not expressed strongly in the cells used here, and other targets of MK2 may be of relevance. Results from this study, together with the information cited above, are summarized in the scheme presented in Fig. 8. According to this model, IL-8 expression is increased by stabilization of its mRNA in response to a signaling pathway in which two consecutively activated kinases, p38 MAP kinase and MK2, impair the functions of distinct mRNA destabilizing proteins, KSRP and TTP, respectively.

IL-8 is an important component of the inflammatory re-

sponse (25). We used HeLa cells stimulated with the inflammatory cytokine IL-1 as an in vitro correlate of an inflammatory reaction for identifying other targets of KSRP besides IL-8.

The microarray data on KSRP-associated mRNAs show enrichment of relevant target mRNAs, such as those of IL-8 itself, IL-6, Cox 2 (PTGS2), CCL20, or CXCL3, which corre-

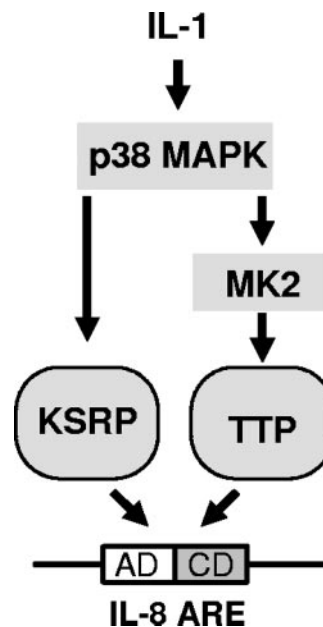


FIG. 8. Scheme of IL-8 ARE-dependent control of mRNA stability. The two destabilizing proteins KSRP and TTP can interact with the IL-8 ARE and promote degradation. Activators of the p38 MAP kinase pathway, like IL-1, can induce stabilization by impairing the function of KSRP via p38 MAP kinase and of TTP via MK2 (for details, see Discussion).

spondingly accumulate in siRNA-mediated knockdown of KSRP (Table 1). However, though ARE-containing mRNAs are enriched in the KSRP-associated population, most of these mRNAs are not listed in the ARED database. This is likely due in part to limitations of the selection criteria which discard transcripts with AREs loosely related to the search pattern or lacking the AUUUA motif. On the other hand, relevant non-ARE targets may be enriched in the pulldown as well. Several studies provide evidence for interaction of KSRP with non-ARE sequences and functions distinct from mRNA destabilization (11, 23, 28, 35). It should be noted that mRNAs were enriched by pulldown of KSRP from cytoplasmic extracts, most likely causing underrepresentation of targets for nucleus-restricted functions of KSRP affecting, e.g., splicing (35) or transcription (11).

Interestingly, among the transcripts associated with KSRP were those of KSRP itself and of its homolog FUBP3. Recently, evidence has been presented for overlapping sets of mRNAs regulated by each of the three FUBPs (11). The association of two of their transcripts with KSRP suggests in addition auto- and cross-regulation of their own expression. RNA-targeted autoregulation of proteins involved in RNA metabolism has been described, e.g., for poly(A)-binding protein (27), for the ARE-binding protein TTP (5, 43), or for the yeast mRNA export factor Yra1p (13). Whether and how KSRP affects its own mRNA and that of FUBP3 will have to await further analysis.

siRNA-induced knockdown of KSRP resulted in >2-fold increased amounts of more than 400 transcripts. These could represent either direct targets of KSRP, like IL-8 mRNA, or indirect targets, upregulated as a consequence of increased expression of a direct target, like a transcription factor or receptor ligand. Further filtering in a rigorous way, including enrichment in KSRP pulldown, rapid degradation in control cells, and increased stability in KSRP knockdown cells as parameters, yielded 10 transcripts which are bona fide direct targets of KSRP-mediated destabilization. Most of them encode cytokines or other proteins connected with inflammatory and immune reactions. Structurally, they mostly are characterized by the presence of AREs. Among them are the mRNAs of IL-6 and CSF2 (also named GM-CSF), which we expected to be identified since IL-6 mRNA and a reporter with the GM-CSF ARE have been found stabilized by IL-1 stimulation or p38 MAP kinase activation in our initial studies (46). Of note, a set of seven KSRP targets was identified most recently by a similar strategy (37) in a different setting, namely, PI3K-AKT activation of pituitary α T3-1 cells. This may explain the lack of overlap with the transcripts identified here by investigating IL-1-stimulated HeLa cells. The true number of targets in the latter has to be considered much higher due to intrinsic limitations of the procedures applied. For example, significant stabilization of highly unstable RNAs might have been missed with the 3-h actinomycin D treatment; IL-1-induced RNAs with half-lives around or longer than 1.5 h would not be enriched twofold within the 2-h stimulation period with IL-1. Therefore, it is expected that more relevant targets are included in Table 1.

These data provide evidence for a role for KSRP in controlling the expression of inflammatory genes by limiting the half-lives of the respective mRNAs. This function of KSRP and its

modulation by p38 MAP kinase may have important consequences for the quality, intensity, and duration of inflammatory reactions.

ACKNOWLEDGMENTS

We thank H. Bujard, A. Stern, P. T. Lomedico, and A.-B. Shyu for previously supplied materials and C.-Y. Chen for monoclonal antibodies against KSRP and the His-KSRP expression vector. We are grateful to Roberto Gherzi for helpful discussion, to Axel Weber for help in the processing of microarray data, and to Heike Schneider for excellent technical assistance.

This work was supported by grants SFB566/A10, SFB566/Z2, and Ho1116/3 from the Deutsche Forschungsgemeinschaft. B.T.K. holds a scholarship of the Hannover Biomedical Research School.

REFERENCES

- Bakheet, T., B. R. Williams, and K. S. Khabar. 2006. ARED 3.0: the large and diverse AU-rich transcriptome. *Nucleic Acids Res.* **34**:D111–D114.
- Ben-Levy, R., S. Hooper, R. Wilson, H. F. Paterson, and C. J. Marshall. 1998. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr. Biol.* **8**:1049–1057.
- Briata, P., S. V. Forcales, M. Ponassi, G. Corte, C. Y. Chen, M. Karin, P. L. Puri, and R. Gherzi. 2005. p38-dependent phosphorylation of the mRNA decay-promoting factor KSRP controls the stability of select myogenic transcripts. *Mol. Cell* **20**:891–903.
- Brook, M., C. R. Tchen, T. Santalucia, J. McIlrath, J. S. Arthur, J. Saklatvala, and A. R. Clark. 2006. Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. *Mol. Cell Biol.* **26**:2408–2418.
- Brooks, S. A., J. E. Connolly, and W. F. Rigby. 2004. The role of mRNA turnover in the regulation of tristetraprolin expression: evidence for an extracellular signal-regulated kinase-specific, AU-rich element-dependent, autoregulatory pathway. *J. Immunol.* **172**:7263–7271.
- Carballo, E., H. Cao, W. S. Lai, E. A. Kennington, D. Campbell, and P. J. Blackshear. 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.
- Chen, C. Y., R. Gherzi, S. E. Ong, E. L. Chan, R. Raijmakers, G. J. Pruijn, G. Stoecklin, C. Moroni, M. Mann, and M. Karin. 2001. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. *Cell* **107**:451–464.
- Chen, C. Y., and A. B. Shyu. 1995. AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci.* **20**:465–470.
- Chou, C. F., A. Mulky, S. Maitra, W. J. Lin, R. Gherzi, J. Kappes, and C. Y. Chen. 2006. Tethering KSRP, a decay-promoting AU-rich element-binding protein, to mRNAs elicits mRNA decay. *Mol. Cell Biol.* **26**:3695–3706.
- Chrestensen, C. A., M. J. Schroeder, J. Shabanowitz, D. F. Hunt, J. W. Pelo, M. T. Worthington, and T. W. Sturgill. 2004. MAPKAP kinase 2 phosphorylates tristetraprolin on *in vivo* sites including Ser178, a site required for 14-3-3 binding. *J. Biol. Chem.* **279**:10176–10184.
- Chung, H. J., J. Liu, M. Dunder, Z. Nie, S. Sanford, and D. Levens. 2006. FBPs are calibrated molecular tools to adjust gene expression. *Mol. Cell Biol.* **26**:6584–6597.
- Dean, J. L., G. Sully, A. R. Clark, and J. Saklatvala. 2004. The involvement of AU-rich element-binding proteins in p38 mitogen-activated protein kinase pathway-mediated mRNA stabilisation. *Cell. Signal.* **16**:1113–1121.
- Dong, S., C. Li, D. Zenklusen, R. H. Singer, A. Jacobson, and F. He. 2007. YRA1 autoregulation requires nuclear export and cytoplasmic Edc3p-mediated degradation of its pre-mRNA. *Mol. Cell* **25**:559–573.
- Engel, K., A. Kotlyarov, and M. Gaestel. 1998. Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. *EMBO J.* **17**:3363–3371.
- Fechir, M., K. Linker, A. Pautz, T. Hubrich, U. Forstermann, F. Rodriguez-Pascual, and H. Kleinert. 2005. Tristetraprolin regulates the expression of the human inducible nitric-oxide synthase gene. *Mol. Pharmacol.* **67**:2148–2161.
- Gaestel, M. 2006. MAPKAP kinases—MKs—two's company, three's a crowd. *Nat. Rev. Mol. Cell Biol.* **7**:120–130.
- Garneau, N. L., J. Wilusz, and C. J. Wilusz. 2007. The highways and byways of mRNA decay. *Nat. Rev. Mol. Cell Biol.* **8**:113–126.
- Gherzi, R., K. Y. Lee, P. Briata, D. Wegmuller, C. Moroni, M. Karin, and C. Y. Chen. 2004. A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Mol. Cell* **14**:571–583.
- Gherzi, R., M. Trabucchi, M. Ponassi, T. Ruggiero, G. Corte, C. Moroni, C. Y. Chen, K. S. Khabar, J. S. Andersen, and P. Briata. 2006. The RNA-binding protein KSRP promotes decay of beta-catenin mRNA and is inactivated by PI3K-AKT signaling. *PLoS Biol.* **5**:e5.
- Gowrishankar, G., R. Winzen, F. Bollig, B. Ghebremedhin, N. Redich, B.

- Ritter, K. Resch, M. Kracht, and H. Holtmann. 2005. Inhibition of mRNA deadenylation and degradation by ultraviolet light. *Biol. Chem.* **386**:1287–1293.
21. Gowrishankar, G., R. Winzen, O. Dittrich-Breiholz, N. Redich, M. Kracht, and H. Holtmann. 2006. Inhibition of mRNA deadenylation and degradation by different types of cell stress. *Biol. Chem.* **387**:323–327.
 22. Green, J., K. S. Khabar, B. C. Koo, B. R. Williams, and S. J. Polyak. 2006. Stability of CXCL-8 and related AU-rich mRNAs in the context of hepatitis C virus replication in vitro. *J. Infect. Dis.* **193**:802–811.
 23. Gu, W., F. Pan, H. Zhang, G. J. Bassell, and R. H. Singer. 2002. A predominantly nuclear protein affecting cytoplasmic localization of beta-actin mRNA in fibroblasts and neurons. *J. Cell Biol.* **156**:41–51.
 24. Hitti, E., T. Iakovleva, M. Brook, S. Deppenmeier, A. D. Gruber, D. Radzioch, A. R. Clark, P. J. Blackshear, A. Kotlyarov, and M. Gaestel. 2006. Mitogen-activated protein kinase-activated protein kinase 2 regulates tumor necrosis factor mRNA stability and translation mainly by altering tristetraprolin expression, stability, and binding to adenine/uridine-rich element. *Mol. Cell. Biol.* **26**:2399–2407.
 25. Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht. 2002. Multiple control of interleukin-8 gene expression. *J. Leukoc. Biol.* **72**:847–855.
 26. Holtmann, H., R. Winzen, P. Holland, S. Eickemeier, E. Hoffmann, D. Wallach, N. L. Malinin, J. A. Cooper, K. Resch, and M. Kracht. 1999. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol. Cell. Biol.* **19**:6742–6753.
 27. Hornstein, E., H. Harel, G. Levy, and O. Meyuhas. 1999. Overexpression of poly(A)-binding protein down-regulates the translation or the abundance of its own mRNA. *FEBS Lett.* **457**:209–213.
 28. Huttelmaier, S., D. Zenklusen, M. Lederer, J. Dichtenberg, M. Lorenz, X. Meng, G. J. Bassell, J. Condeelis, and R. H. Singer. 2005. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature* **438**:512–515.
 29. Junttila, M. R., S. Saarinen, T. Schmidt, J. Kast, and J. Westermarck. 2005. Single-step Strep-tag purification for the isolation and identification of protein complexes from mammalian cells. *Proteomics* **5**:1199–1203.
 30. Kolev, N. G., and P. W. Huber. 2003. VgRBP71 stimulates cleavage at a polyadenylation signal in Vg1 mRNA, resulting in the removal of a cis-acting element that represses translation. *Mol. Cell* **11**:745–755.
 31. Lai, W. S., E. Carballo, J. M. Thorn, E. A. Kennington, and P. J. Blackshear. 2000. Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to AU-rich elements and destabilization of mRNA. *J. Biol. Chem.* **275**:17827–17837.
 32. Linker, K., A. Pautz, M. Fehlich, T. Hubrich, J. Greeve, and H. Kleinert. 2005. Involvement of KSRP in the post-transcriptional regulation of human iNOS expression-complex interplay of KSRP with TTP and HuR. *Nucleic Acids Res.* **33**:4813–4827.
 33. Lykke-Andersen, J., and E. Wagner. 2005. Recruitment and activation of mRNA decay enzymes by two ARE-mediated decay activation domains in the proteins TTP and BRF-1. *Genes Dev.* **19**:351–361.
 34. Mahtani, K. R., M. Brook, J. L. Dean, G. Sully, J. Saklatvala, and A. R. Clark. 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.
 35. Min, H., C. W. Turck, J. M. Nikolic, and D. L. Black. 1997. A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. *Genes Dev.* **11**:1023–1036.
 36. Rehbein, M., K. Wege, F. Buck, M. Schweizer, D. Richter, and S. Kindler. 2002. Molecular characterization of MARTA1, a protein interacting with the dendritic targeting element of MAP2 mRNAs. *J. Neurochem.* **82**:1039–1046.
 37. Ruggiero, T., M. Trabucchi, M. Ponassi, G. Corte, C. Y. Chen, L. al-Hajj, K. S. Khabar, P. Briata, and R. Gherzi. 2007. Identification of a set of KSRP target transcripts upregulated by PI3K-AKT signaling. *BMC Mol. Biol.* **8**:28.
 38. Rydziel, S., A. M. Delany, and E. Canalis. 2004. AU-rich elements in the collagenase 3 mRNA mediate stabilization of the transcript by cortisol in osteoblasts. *J. Biol. Chem.* **279**:5397–5404.
 39. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* **46**:659–667.
 40. Snee, M., G. J. Kidd, T. P. Munro, and R. Smith. 2002. RNA trafficking and stabilization elements associate with multiple brain proteins. *J. Cell Sci.* **115**:4661–4669.
 41. Stoecklin, G., T. Mayo, and P. Anderson. 2006. ARE-mRNA degradation requires the 5'-3' decay pathway. *EMBO Rep.* **7**:72–77.
 42. Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W. F. Rigby, T. K. Blackwell, and P. Anderson. 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. *EMBO J.* **23**:1313–1324.
 43. Tchen, C. R., M. Brook, J. Saklatvala, and A. R. Clark. 2004. The stability of tristetraprolin mRNA is regulated by mitogen-activated protein kinase p38 and by tristetraprolin itself. *J. Biol. Chem.* **279**:32393–32400.
 44. Winzen, R., G. Gowrishankar, F. Bollig, N. Redich, K. Resch, and H. Holtmann. 2004. Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. *Mol. Cell. Biol.* **24**:4835–4847.
 45. Winzen, R., S. Kafert, B. Preiss, H. A. Mylius-Spencker, K. Resch, and H. Holtmann. 1996. Interaction between the mRNA of the 55-kDa tumor necrosis factor receptor and cellular proteins. Possible involvement in post-transcriptional regulation of receptor expression. *J. Biol. Chem.* **271**:13461–13467.
 46. Winzen, R., M. Kracht, B. Ritter, A. Wilhelm, C. Y. Chen, A. B. Shyu, M. Muller, M. Gaestel, K. Resch, and H. Holtmann. 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.
 47. Yu, J. Y., S. L. DeRuiter, and D. L. Turner. 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**:6047–6052.