Interleukin-1 Activates Synthesis of Interleukin-6 by Interfering with a KH-type Splicing Regulatory Protein (KSRP)-dependent Translational Silencing Mechanism*§

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Post-transcriptional mechanisms play an important role in the control of inflammatory gene expression. The heterogeneous nuclear ribonucleoprotein K homology (KH)-type splicing regulatory protein (KSRP) triggers rapid degradation of mRNAs for various cytokines, chemokines, and other inflammation-related proteins by interacting with AU-rich elements (AREs) in the 3′-untranslated mRNA regions. In addition to destabilizing mRNAs, AU-rich elements can restrict their translation. Evidence that KSRP also participates in translational silencing was obtained in a screen comparing the polysome profiles of cells with siRNA-mediated depletion of KSRP with that of control cells. Among the group of mRNAs showing increased polysome association upon KSRP depletion are those of interleukin (IL)-6 and IL-1α as well as other ARE-containing transcripts. Redistribution of IL-6 mRNA to polysomes was associated with increased IL-6 protein secretion by the KSRP-depleted cells. Silencing of IL-6 and IL-1α mRNAs depended on their 3′-untranslated regions. The sequence essential for translational control of IL-6 mRNA and its interaction with KSRP was located to an ARE. KSRP-dependent silencing was reversed by IL-1, a strong inducer of IL-6 mRNA and protein expression. The results identify KSRP as a protein involved in ARE-mediated translational silencing. They suggest that KSRP restricts inflammatory gene expression not only by enhancing degradation of mRNAs but also by inhibiting translation, both functions that are counteracted by the proinflammatory cytokine IL-1.

Inflammatory gene expression is controlled by post-transcriptional mechanisms. Many relevant transcripts contain AU-rich elements (AREs) that can impose rapid degradation and restrict translation (1–3). Several proteins that interact with and mediate the effects of AREs have been identified. One of them, heterogeneous nuclear ribonucleoprotein K homology (KH)-type splicing regulatory protein (KSRP) is a member of the far upstream sequence element-binding proteins (4). It is a single-stranded nucleic acid-binding protein that contains four KH domains and has been reported to participate in transcription as well as splicing, editing, localization, and degradation of mRNAs and maturation of microRNA precursors (for a review, see Ref. 5). Most recently it has been found to contribute to regulation of RNA 3′-end processing (6).

KSRP has been shown to facilitate mRNA degradation by directly binding to AREs and recruiting mRNA-degrading enzymes (7, 8). Ksrp−/− cells and mice produce more type I interferons as a result of decreased mRNA decay and are refractory to viral infection (9). Different conditions of cellular activation or differentiation have been described to reduce KSRP binding and increase stability of specific mRNAs (e.g. Refs. 10–13). The proinflammatory cytokine IL-1 can induce stabilization of KSRP target mRNAs, including that of IL-6 (14, 15), by reducing interaction of KSRP with the ARE presumably as a result of KSRP phosphorylation by p38 MAP kinase (10).

IL-6, originally cloned as 26-kDa protein (16), IFN-β (17), and BSF-2 (18), is a pleiotropic cytokine with important roles in inflammation, immune regulation, oncogenesis, and other physiological and pathological processes. Accordingly, interfering with IL-6 action is the aim of therapeutic strategies (19, 20). IL-6 is induced in various cell types by a plethora of activators. Among the strongest and first identified are the proinflammatory cytokines tumor necrosis factor (TNF) (21) and IL-1 (22). Expression of IL-6 is controlled by transcriptional and post-transcriptional mechanisms. The latter involve AREs and a putative stem-loop structure that cause rapid degradation of the mRNA (23). Activators like IL-1 and lipopolysaccharide induce IL-6 mRNA stabilization through p38 MAPK/MK2 signaling (14, 24). IL-6 expression is also controlled by miRNAs (25, 26).

Our recent data showed that, in addition to stabilizing mRNAs, IL-1 can activate translation of mRNAs, including that of IL-6 (27). We now provide evidence that repressed basal translation of IL-6 and a group of other mRNAs depends on KSRP. Interaction of KSRP with the IL-6 ARE is direct and is decreased in response to IL-1, suggesting a novel, miRNA-independent function for KSRP in translation.
**Experimental Procedures**

Plasmids—The complete cDNA of human IL-6 was cloned into the BamHI site of expression vector pUHD10-3 (kindly provided by Hermann Bujard) to obtain pUHD10/IL6-IL6-IL6. Silent mutations were inserted in pUHD10/IL6-IL6-IL6 with the QuikChange site-directed mutagenesis kit (Stratagene) to allow distinction from endogenous IL-6 mRNA. Plasmids for expression of IL-6-β-globin chimeric mRNAs were derived from this plasmid as follows. After deletion of the upstream BamHI site and generation of an additional BamHI site after the stop codon, the IL-6 3′-UTR was excised by BamHI and replaced by that of rabbit β-globin, creating pUHD10/IL6-IL6-β. For generation of pUHD10/IL6-β-IL6, BglII sites were introduced before the stop codon and after the stop codon of IL-6. The coding region was excised with BglII and replaced with that of β-globin. pUHD10/B-IL6-IL6 was cloned by inserting the PCR-amplified fragment spanning the coding region and the 3′-UTR of IL-6 into the BamHI site of pUHD10-3. The 5′-UTR of β-globin was then cloned into the EcoRI site upstream of the IL-6 fragment. pUHD10/B-β-IL6 was cloned by inserting the PCR-amplified β-globin coding region-IL-6 3′-UTR fragment of pUHD10/IL6-β-IL6 into the BamHI site of pUHD10-3 followed by inserting the 5′-UTR of β-globin into the EcoRI site upstream of the β-globin coding region. Plasmids with different regions of the IL-6 3′-UTR were generated with overlap extension PCR cloning as described (28) (see primer sequences in the supplemental data). In pUHD10/IL6-ΔARE, nucleotides (nt) 828–1002 of the IL-6 3′-UTR were deleted (numbering according to GenBank™ accession number NM_000600); pUHD10/IL6-ARE I and -II contain only base pairs 816–1024 and 857–987, respectively, of the IL-6 3′-UTR. The seed region for miR-26 binding, ACTTGAA, was mutated to ACCTGAA in pUHD10/IL6-ΔARE by using the QuikChange procedure (Stratagene). For in vitro transcription of polyadenylated mRNA the IL-6-IL6-IL6 and IL-6-IL6-B sequences were subcloned into a modified pBluescript plasmid 5′-to a 54-nt poly(A) stretch. The IL-1α mRNA fragment spanning its 5′-UTR and coding region was amplified by RT-PCR and cloned into the BamHI site of pUHD10-3. The 3′-UTR of IL-1α or the 3′-UTR of β-globin was cloned immediately downstream of the IL-1α coding region to generate pUHD10/IL1-IL1-IL1 or pUHD10/IL1-IL1-β, respectively. Plasmids for expression of Strep-tagged GFP and KSRP have been described previously (15).

**Cells and Materials**—HeLa cells were cultured and transfected with plasmids (see above) or with siRNAs specific for GFP or KSRP mRNAs (Qiagen) by the calcium phosphate method as described (15). Recombinant human IL-1α was obtained from Promocell.

Sucrose Gradient Fractionation and RNA Isolation—Cytoplasmic lysates of HeLa cells were prepared and subjected to centrifugation through linear sucrose gradients (10–50% sucrose), fractions were collected, and RNA was isolated as described (27). Results shown were confirmed in at least two independent experiments.

mRNA Detection—Northern blot analysis of IL-6 and β-actin mRNA was performed as described previously (29) using digoxigenin-labeled antisense probes. Reverse transcription and quantitative PCR (RT-qPCR) were carried out as described (27) using TaqMan kits (Applied Biosystems assay identification numbers Hs00174131_m1 for IL-6 mRNA, Hs00174092_m1 for IL-1α mRNA, Hs99999905_m1 for GAPDH mRNA, and Hs00174103_m1 for IL-8 mRNA and a custom-made assay for rabbit β-globin mRNA). Ectopically expressed IL-6 mRNA and its derivatives were quantified by SYBR Green-based detection using a PCR primer pair with a sense primer specific for the mutated sequence that allows distinction from endogenous IL-6 mRNA (sense, 5′-CCCTCA-GAATAGGTTCGAAAGCAGC (mutated positions underlined); antisense, 5′-CCTGCAGCCACTGGTCTCGT). Values with standard deviations >0.2 cycles were excluded from the analysis.

Microarray Analysis—RNA of pooled sucrose gradient fractions was subjected to quality control and analyzed using whole human genome oligonucleotide microarrays (G4112F, identification number 014850, Agilent Technologies) in essence as described (15). Data were filtered according to a stringent multistep approach that accounted for 1) the quality of the measurements (hybridization performance), 2) the consistency among replicate assays, 3) the intensity range, and 4) fold-change values (see also the legend to Table 1). A detailed protocol is available upon request.

In Vitro Transcription and Electrophoretic Mobility Shift Assays—Templates were generated by PCR with T7-flanked sense primers. Radiolabeled RNA probes spanning the IL-6 3′-UTR with or without nt 828–1002 were in vitro transcribed with the T7 Megascript kit (Ambion) using 32P-labeled UTP. Purification of the labeled RNA probes, incubation with cytoplasmic extracts or purified Strep-tagged proteins, and separation of the samples on non-denaturing gels were performed as described (15).

In Vitro Translation—Polyadenylated RNAs were in vitro transcribed from linearized plasmids and purified using Mini Quick Spin RNA columns (Roche Applied Science) followed by LiCl precipitation. Translation reactions were performed using nucleate-treated rabbit reticulocyte lysate (Promega) in the presence of 10 μCi of [35S]methionine (1000 Ci/mmol) according to the manufacturer’s protocol, 0.8 pmol of test RNAs, and 0.2 pmol of control firefly luciferase RNA per 10-μl reaction. Reactions were stopped with SDS loading buffer and resolved by 12% SDS-PAGE, and products were detected by autoradiography.

**Nuclear-Cytoplasmic Fractionation and Western Blot**—Nuclear and cytoplasmic fractions were isolated using the NE-PER kit (Thermo Scientific) following the manufacturer’s protocol. Equivalent amounts of nuclear or cytoplasmic fractions or total cell lysates were separated by SDS-PAGE (NuPAGE gels, Invitrogen; 10% acrylamide). Proteins were blotted onto PVDF membranes. After blocking with 5% dried milk in Tris-buffered saline, the blots were incubated with antibodies against KSRP (murine monoclonal antibody kindly donated by Ching-Yi Chen), ATF1 (Santa Cruz Biotechnology), NF-κB p65 (Cell Signaling Technology), or α-tubulin (Sigma-Aldrich) followed by peroxidase-coupled secondary antibodies. Chemiluminescence was detected by the LAS 3000 imaging system (Fujifilm).
ELISA—IL-6 or IL-1α protein released into the culture medium was quantified by sandwich ELISA (DuoSet, R&D Systems) following the instructions supplied by the manufacturer.

Pulldown Assay—Cells expressing Strep-tagged KSRP or GFP were lysed in buffer (20 mM β-glycerophosphate (pH 7.4), 150 mM KCl, 5 mM MgCl₂, 0.25 mM DTT, 1 mM sodium orthovanadate, 0.5% (v/v) Nonidet P-40, protease inhibitor mixture (Roche Applied Science)), and the cytoplasmic extract was adsorbed to Strep-Tactin agarose beads (IBA) essentially as described (15). Co-purified mRNAs eluted from the beads and mRNAs in the cytoplasmic extracts before adsorption (input, 10% of the sample) were quantified by RT-qPCR.

RESULTS

Translation of Individual mRNAs Is Suppressed by KSRP—As reported previously (27), IL-1 induces an increase in polysome association of several mRNAs, including that of IL-6. Because IL-6 mRNA is also among the target mRNAs for the destabilizing RNA-binding protein KSRP (15), we asked whether KSRP contributes to translational control of IL-6 mRNA. KSRP expression was suppressed in HeLa cells by specific siRNAs (Fig. 1A). mRNAs from cytoplasmic extracts of IL-1-treated cells were separated according to their ribosome occupancy by density gradient centrifugation (Fig. 1B), and the distribution profile of IL-6 mRNA was analyzed by Northern blotting (Fig. 1, C and D). Whereas in the control cells transfected with irrele-
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vant siRNAs a large part of the IL-6 mRNA was detected in subpolysomal fractions, depletion of KSRP induced a shift to polysomes, indicating increased translation. No such change was observed for β-actin mRNA analyzed as a housekeeping control mRNA that preferentially localized to polysomes. A similar shift to polysomes was also observed with RT-qPCR for IL-6 mRNA in cells not stimulated by IL-1 in which IL-6 mRNA was below the level detected in Northern blots (Fig. 2A). Total IL-6 mRNA in KSRP-depleted cells was slightly increased as expected from the role of KSRP in mRNA degradation (Fig. 1E). The amount of IL-6 protein determined by ELISA was increased more strongly (Fig. 1E), confirming that translation of IL-6 mRNA was increased by KSRP depletion.

After separating mRNAs of control and KSRP-depleted cells as above, fractions 2–4 containing non-translated mRNAs and fractions 6–9 containing polysome-associated mRNAs were pooled and subjected to microarray analysis. In two independent experiments, the distribution of a group of mRNAs shifted to the polysomal pool. The top 50 mRNAs showing the strongest change in distribution are shown in Table 1. This indicated that besides IL-6 other mRNAs are translationally suppressed by KSRP. Of note, among the mRNAs in Table 1, 40% contain AU-rich elements according to the ARED database criteria (30). We previously defined 100 target mRNAs for KSRP in HeLa cells based on association in pulldown assays and increased amounts in KSRP-depleted cells (15). Eight of these mRNAs, including that of IL-6, also were redistributed to polysomes upon KSRP depletion (Table 1). As a whole, the mRNAs affected in this way by KSRP depletion are enriched for species relevant for defense reactions as analyzed by the GO-TermFinder program (supplemental Table S1).

The 3′-UTRs of IL-6 and IL-1α mRNA Mediate Translational Silencing—To identify regions in the IL-6 mRNA that mediate translational control, a vector was constructed that expresses wild type IL-6 protein but carries silent mutations that allow detection by RT-qPCR without interference of endogenous IL-6 mRNA. The mRNA encoded by this vector recapitulated the behavior of endogenous IL-6 mRNA: it localized primarily to subpolysomal fractions in untreated cells and was shifted to polysomes by KSRP depletion (Fig. 2A). The behavior of chimeric mRNAs in which parts of the IL-6 sequences were replaced by the corresponding sequences of rabbit β-globin mRNA was analyzed (see Fig. 2B, scheme). Although replacing the 5′-UTR or the coding region did not alter mRNA distribution in density gradients (Fig. 2B, B-IL6-IL6 and IL6-B-IL6), replacing the 3′-UTR with that of β-globin mRNA resulted in preferential localization of the chimeric mRNA in the polysome fractions (Fig. 2B, IL6-IL6-B). This suggested that RNA sequences essential for translational control of IL-6 mRNA are located in the 3′-UTR. Distribution of a chimeric mRNA consisting of the β-globin 5′-UTR and coding region and the IL-6 3′-UTR (Fig. 2B, B-B-IL6) was similar to the distribution of endogenous IL-6 mRNA, indicating that 3′-UTR sequences were sufficient for translational control. The IL-6 protein/RNA ratio for cells expressing the chimeric IL-6 mRNA lacking its 3′-UTR (IL6-IL6-B) increased as compared with cells expressing the complete IL-6 mRNA (Fig. 2C). This indicated that the IL-6 3′-UTR indeed suppresses translation. Correspondingly, in an in vitro translation assay with reticulocyte lysate, IL-6 mRNA lacking its 3′-UTR yielded more protein than its wild type counterpart (Fig. 2D).

Basaically similar results were obtained for IL-1α mRNA, which is among the transcripts most strongly shifted to polysomes upon KSRP depletion (Table 1). RT-qPCR determination in individual fractions from IL-1-stimulated cells as in Fig. 1C confirmed redistribution to polysomes (Fig. 3A). IL-1α mRNA and protein were not detected in significant amounts in unstimulated cells. Therefore, plasmids were generated to express the complete IL-1α mRNA or a chimeric mRNA in which the IL-1α 3′-UTR was exchanged by that of β-globin. Ectopically expressed complete IL-1α mRNA localized mainly to subpolysomal fractions, whereas its chimeric counterpart containing the β-globin 3′-UTR localized to polysome fractions (Fig. 3B). Cells expressing the chimeric mRNA, which lacks the IL-1α 3′-UTR, contained more RNA and released more IL-1α protein into the supernatant as compared with cells expressing the complete IL-1α mRNA (Fig. 3C). As for IL-6, the increase in protein markedly exceeded that of the mRNA. IL-1α protein in the cell lysate was also increased (5-fold; not shown). This indicated that, similar to IL-6, repression of IL-1α translation by KSRP depends on sequences in its 3′-UTR.

Translational Silencing of IL-6 mRNA Depends on AU-rich Sequences and Is Counteracted by IL-1—As depicted in Fig. 4A, the IL-6 3′-UTR contains AU-rich sequences and a stem-loop-forming sequence that contribute to rapid degradation (23). Furthermore, IL-6 expression has been reported to be controlled by microRNAs miR-26 (26) and let-7 (25). Deleting a region that contains the predicted stem-loop structure, ARE sequences, and the miR-26 target region abolished silencing (Fig. 4B, IL6ΔARE). Correspondingly, a transcript that contains only these sequences in its 3′-UTR was translationally silenced (IL6 ARE). Thus, the let-7 binding region and ARE sequences located 3′-of that region are dispensable for the translational control observed here. Further shortening showed that an mRNA that also lacks the predicted stem-loop structure still was repressed albeit somewhat less pronounced (Fig. 4C, IL6 ARE II). Importantly, this mRNA was shifted to polysomes in response to IL-1 as observed for endogenous and ectopically expressed full-length IL-6 mRNA (Fig. 4C and Ref. 27). To address the role of miR-26 in KSRP-dependent silencing and its modulation by IL-1, the seed region of its target sequence was mutated in three positions. As shown in Fig. 4D, this did not result in any significant increase in polysome association. Thus, miR-26 is unlikely to play a role in the regulation of translation observed here. Furthermore, the mutated mRNA was still redistributed to polysomes in response to IL-1.

Direct Interaction between KSRP and IL-6 mRNA—Association of KSRP with its target mRNAs IL-6 and IL-1α was demonstrated in pulldown assays. A specific increase in these mRNAs as well as the previously defined target mRNA IL-8 was observed after pulldown with Strept-Tactin-coated beads from lysates of cells expressing Strept-tagged KSRP as compared with Strept-tagged GFP (Fig. 5A). GAPDH mRNA as a housekeeping control was not significantly enriched. IL-1 treatment of the cells reduced the interaction between KSRP and its target mRNAs. Thus, IL-1 appears to increase IL-6
FIGURE 2. The 3′-UTR of IL-6 mRNA suppresses translation. A, cells were transfected with siRNAs specific for GFP or KSRP mRNAs as in Fig. 1 but left without IL-1 treatment. Polysome profiles for endogenous IL-6 mRNA (end. IL-6) or ectopically expressed IL-6 mRNA (ectop. IL-6) were obtained by RT-qPCR. B, distribution profiles of chimeric mRNAs in which the 5′-UTR, coding sequence (CDS), or 3′-UTR of IL-6 was replaced by the corresponding part of β-globin mRNA (see scheme) were obtained by RT-qPCR for IL-6 mRNA or for β-globin mRNA (IL6-B-IL6 and B-B-IL6). The distribution of GAPDH mRNA is shown to allow comparison of the gradient separations. C, cells were transfected with expression vectors for IL-6 with its own (IL6-IL6-IL6) or β-globin 3′-UTR (IL6-IL6-B). The next day medium was exchanged. 2 h later, the culture medium was collected, and IL-6 protein release was quantified by ELISA. The cells were lysed, and IL-6 mRNA was determined by RT-qPCR. Results show the fold change for the amounts of RNA and protein for IL6-IL6-B as compared with those of IL6-IL6-IL6 (mean ± S.D., n = 5). D, in vitro transcribed IL-6 RNA containing its own (IL6-IL6-IL6) or the β-globin 3′-UTR (IL6-IL6-B) or firefly luciferase RNA were subjected to in vitro translation reactions with reticulocyte lysates and [35S]methionine. The samples were separated by SDS-PAGE, and proteins were visualized by autoradiography. Results from one of two independent assays with similar results are shown.
mRNA translation by reducing binding of the translational silencing protein KSRP. Of note, residual binding of KSRP was still observed after IL-1 treatment. This correlates to the IL-1-induced redistribution of IL-6 mRNA in polysome profiles where some amount of IL-6 mRNA still remained in the untranslated fractions.

**TABLE 1**

| Gene symbol | Description | Fold-change | Target of destabilization by KSRP | ARE class |
|-------------|-------------|-------------|----------------------------------|-----------|
| CLDN12      | Claudin 12 (CLDN12), mRNA (NM_012129) | 14.0         | +                                 | 1         |
| IL1A        | Interleukin-1, α (IL1A), mRNA (NM_000575) | 9.8          | +                                 | 1         |
| MRFC2       | Mannose receptor, C type 2 (MRFC2), mRNA (NM_006039) | 8.9          | +                                 | 1         |
| EDN1        | Endothelin 1 (EDN1), mRNA (NM_001955) | 8.2          | +                                 | 1         |
| FAM46B      | Family with sequence similarity 46, member B (FAM46B), mRNA (NM_025943) | 8.0          | +                                 | 1         |
| HFN1        | Interferon, β1, fibroblast (HFN1), mRNA (NM_00176) | 7.7          | 2                                 | 1         |
| PLSCR4      | Phospholipid scramblase 4 (PLSCR4), mRNA (NM_020353) | 7.4          | +                                 | 1         |
| IL6         | Interleukin-6 (interferon, β2) (IL6), mRNA (NM_000600) | 7.3          | +                                 | 1         |
| GJB3        | Gap junction protein, β3 (connexin 31.1) (GJB3), mRNA (NM_005268) | 6.4          | +                                 | 1         |
| SOD2        | Superoxide dismutase 2, mitochondrial, mRNA (cDNA clone MG2:21350 IMAGE:4184203), complete cds (BC016934) | 6.4          | +                                 | 1         |
| ATF3        | Activating transcription factor 3 (ATF3), transcript variant 2, mRNA (NM_004024) | 6.4          | +                                 | 1         |
| ADM         | Adrenomedullin (ADM), mRNA (NM_001124) | 6.1          | +                                 | 1         |
| EDN2        | Endothelin 2 (EDN2), mRNA (NM_001956) | 6.1          | +                                 | 1         |
| BDKRB1      | Bradykinin receptor B1 (BDKRB1), mRNA (NM_000710) | 5.8          | +                                 | 1         |
| CLDN1       | Claudin 1 (CLDN1), mRNA (NM_021101) | 5.8          | +                                 | 1         |
| IL23A       | Interleukin-23, α subunit p19 (IL23A), mRNA (NM_016584) | 5.7          | +                                 | 1         |
| SYNE1       | Spectrin repeat-containing, nuclear envelope 1 (SYNE1), transcript variant longer, mRNA (NM_030701) | 5.4          | 1                                 | 1         |
| JUN         | v-jun sarcoma virus 1 oncogene homolog (avian) (JUN), mRNA (NM_002228) | 5.4          | 1                                 | 1         |
| NRD1        | Nuclear receptor subfamily 1, group D, member 1 (NRD1), mRNA (NM_021724) | 5.3          | 1                                 | 1         |
| CYR61       | Cysteine-rich, angiogenic inducer, 61 (CYR61), mRNA (NM_001554) | 5.3          | 1                                 | 1         |
| CTH         | Cystathionase (cystathionine γ-lyase) (CATH), transcript variant 1, mRNA (NM_001902) | 5.2          | 1                                 | 1         |
| ZCCHC6      | Zinc finger, CCHC domain-containing 6 (ZCCHC6), mRNA (NM_024617) | 5.2          | 1                                 | 1         |
| STC2        | Stanniocalcin 2 (STC2), mRNA (NM_003714) | 5.2          | 1                                 | 1         |
| SERPINE1    | Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1 (SERPINE1), mRNA (NM_000602) | 5.1          | 1                                 | 1         |
| ALCAM       | Activated leukocyte cell adhesion molecule (ALCAM), mRNA (NM_001627) | 5.1          | 1                                 | 1         |
| NIPBL       | Nippl-B homolog (Drosophila) (NIPBL), transcript variant B, mRNA (NM_015384) | 5.1          | 1                                 | 1         |
| ETS1        | v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS1), mRNA (NM_005238) | 5.0          | 1                                 | 1         |
| ADAM9       | ADAM metalloprotease domain 9 (meltrin γ) (ADAM9), transcript variant 2, mRNA (NM_001058465) | 5.0          | 1                                 | 1         |
| AXD1D1      | AXD1 up-regulated 1 (AXD1D1), mRNA (NM_030327) | 5.0          | 1                                 | 1         |
| SLC25A25    | Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25 (SLC25A25), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA (NM_001006641) | 5.0          | 1                                 | 1         |
| DUSP2       | Dual specificity phosphatase 2 (DUSP2), mRNA (NM_004418) | 4.9          | 1                                 | 1         |
| PTX3        | Pentraxin-related gene, rapidly induced by IL-1, β (PTX3), mRNA (NM_002852) | 5.0          | 1                                 | 1         |
| TNFRSF9     | Tumor necrosis factor receptor superfamily, member 9 (TNFRSF9), mRNA (NM_003161) | 4.9          | 1                                 | 1         |
| SELE        | Selectin E (endothelial adhesion molecule 1) (SELE), mRNA (NM_000450) | 4.8          | +                                 | 2         |
| IL6R        | Interleukin-6 receptor (IL6R), transcript variant 1, mRNA (NM_000565) | 4.8          | +                                 | 2         |
| P18SRP      | P18SRP protein (P18SRP), mRNA (NM_173829) | 4.8          | 1                                 | 1         |
| RP2         | Retinitis pigmentosa 2 (X-linked recessive) (RP2), mRNA (NM_0006915) | 4.7          | 1                                 | 1         |
| TNFAIP3     | Tumor necrosis factor, α-induced protein 3 (TNFAIP3), mRNA (NM_002970) | 4.7          | 1                                 | 1         |
| KCNJ12      | Potassium inwardly rectifying channel, subfamily J, member 12 (KCNJ12), mRNA (NM_021012) | 4.6          | 1                                 | 1         |
| RUVBL1      | RuVBL-1 (Retinoblastoma homolog 1) (RUVBL1), mRNA (NM_003707) | 4.6          | 1                                 | 1         |
| NTF         | Tumor necrosis factor (TFN superfamily, member 2) (TNF), mRNA (NM_000594) | 4.6          | 1                                 | 1         |
| Btg2        | Btg family, member 2 (Btg2), mRNA (NM_006763) | 4.6          | 1                                 | 1         |
| TipARP      | TCD-inducible poly(ADP-ribose) polymerase (TipARP), mRNA (NM_015508) | 4.6          | 1                                 | 1         |
| Kif27       | Kinesin family member 27 (Kif27), mRNA (NM_017576) | 4.5          | 1                                 | 1         |
| Hist2H2Be   | Histone, H2be (Hist2H2Be), mRNA (NM_005328) | 4.5          | 1                                 | 1         |
| Irf1        | Interferon regulatory factor 1 (Irf1), mRNA (NM_001918) | 4.5          | 1                                 | 1         |
| Hm13        | Histocompatibility (minor) 13 (Hm13), transcript variant 4, mRNA (NM_007532) | 4.5          | 1                                 | 1         |
| Gadd45b     | Growth arrest- and DNA damage-inducible, β (Gadd45b), mRNA (NM_015675) | 4.4          | 1                                 | 1         |
| Loc153222   | Adult retina protein (Loc153222), mRNA (NM_153607) | 4.4          | 1                                 | 1         |
| Pvr         | Poliovirus receptor (Pvr), mRNA (NM_005605) | 4.3          | 1                                 | 1         |
IL-1 treatment did not result in a detectable change in the level or in the cellular distribution of KSRP as observed by Western blot of cytoplasmic and nuclear fractions (Fig. 5B), whereas translocation of the p65 isoform of NF-κB to the nucleus as a positive control was clearly detectable. This argues against a mere decrease in cytoplasmic KSRP as an explanation for decreased binding and silencing of IL-6 mRNA.

Association with KSRP in pulldown assays was strongly reduced for IL-6 mRNA lacking the 3′-UTR or the ARE region (Fig. 5C). In electrophoretic mobility shift assays using radiolabeled RNA probes, proteins from HeLa lysate formed a prominent complex with the complete 3′-UTR, whereas no such complex was detected with 3′-UTR RNA lacking the ARE region (Fig. 5D). Similarly to HeLa lysate, purified KSRP protein formed a complex with the complete 3′-UTR but not with the ARE-deleted RNA. This suggests that KSRP exerts its effect on translation of IL-6 mRNA through direct interaction with AU-rich sequences in the 3′-UTR.

**DISCUSSION**

KSRP participates in the control of gene expression in different ways, including the ARE-dependent rapid degradation of mRNAs and the maturation of miRNAs (5). By studying IL-6 mRNA, we obtained evidence that KSRP also limits translation of mRNAs through direct interaction with AREs. siRNA-mediated knockdown of KSRP resulted in increased ribosome association of IL-6 mRNA according to polysome profiling by density gradient centrifugation (Fig. 1C). Furthermore, KSRP depletion caused a marked increase in IL-6 protein that clearly surpassed that of its mRNA (Fig. 1E). IL-6 mRNA contains AU-rich sequences that contribute to its rapid degradation (14, 23, 24, 31). The observations that KSRP-dependent translational silencing required an ARE (Fig. 4) and that KSRP interacted directly with the IL-6 mRNA in an ARE-dependent manner (Fig. 5D) strongly suggest that KSRP binding to the ARE suppresses translation.

Translational repression by AREs was first observed for RNAs injected into *Xenopus* oocytes under conditions in which the RNAs were stable (32). The RNA-binding protein TIA-1 was identified as a translational silencer selective for TNF-α mRNA, whereas its deletion hardly affected levels of certain other cytokines, including IL-6 (33). Activation by lipopolysaccharide can release translational silencing of TNF-α mRNA through p38 MAP kinase and its substrate kinase MK2 (34, 35). Of note, decreased polysome association of TNF-α mRNA upon inhibition of p38 MAP kinase was still observed in cells lacking TIA-1 (33), indicating that TIA-1 is not the target of signals increasing translation. It is tempting to speculate that KSRP, a substrate of p38 MAP kinase (10), represents a signal-controlled component of translational silencing of ARE-containing mRNAs. In fact, interaction between TIA-1 and KSRP has been reported (36). Besides TIA-1, other proteins have been

**FIGURE 3.** Translational silencing of IL-1α mRNA depends on its 3′-UTR and KSRP. A, cytoplasmic lysates of cells transfected with siRNAs specific for KSRP or GFP were separated by density gradient centrifugation, and polysome profiles of IL-1α mRNA were obtained by RT-qPCR of individual fractions. B, polysome profiles of ectopically expressed IL-1α mRNA with its own 3′-UTR (IL1-IL1-IL1) or with the β-globin 3′-UTR (IL1-IL1-B). C, cells were transfected with expression vectors IL1-IL1-IL1 or IL1-IL1-B as in B. IL-1α mRNA was quantified by RT-qPCR, and protein release into the supernatant was quantified by ELISA. Results are expressed as fold change in the amounts of RNA and protein for IL1-IL1-B compared with IL1-IL1-IL1 (mean ± S.D. of duplicates). Similar results were obtained in two independent assays.
observed to influence translation of ARE-containing mRNAs (Refs. 1–3 and references therein).

KSRP has been demonstrated to regulate maturation of a group of miRNAs, including miR-26b and let-7a (37). We did not obtain evidence for the contribution of these miRNAs, which have been reported to control IL-6 expression (25, 26), to restricted translation of IL-6 mRNA in our experimental setting. However, it is well possible that under different condi-

FIGURE 4. Localization of sequences involved in translational silencing of IL-6 mRNA. A, scheme of IL-6 mRNA and constructs expressed. Numbers indicate nt positions at the ends of deleted or inserted sequences. B, polysome profiles of IL-6 mRNA lacking nt 828–1002 (IL6ΔARE) or containing only nt 816–1024 of its 3′-UTR (IL6 ARE I) were obtained as described for Fig. 2B. For comparison of the gradients, the corresponding profiles of GAPDH mRNA are shown in the lower panel. C, polysome profiles of endogenous IL-6 mRNA (end. IL-6), ectopically expressed IL-6 mRNA containing the complete 3′-UTR (ectop. IL-6), or its derivative containing only nt 857–987 of its 3′-UTR (IL-6ARE II) were compared for cells incubated without (con) or with IL-1α (2 ng/ml) (IL-1) for 1 h. D, comparison of polysome profiles for ectopically expressed IL-6 mRNA without (IL-6) or with mutations that destroy the seed region for miR-126 (IL-6 miR-mu) (upper panel) and for IL-6 miR-mu mRNA in cells incubated without (con) or with IL-1α (IL-1) for 1 h (lower panel).
KSRP-dependent Translational Silencing of ARE mRNA

FIGURE 5. Direct and ARE-dependent interaction of KSRP with IL-6 mRNA. A, cells expressing Strep-tagged KSRP (stKSRP) or GFP (stGFP) were incubated without (control) or with IL-1α (IL-1) for 30 min and lysed, and tagged proteins were enriched by pulldown with Strep-Tactin-coated beads (see “Experimental Procedures”). Shown are the ratios of the amounts of the indicated mRNAs determined by RT-qPCR in the pulldown and input samples. Note that IL-1α mRNA was not detected in untreated HeLa cells (nd, not detected). B, after stimulation of cells with IL-1α for the indicated times, cytoplasmic and nuclear fractions were prepared and analyzed by Western blotting using antibodies against KSRP, against α-tubulin and ATF1 as controls for fractionation quality, and against NF-κB p65 to control IL-1 responsiveness. C, enrichment of complete IL-6 mRNA or of IL-6 mRNA in which its 3′-UTR was exchanged with that of β-globin (IL6-IL6-B) or that lacks nt 828–1002 (ΔARE) after pulldown with Strep-tagged GFP or Strep-tagged KSRP was determined as in A. D, cytoplasmic (cyl.) lysate or purified Strep-tagged KSRP (200 ng) was incubated with in vitro transcribed radiolabeled RNA comprising the IL-6 3′-UTR with (3′ UTR) or without nt 828 to 1002 (ΔARE). Samples were subjected to non-denaturing gel electrophoresis. The autoradiograph shows protein-RNA complexes and part of the free RNA products.

In an attempt to identify other targets regulated by KSRP, pools of polysomal and subpolysomal fractions from KSRP-depleted and control cells were compared by microarray analysis. Cells were treated with IL-1 to include analysis of mRNAs that are only expressed in response to inflammatory stimuli. Although IL-1 can already increase polysome association, a further increase upon KSRP depletion was noted for IL-6, IL-1α, and other mRNAs. A likely explanation is that the IL-1-induced redistribution to polysomes and decrease in KSRP binding are partial (Figs. 4C and 5A, respectively). Furthermore, IL-1 treatment was for 2 h in these assays, a period after which some effects of IL-1 are already reversed (not shown). However, it is possible that the effect of IL-1 is stronger for certain mRNAs that therefore might remain undetected in our screen.

KSRP targets defined by association with KSRP in pulldown experiments and increased amounts upon KSRP depletion (15) are enriched among the mRNAs that show increased polysome association in KSRP-depleted cells. This argues for a mechanistic link between translational silencing and destabilization. On the other hand, most of the transcripts in Table 1 are not among the targets of KSRP destabilization identified earlier. This is not unexpected because the screen for increased ribosomal association following KSRP depletion should not only detect mRNAs controlled by KSRP directly but also mRNAs that KSRP regulates indirectly by promoting maturation of miRNAs that target them (37, 39). To what extent KSRP can restrict mRNA translation also independently of AREs and destabilization and of its effect on miRNA maturation is not clear at present. According to one report, KSRP negatively regulates viral translation by interacting with an internal ribosomal entry site (40). Recently, regulation of translation has also been reported for the other members of the far upstream sequence element-binding proteins FUBP1 (41) and FUBP3 (42).

Several groups including our own have shown that IL-1 can increase inflammatory gene expression by stabilizing mRNAs (Refs. 13, 14, and 43) and references therein). Among them are IL-6 mRNA and other targets of KSRP-dependent destabilization (15). Recently, we have provided evidence that IL-1 can exert a dual effect on post-transcriptional control of several mRNAs: stabilization and increased translation (27). IL-1 interfered with KSRP-dependent translational silencing of IL-6 mRNA (Fig. 4). Like IL-1-induced stabilization, this might be explained by decreased KSRP binding through activation of p38 MAP kinase. However, other mRNAs regulated in both ways by IL-1 were not significantly affected by KSRP knockdown and thus are unlikely to be targets of KSRP (data not shown).

Taken together, the results presented here demonstrate a role for KSRP in the ARE-dependent translational silencing of IL-6 and IL-1α mRNA and suggest that IL-1-induced interference with KSRP function not only contributes to mRNA stabilization but also to the recently observed translational activa-
tion exerted by this cytokine. These results support the importance of KSRP as a negative regulator of inflammation.

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