Similar Regulation of Human Inducible Nitric-oxide Synthase Expression by Different Isoforms of the RNA-binding Protein AUF1*§

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The ARE/poly-(U) binding factor 1 (AUF1), a protein family consisting of four isoforms, is believed to mediate mRNA degradation by binding to AU-rich elements (ARE). However, evidence exists that individual AUF1 isoforms may stabilize ARE-containing mRNAs. The 3′-untranslated region of the human inducible nitric-oxide synthase (iNOS) contains five AREs, which promote RNA degradation. We have recently shown that the RNA-binding protein KSRP is critically involved in the decay of the iNOS mRNA. In this study we examined the effects of the individual AUF1 isoforms on iNOS expression. Overexpression of each AUF1 isoform reduces iNOS expression on mRNA and protein levels to the same extent by modulation of mRNA stability. Accordingly, knockdown of all or individual AUF1 isoforms by an RNA interference approach enhances iNOS expression. The AUF1 effect on iNOS expression is dependent on the iNOS 3′-untranslated region sequence, as demonstrated in transfection experiments with a reporter mRNA. Binding studies showed that all AUF1 isoforms interact with the same AU-rich region in the iNOS 3′-untranslated region. Cytokine stimulation altered intracellular AUF1 binding activities. These data demonstrate that AUF1 is an important factor that promotes iNOS mRNA degradation. Furthermore, all individual AUF1 isoforms act in a similar manner.

Modulation of mRNA stability is an important mechanism in the post-transcriptional regulation of gene expression. It permits stringent control of intracellular mRNA levels and, thus, contributes to regulation of protein expression (1–3). AU-rich elements (ARE)2 are well characterized cis-acting elements involved in the regulation of mRNA stability (4–7). They are often located in the 3′-untranslated regions (3′-UTR) of many inherently unstable mRNAs that code for cytokines, growth factors, and proto-oncogenes, and they serve as binding sites for trans-acting proteins. Normally, ARE-containing mRNAs are rapidly degraded, but they can be stabilized upon exposure to various exogenous stimuli. Defects in the regulation of mRNA stability have been reported to be associated with different human diseases like cancer (8, 9) or chronic inflammatory diseases (9, 10).

In recent years several proteins have been identified that are able to interact with AREs and thereby mediate changes in mRNA stability. The members of the embryonic lethal abnormal vision (ELAV) protein family, especially HuR (11), have been described to stabilize ARE-containing mRNAs and to modulate their translational efficiency (12). The closely related proteins T cell-restricted intracellular antigen (TIA)-1 and the TIA-related protein (TIAR) destabilize mRNAs and interfere with translation by binding to ARE sequences (13, 14). The KH-type splicing regulatory protein (KSRP) (15), the heteronuclear ribonucleoprotein A1 (16), and tristetraprolin (TTP) (17) promote mRNA degradation. The mechanism in which way these RNA-binding proteins mediate mRNA decay is not yet fully understood. It is generally believed that mRNA decay starts with shortening of the poly-A tail followed by degradation of the mRNA by the exosome (multiprotein complex with 3′ to 5′ nuclease activity). Because the exosome is not able to bind directly to the ARE-mRNA it requires the RNA-binding proteins KSRP and TTP for this interaction (18). In addition, also the 5′-3′ decay pathway is important for the degradation of ARE-containing mRNAs. Here the process starts with the decapping of the mRNA by Dcp1 followed by Xrn1-mediated 5′-3′ degradation of the mRNA body. It has been published that TTP is also able to target ARE-mRNAs to this decay pathway (19).

The ARE/poly(U) binding/degradation factor 1 (AUF1, also named heteronuclear ribonucleoprotein D) is a RNA-binding mixture; Dox, doxycycline; GFP, green fluorescence protein; EGFP, enhanced GFP; GST, glutathione S-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HuR, human antigen R; Luc, luciferase; KSRP, KH-type splicing regulatory protein; iNOS, inducible NO synthase; RT, reverse transcription; qRT, quantitative real time RT; BP, binding protein; PTB, polypyrimidine tract binding protein; siRNA, small interfering RNA; shRNA, short hairpin RNA; TTP, tristetraprolin; DRB, 6-dichloro-1-ribofuranosylbenzimidazole.

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§ The abbreviations used are: ARE, AU-rich element; UTR, untranslated region; AUF1, AU-rich element RNA-binding protein 1; CM, cytokine...
protein with a dual function in terms of regulation of mRNA stability (20). On the one hand a destabilizing effect of AUF1 has been described, but on the other hand some reports demonstrate an mRNA-stabilizing role for this protein (21–23). Functional analysis of AUF1 in mRNA degradation is further complicated due to the existence of four different isoforms, which are generated by alternative splicing of one heteronuclear RNA. The family consists of a 45-kDa protein (p45AUF1) which contains all exons, a 42-kDa protein (p42AUF1) with deleted exon 2, a 40-kDa protein (p40AUF1) which lacks exon 7, and a 37-kDa protein (p37AUF1) with both exon 2 and exon 7 deleted (see Fig. 1A). According to the literature, all isoforms display different RNA binding activities, whereas p37AUF1 possesses the highest and p40AUF1 the lowest RNA binding affinity (see Fig. 1A) (24). Furthermore, some evidence exists that the individual AUF1 isoforms exert different effects in the regulation of mRNA stability depending on their expressional level (25–27). In addition to these post-transcriptional effects, also AUF1-dependent transcriptional regulation mechanisms have been published (28, 29).

In the innate immune system nitric oxide generated by the human inducible nitric-oxide synthase (iNOS) is important for the defense of bacteria, viruses, parasites, and tumor cells. The expression of the enzyme is induced by lipopolysaccharide or proinflammatory cytokines (30). The 3′-UTR of the human iNOS mRNA contains five ARE sequences. Transfection experiments in A549/8- or DLD-1 cells showed that these sequences are able to destabilize the mRNA of a heterologous reporter gene (31). Therefore, post-transcriptional mechanisms are important for the regulation of iNOS gene expression. In recent studies we demonstrated that the ARE-binding proteins KSRP, HuR, PTB, and tristetraprolin are critically involved in the post-transcriptional regulation of human iNOS expression (32–34). Functional analysis of AUF1 in mRNA degradation is further complicated due to the existence of four different isoforms, which are generated by alternative splicing of one heteronuclear RNA. The family consists of a 45-kDa protein (p45AUF1) which contains all exons, a 42-kDa protein (p42AUF1) with deleted exon 2, a 40-kDa protein (p40AUF1) which lacks exon 7, and a 37-kDa protein (p37AUF1) with both exon 2 and exon 7 deleted (see Fig. 1A). According to the literature, all isoforms display different RNA binding activities, whereas p37AUF1 possesses the highest and p40AUF1 the lowest RNA binding affinity (see Fig. 1A) (24). Furthermore, some evidence exists that the individual AUF1 isoforms exert different effects in the regulation of mRNA stability depending on their expressional level (25–27). In addition to these post-transcriptional effects, also AUF1-dependent transcriptional regulation mechanisms have been published (28, 29).

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In the present study we investigated the involvement of AUF1 in the post-transcriptional regulation of human iNOS expression. Moreover, we were interested if all AUF1 isoforms regulate iNOS expression in the same way or if some isoforms exert opposite effects. Overexpression or siRNA-mediated down-regulation of AUF1 indicates that all AUF1 isoforms reduce iNOS expression to a similar extent by decreasing iNOS mRNA stability. All AUF1 isoforms display the same binding pattern to the iNOS 3′-UTR sequence. Transfection data indicate that the four different AUF1 proteins mediate their effects on human iNOS expression via this binding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin, glutamine, and pyruvate solutions, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, agarose, isopropyl 1-thio-β-d-galactopyranoside, tRNA, bovine serum albumin, horseradish peroxidase-coupled anti-mouse IgG were purchased from Sigma. Isotopes were obtained from Hartmann Analytic, Braunschweig, Germany. Restriction enzymes, Taq polymerase, Klenow DNA polymerase, dNTPs, pGEX-2T, and glutathione-agarose affinity beads were purchased from Ameri-sham Biosciences. The monoclonal anti-GFP antibody, calf intestine alkaline phosphatase, RNase A, RNase T1, DNase I, and T3 and T7 RNA polymerase were obtained from Roche Diagnostics. The QuantiTect Probe RT-PCR kit was from Qiagen, Hilden, Germany. All oligonucleotides and dual-labeled probes were from MWG Biotech, Ebersberg, Germany. Human interferon-γ, interleukin-1β, and tumor necrosis factor-α were obtained from Strathmann, Hannover, Germany. Fetal calf serum and Dulbecco’s modified Eagle’s medium were purchased from PAN-Systems, Nürnberg, Germany. Zeocin and psiRNAH1-GFPzeo were purchased from InvivoGen, San Diego, CA. pcDNA3, pZeo SV2(−), and pcDNA4/T4 were purchased from Invitrogen. pEGFP-C1 was from BD-Clontech, Heidelberg, Germany. pCR-Script was from Stratagene, Heidelberg, Germany. The Bradford reagent mix for determination of protein concentration was obtained from Bio-Rad. The monoclonal anti-AUF1 antibody was a kind gift of Dr. Gideon Dreyfuss (Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA). The DLD-1–TR7 cells and the pTER vector were a kind gift of Dr. M. Weitering, Center for Biomedical Genetics, Utrecht, Netherlands.

**Cell Culture, Cytokine Treatment, and RNA Isolation**—Human colon carcinoma DLD-1 cells were grown in Dulbecco’s modified Eagle’s medium with 2 mm L-glutamine, penicillin, and streptomycin and 10% heat-inactivated fetal bovine serum. Eighteen hours before cytokine induction, cells were washed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium containing 2 mm L-glutamine in the absence of serum and phenol red. iNOS expression in cells was induced with a cytokine mixture (CM) containing human interferon-γ (100 units/ml), interleukin-1β (50 units/ml), and tumor necrosis factor-α (10 ng/ml) for the corresponding time periods. The cells were washed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium containing 2 mm L-glutamine in the absence of serum and phenol red. iNOS expression in cells was induced with a cytokine mixture (CM) containing human interferon-γ (100 units/ml), interleukin-1β (50 units/ml), and tumor necrosis factor-α (10 ng/ml) for the corresponding time periods. The cells were washed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium containing 2 mm L-glutamine in the absence of serum and phenol red. iNOS expression in cells was induced with a cytokine mixture (CM) containing human interferon-γ (100 units/ml), interleukin-1β (50 units/ml), and tumor necrosis factor-α (10 ng/ml) for the corresponding time periods. The cells were washed with phosphate-buffered saline and incubated with Dulbecco’s modified Eagle’s medium containing 2 mm L-glutamine in the absence of serum and phenol red. iNOS expression in cells was induced with a cytokine mixture (CM) containing human interferon-γ (100 units/ml), interleukin-1β (50 units/ml), and tumor necrosis factor-α (10 ng/ml) for the corresponding time periods.

**Establishment of Cell Lines Expressing EGFP-AUF1 Fusion Proteins**—To generate DLD-1 cells overexpressing EGFP-AUF1 fusion proteins, cells were transfected with 5 μg of pZeo-EGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 (20) with FuGENE according to the manufacturer’s recommendations. Stable transfectants (DLD-1-pZeo-EGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1) were selected with zeocin (0.2 mg/ml). As a control, DLD-1 cells stably transfected with the pZeo-EGFP-C1 vector were also generated (DLD-1-pZeo-EGFP-C1). The pZeo-EGFP-C1 and pZeo-EGFP-AUF1 constructs code for an EGFP protein or EGFP-AUF1 fusion protein, the zeocin-resistant cell pools were also selected for EGFP expression by fluorescence activated cell sorting. These sorted cell pools were characterized for the expression of EGFP-AUF1 by Western blots using a monoclonal anti-EGFP antibody.

**Quantitative Reverse Transcription (qRT)-PCR**—Gene expression was quantified in a two-step real-time RT-PCR. The cDNA was reverse-transcribed from total RNA samples using the High Capacity cDNA Reverse Transcription kit (Applied
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Biosystems, Darmstadt, Germany) according to the manufacturer’s recommendations. Real-time PCR was performed in a total volume of 25 μl in a 96-well spectrofluorometric thermal cycler (iCycler, Bio-Rad). For all genes the final reaction mix contained 2.5 units of Taq-DNA polymerase, Taq-DNA polymerase buffer, and dNTPs (final concentration, 2.5 μM each), forward and reverse primers at final concentrations of 0.4 μM for each primer, the corresponding probe at the final concentration of 0.2 μM, and 2 μl of cDNA.

For real-time PCR (40 cycles of 15 s 94 °C, 60 s 60 °C), the oligonucleotides listed below served as sense and antisense primers and Taqman hybridization probes: AUF1, sense 5’-GCCCCTTCCCCAGATAACACCTGAA-3’, antisense 5’-CTTTATGCTCTTGGTCCATGG-3’, probe 5’-CGCCCCTCGTCCATGG-3’; GAPDH, sense 5’-TGGGACACGACTTCTGC-3’, antisense 5’-CTCTGCCTGGTTGTCCATGG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’; renilla, sense 5’-CTGCTGCGGTAAGGATTTAGC-3’, antisense 5’-GGTAGCCCGGAGTATGAGTCCTTCCACG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’; iNOS, sense 5’-TTTTGCTGGTGAGGTGGAATCCATA-3’, antisense 5’-ATTGGTCTTGTTGTCCATGGG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’; GAPDH, sense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, antisense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’; GAPDH, sense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, antisense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’; GAPDH, sense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, antisense 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’, probe 5’-CCATGACTTCGAAAGTTTATGATCCAG-3’.

Each experimental reaction was performed in triplicate. All primer/probes sets had efficiencies of 100% (±10%). To calculate the relative expression of iNOS or AUF1 mRNA the 2^(-ΔΔCt(T)) method (37) was used. The values of untreated cell samples were set 100%, and the percentage of iNOS or AUF1 mRNA expression was calculated.

Analysis of Human iNOS Promoter Activity in Stably Transfected Cells—To investigate the effect of the overexpression of each AUF1 isoform on cytokine-induced iNOS promoter activity, DLD-1–16kb cells (containing a 16-kilobase fragment of each AUF1 isoform on cytokine-induced iNOS promoter activity) were transiently transfected as described above. To induce luciferase expression, cells were incubated with 10 ng/ml doxycycline for 24 h. Then cells were lysed, and firefly and renilla luciferase mRNA expression and activities were determined as described above.

6-Dichloro-1-ribofuranosylbenzimidazole (DRB) Experiments—To examine the influence of the different AUF1 isoforms on iNOS mRNA stability, DLD-1-overexpressing AUF1 cells were incubated as indicated, and iNOS expression was induced by cytokines for 4 h. Then 25 μg/ml DRB (Sigma) was added, and RNAs were prepared 0–4 h thereafter. Relative iNOS and GAPDH mRNA amounts were determined by qRT-PCR, and iNOS mRNA was normalized to GAPDH mRNA. The relative amount of iNOS mRNA at 0 h DRB was set at 100%. Curve fittings of the resulting DRB time curves were performed by nonlinear regression using Graph-Pad Prism 3.0 (GraphPad Software, San Diego, CA).

Immunoprecipitation-qRT-PCR Assay—For determination of intracellular protein-RNA interactions, EGFP-, EGFP-PTB, and EGFP-AUF1 isofrom overexpressing DLD-1 cells were incubated for 4 h with or without the cytokine mixture. Immunoprecipitation was performed with a monoclonal anti-EGFP antibody. All further steps were performed as described before (33).

Purification of Glutathione S-Transferase (GST)-AUF1 Proteins—Procaroyctic expression vectors (pGEX2T-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1) were combined with 0.5 μg of the renilla reporter gene plasmid pEF-1α-renilla (38) for normalization of transfection efficiency. After overnight incubation, cells were stimulated for corresponding time points with or without CM. Then the cells were lysed in 1× Passive Lysis Buffer provided by the Dual Luciferase reporter assay system, and firefly and renilla luciferase activities were determined in 40 and 20 μl of the extracts, respectively. The light units of the firefly luciferase were normalized by those of renilla luciferase after subtraction of extract background.

Western Blot Experiments—To study the protein expression in DLD-1 cells, total cell protein was isolated as described (33). To study the expression of human AUF1, EGFP-AUF1 fusion protein, or iNOS, extracts (10–50 μg protein) were separated on SDS-PAGE and transferred to nitrocellulose membrane by semi-dry electrobolting. All further steps were performed as described (31). For detection of AUF1, EGFP-AUF1 fusion protein or iNOS monoclonal anti-AUF1-, anti-EGFP, or anti-iNOS antibodies were used. The immunoreactive proteins on the blots were visualized by the enhanced chemiluminescence detection system (ECL, PerkinElmer Life Sciences).

Analysis of mRNA Stability Using a Tetracycline-inducible Expression Vector—A pcDNA4/TO-based luciferase expression vector (5’-UTR-Luc) containing the first exon, the first intron, and part of the second exon of the human iNOS gene was generated. Transfection of this clone in cells resulted in the expression of an mRNA containing the 5’-UTR of the human iNOS mRNA fused to the luciferase coding region. Integration of the 3’-UTR sequence of the human iNOS mRNA into this vector (3’ to the luciferase stop codon) resulted in the vector 5’-UTR-Luc-3’-UTR (see supplemental Fig. S2). To study binding properties of the different AUF1 isoforms to the iNOS 3’-UTR, a pcDNA4/TO-based luciferase expression vector with the iNOS 5’-UTR and an iNOS 3’-UTR lacking the first, second, and third AU-rich elements was generated (5’-UTR-Luc-3’-UTR without ARE 1–3). DNA sequences of the clones were determined using the dideoxy chain termination method with a sequencing kit from Amersham Biosciences.

To analyze the effect of the different AUF1 isoforms on the expression of these luciferase reporter mRNAs, DLD-1-TR7 cells were transiently transfected as described above. To induce luciferase expression, cells were incubated with 10 ng/ml doxycycline for 24 h. Then cells were lysed, and firefly and renilla luciferase mRNA expression and activities were determined as described above.

UV Cross-linking Experiments—cDNAs encoding subfragments of the human iNOS 3’-UTR have been described previ-
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FIGURE 1. Overexpression of EGFP-AUF1 fusion proteins in DLD-1 cells. Plasmid constructs (pEGFP-p37AUF1, p40AUF1, p42AUF1, or p45AUF1) allowing high level expression of EGFP-AUF1 fusion proteins were stably transfected into DLD-1 cells (p37, p40, p42, p45). Cells transfected with the pEGFP-C1 vector backbone (C1) were used as control. For analysis of EGFP-AUF1, expression pools of stably transfected cells were preincubated for 18 h in medium without fetal calf serum and phenol red. Then cells were incubated with (CM) or without the cytokine mixture for 6 h, and protein extracts were isolated. Panel A, scheme of the alternative splice products of the AUF1 mRNA coding for the different AUF1 proteins and their RNA binding activities (24). Panel B, analysis of EGFP-p37AUF1, p40AUF1, p42AUF1, or p45AUF1 and β-tubulin protein expression in DLD-1 EGFP-p37AUF1, p40AUF1, p42AUF1, or p45AUF1 cells (p37, p40, p42, p45) or pEGFP-C1 cells (C1) cells by Western blot using monoclonal anti-EGFP- and β-tubulin antibodies and cytoplasmic extracts from stably transfected DLD-1 cells. The position of β-tubulin and EGFP-AUF1 is indicated. This blot is representative of three other blots showing similar results. Panel C, analysis of protein expression of endogenous AUF1 and β-tubulin antibodies and cytoplasmic extracts from stably transfected DLD-1 cells. The position of β-tubulin and the different AUF1 isoforms are indicated. This blot is representative of three other blots showing similar results.

AUF1 Isoform-specific qRT-PCR

DLD-1 cells were transiently transfected with psiRNAhH1-GFPzeo vectors coding for the same shRNAs directed against AUF1-exon 1, AUF1-exon 2, and AUF1-exon 7 as described above. As control, a psiRNAhH1-GFPzeo vector coding for a shRNA targeting GL2-luciferase was used. 48 h after transfection RNA was isolated.

To distinguish the four different AUF1 isoforms by two-step qRT-PCR, two separate reverse transcription reactions were performed. A primer located in AUF1-exon 7 (5′-GTGTCGACGGCAGCTTAGT-GCCCACTGGTGTCGCACTGCCACCCAGGACAGGCACCC-

GAGTGTCG-3′) allows isoform-specific transcription of p42AUF1 and the p45AUF1 cDNA. With a primer targeting a region spanning AUF1-exon 6 to AUF1-exon 8 (5′-CCACCTCGTGTGTCGACCCAGC-3′), only p37AUF1 and p40AUF1 mRNAs were transcribed into cDNA. To analyze the expression of GAPDH, a reverse transcription with a random primer was performed.

In qRT-PCR experiments relative amounts of p37AUF1 and p42AUF1 were detected with a sense primer located in AUF1-exon 1 (5′-GTCCTGAGCCACCCAGG-3′), an antisense primer binding to AUF1-exon 3 (5′-TGATAGGATCTAATCTTACATGGTCACTGG-3′), and a probe corresponding to a sequence in AUF1-exon 3 (5′-AAGATGGACCCAGTTAA-GAAGGAGGAGG-3′). p40AUF1 and p45AUF1 were distinguished in qRT-PCR experiments using a sense primer located in AUF1-exon 1 (5′-GTCCTGAGCCACCCAGG-3′), an antisense primer located in AUF1-exon 2 (5′-CCGCTGTGCTGAGGTTC-3′), and a probe complementary to a region in AUF1-exon 2 (5′-CGAGGGAGGATGAGGCCCATCAA-CTG-3′). The reverse transcription and real-time PCR were performed as described above.
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RESULTS

Overexpression of Each AUF1 Isoform Reduces Cytokine-induced iNOS Expression in DLD-1 Cells—To determine whether AUF1 is involved in the regulation of iNOS expression, we generated DLD-1 cells constitutively expressing each AUF1 isoform as an EGFP-AUF1 fusion protein (p37, p40, p42, and p45). DLD-1 cells stably expressing EGFP (C1) were used as control. All cells were analyzed for EGFP expression by fluorescent activated cell sorting analysis (data not shown), and overexpression of each AUF1 isoform was confirmed in Western blot experiments (Fig. 1B). Overexpression of the different AUF1 isoforms did not modify endogenous AUF1 expression (Fig. 1C).

To induce iNOS expression DLD-1-AUF1 cells were incubated with a cytokine mixture (containing interleukin-1β, human interferon-γ, and tumor necrosis factor-α), and total mRNA and protein were isolated. iNOS-mediated NO production was determined by measuring the nitrite content in the supernatant of the cells. Overexpression of each EGFP-AUF1 fusion protein impaired cytokine-induced iNOS mRNA and protein expression (Fig. 2, A and B) as well as iNOS-mediated NO production (Fig. 2C). However, cytokine incubation did not alter the expression of endogenous AUF1 or EGFP-AUF1 fusion proteins (Figs. 1, B and C). Also, subcellular localization of EGFP-AUF1 fusion protein was not significantly altered upon cytokine stimulation (see supplemental Fig. S1).

Down-regulation of AUF1 Expression Enhances Cytokine-induced iNOS Expression—To confirm these results we tested whether down-regulation of endogenous AUF1 expression using the RNA interference technique resulted in enhanced iNOS expression. Experiments were performed with siRNAs targeting different exons of the AUF1 mRNA. siRNA directed against exon 1 down-regulated expression of all AUF1 isoforms. siRNA against exon 2 reduced expression of p40AUF1 and p45AUF1, whereas siRNA targeting exon 7 selectively decreased p42AUF1 and p45AUF1 expression. DLD-1 cells were transiently transfected with the different AUF1 siRNAs or a luciferase siRNA as control. After transfection cells were incubated in the presence or absence of a cytokine mixture (containing interleukin-1β, human interferon-γ, and tumor necrosis factor-α), and total mRNA and protein were isolated. iNOS-mediated NO production was determined by measuring the nitrite content in the supernatant of the cells. Overexpression of each EGFP-AUF1 fusion protein impaired cytokine-induced iNOS mRNA and protein expression (Fig. 2, A and B) as well as iNOS-mediated NO production (Fig. 2C). However, cytokine incubation did not alter the expression of endogenous AUF1 or EGFP-AUF1 fusion proteins (Figs. 1, B and C). Also, subcellular localization of EGFP-AUF1 fusion protein was not significantly altered upon cytokine stimulation (see supplemental Fig. S1).

FIGURE 2. Overexpression of each EGFP-AUF1 isoform reduces cytokine-induced iNOS mRNA expression and iNOS-dependent NO production. Plasmid constructs (pEGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1) allowing high level expression of EGFP-AUF1 fusion proteins were stably transfected into DLD-1 cells (p37, p40, p42, p45). Cells transfected with the pEGFP-C1 vector backbone (C1) were used as controls. For analysis of iNOS expression pools of stably transfected cells were preincubated for 18 h in medium without fetal calf serum and phenol red. Then cells were incubated with (CM) or without the cytokine mixture for 6 h, and total RNA was isolated. To determine iNOS protein expression and iNOS-mediated NO production, cells were incubated for 9 h with or without CM. Then protein extracts were prepared, and the supernatant of the cells was analyzed for nitrite content. Panel A, a summary of 12 qRT-PCR analyses is shown using RNAs from DLD-1-pEGFP-C1 (C1) or DLD-1-pEGFP p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 cells (p37, p40, p42, p45). Data (means ± S.E.) represent relative iNOS mRNA levels (***, p < 0.001 versus CM-treated pEGFP-C1 cells). Panel B, NO and β-tubulin protein expression in DLD-1-pEGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 cells (p37, p40, p42, p45) or DLD-1-pEGFP-C1 (C1) cells were analyzed in Western blot experiments using monoclonal anti-iNOS- and β-tubulin antibodies and cytoplasmic extracts from stably transfected DLD-1 cells. The position of β-tubulin and iNOS is indicated. This blot is representative of three other blots showing similar results. Panel C, a summary of 5 nitrite analyses using supernatants from DLD-1-pEGFP-C1 (C1), DLD-1-EGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 cells (p37, p40, p42, p45) is shown. The nitrite concentration in the supernatant of untreated DLD-1 cells was 0.57 ± 0.06 μM. After cytokine induction the nitrite concentration increased to 12.32 ± 0.15 μM. Data (means ± S.E.) represent relative nitrite levels (*, p < 0.05; **, p < 0.01; ***, p < 0.001 versus CM-treated pEGFP-C1 cells).

Statistics—Data represent the means ± S.E. Statistical differences were determined by factorial analysis of variance followed by Fisher’s protected least-significant-difference test for comparison of multiple means.

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directed against exon 1, 2, and 7 (Fig. 3C). Analysis of iNOS mRNA and protein expression in DLD-1 cells transfected with the different AUF1 siRNAs showed that down-regulation of each AUF1 isoform enhances cytokine-induced iNOS expression (Fig. 3, D and E). In summary, these data indicate that all AUF1 isoforms decrease human iNOS expression.
Activity—Besides its post-transcriptional effects, AUF1 has the AUF1-exon 1, AUF1-exon 2, or AUF-exon 7 siRNA by Western blot using monoclonal anti-AUF1- and

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FIGURE 3.

Down-regulation of AUF1 expression by siRNA enhances cytokine-induced iNOS mRNA and protein expression. FIGURE 4.

Effect of AUF1 expression on human iNOS promoter activity. DLD-1–16kb cells (DLD-1 cells stably transfected with a luciferase reporter gene under the control of the 16-kilobase human iNOS promoter) were transfected with plasmids coding for the different EGFP-AUF1 fusion proteins (p37, p40, p42, p45) or the pEGFP-C1 plasmid (C1) as control. After transfection, cells were incubated with (CO) or without a mixture of cytokines (CM) for 6 h. Columns (mean ± S.E.) represent the cytokine-induced activation of the iNOS promoter expressed as a percentage of the corresponding basal values determined in the absence of cytokines (ns, not significant versus CM induced pEGFP-C1 control cells).

None of the AUF1 Isoforms Changes Human iNOS Promoter Activity—Besides its post-transcriptional effects, AUF1 has been described to regulate the promoter activity of genes like α-fetoprotein or enkephalin by binding to AT-rich DNA sequences (28, 29, 40). Therefore, we investigated if the AUF1 isoforms mediate their effect on iNOS expression by modulation of human iNOS promoter activity. We used DLD-1 cells stably transfected with a construct containing a 16-kilobase human iNOS promoter fragment cloned in front of a luciferase reporter gene (DLD-1–16kb cells) (32). These cells were transiently transfected with plasmids coding for the different EGFP-AUF1 fusion proteins and a renilla luciferase plasmid (pEF-1α-renilla) (38) for normalization of transfection efficiency. After transfection the cells were incubated for 6 h with or without cytokines, and luciferase activities were measured in the extracts. These data demonstrate that cytokine incubation induces iNOS promoter activity in all cells to a similar extent (Fig. 4). Therefore, none of the AUF1 isoforms is involved in the regulation of human iNOS promoter activity. Therefore, it is very likely that each AUF1 isoform reduces iNOS expression by decreasing iNOS mRNA stability.

Overexpression of Each AUF1 Isoform Modulates Human iNOS mRNA Stability—To test if the reducing effect of AUF1 on iNOS expression resulted from AUF1-mediated changes in the stability of the human iNOS mRNA, we performed experiments using DRB to block the RNA polymerase II-dependent transcription. DLD-1–EGFP-C1 (C1) or DLD-1 EGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 cells (p37, p40, p42, p45) were incubated with a cytokine mixture for 4 h. Then 25 μg/ml DRB was added, and RNAs were prepared after 0–4 h. iNOS mRNA and GAPDH mRNA concentrations were determined by qRT-PCR, and iNOS mRNA was normalized to GAPDH mRNA. A summary of seven qRT-PCR analyses is shown. Data (means ± S.E.) represent relative iNOS mRNA levels (*, p < 0.05; versus 0-h DRB). Curve-fitting was performed using GraphPad Prism for Macintosh.

AUF1 Regulates Human iNOS Expression

FIGURE 5.

Effect of AUF1 expression on human iNOS mRNA stability. DLD-1–EGFP-C1 control cells (C1) or DLD-1–EGFP-p37AUF1, -p40AUF1, -p42AUF1, or -p45AUF1 cells (p37, p40, p42, p45) were incubated with a cytokine mixture for 4 h. Then 25 μg/ml DRB was added, and RNAs were prepared after 0–4 h. The iNOS mRNA and GAPDH mRNA concentrations were determined by qRT-PCR, and iNOS mRNA was normalized to GAPDH mRNA. A summary of seven qRT-PCR analyses is shown. Data (means ± S.E.) represent relative iNOS mRNA levels (*, p < 0.05; versus 0-h DRB). Curve-fitting was performed using GraphPad Prism for Macintosh.
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Figure 6. Overexpression of each AUF1 isoform reduces the stability of an iNOS-5’-UTR-luciferase-iNOS-3’-UTR mRNA in human DLD-1 cells. DLD-1-TR7 cells (expressing a tetracycline repressor) were transfected with pcDNA4/TO-5’-UTR-Luc (5’-UTR-Luc) or pcDNA4/TO-5’-UTR-luc-3’-UTR (5’-UTR-Luc-3’-UTR) separately or together with pEGFP-C1 (C1) or the different pEGFP-AUF1 isoform containing plasmids (p37, p40, p42, p45). The renilla luciferase expression plasmid pEF-1α-renilla was cotransfected for normalization. After transfection cells were treated without (Co) or with 10 ng/ml Dox to induce firefly luciferase expression. After 24 h cells were lysed, and either firefly luciferase- and renilla luciferase mRNA expression or activities were determined. Panel A, columns (mean ± S.E.) represent the relative doxycycline-induced firefly luciferase mRNA expression as a percentage of the Dox-induced luciferase mRNA in cells transfected with pcDNA4/TO-5’-UTR-Luc or pcDNA4/TO-5’-UTR-luc-3’-UTR (*, p < 0.05 versus Dox-induced pcDNA4/TO-5’-UTR-Luc and pcDNA4/TO-5’-UTR-luc-3’-UTR-transfected cells). Panel B, columns (mean ± S.E.) represent the relative doxycycline-induced renilla luciferase activities expressed as a percentage of the Dox-induced renilla luciferase activities in cells transfected with pcDNA4/TO-5’-UTR-Luc and pcDNA4/TO-5’-UTR-luc-3’-UTR-transfected cells). Panel C, columns (mean ± S.E.) represent the relative doxycycline-induced firefly luciferase mRNA expression as a percentage of the Dox-induced luciferase activity in cells transfected with pcDNA4/TO-5’-UTR-Luc or pcDNA4/TO-5’-UTR-luc-3’-UTR and pEGFP-C1 (*, p < 0.05; **, p < 0.01 versus Dox-induced pEGFP-C1 and pcDNA4/TO-5’-UTR-luc-3’-UTR-transfected cells). Panel D, columns (mean ± S.E.) represent the relative doxycycline-induced luciferase activities expressed as a percentage of the Dox-induced normalized luciferase activities in cells transfected with pcDNA4/TO-5’-UTR-Luc-3’-UTR and pEGFP-C1 (***, p < 0.001 versus Dox-induced pEGFP-C1 and pcDNA4/TO-5’-UTR-Luc-3’-UTR-transfected cells).

sequence generating pcDNA/TO-5’-UTR-Luc-3’-UTR (5’-UTR-Luc-3’-UTR). These luciferase expression vectors together with plasmids coding for EGFP or EGFP-AUF1 isoforms and pEF-1α-renilla (renilla luciferase expression vector, normalization of transfection efficiency) were transiently transfected into the DLD-1-TR7 cells. After 24 h of incubation with doxycycline (Dox) to induce luciferase expression, cells were lysed, and relative luciferase activities (normalized to renilla luciferase activity) and expression of luciferase mRNA (normalized to renilla mRNA) were determined. As shown in Fig. 6, A and B, Dox incubation resulted in a marked enhancement of firefly luciferase mRNA and activity in cells transfected with the pcDNA/TO-5’-UTR-luc plasmid. The presence of the 3’-UTR of the human iNOS mRNA 3’ to the firefly luciferase stop codon markedly reduced luciferase expression, demonstrating the destabilizing effect of this sequence (comparison of 5’-UTR-Luc with 5’-UTR-Luc-3’-UTR-transfected cells). Overexpression of each AUF1 isoform in this system resulted in further reduction of firefly luciferase mRNA and activity in cells transfected with pcDNA4/TO-5’-UTR-Luc-3’-UTR (Fig. 6, C and D). Therefore, the effect of each AUF1 isoform on the human iNOS mRNA is likely to result from the binding of the isoforms to the human iNOS 3’-UTR sequence.

All AUF1 Isoforms Bind to the 3’-UTR of the Human iNOS mRNA—The ARE sequences in the human iNOS 3’-UTR are putative AUF1 binding sites. To examine the definite binding site of each AUF1 isoform to the human iNOS 3’-UTR, recombinant GST-AUF1 fusion proteins were incubated with 32P-labeled transcripts comprising different nucleotide regions of the iNOS 3’-UTR (see Fig. 7A). Complex formation was assayed by UV cross-linking experiments. Each recombinant GST-AUF1 isoform protein interacted with the whole 3’-UTR transcript. This binding activity was not observed with the GST protein (Fig. 7B). To localize the binding sites of the different AUF1 isoforms within the iNOS 3’-UTR, the region was dissected into a non-AU fragment without ARE sequences and an AU fragment containing all five AU repeats. Complex formation only appeared between the AU subfragment and all GST-AUF1 proteins (Fig. 6, panel B). Subsequently, the AU fragment was dissected into three subfragments: subfragment A (323–329), subfragment B (327–428, three AREs), and subfragment C (387–477, two AREs). As shown in Fig. 7C, all GST-AUF1 proteins displayed a marked binding activity to fragment B and a minor binding activity to fragment C. Mutation of the three AU-repeats in subfragment B resulted in the abolishment of the binding of all GST-AUF1 proteins to the radiolabeled B mutant RNA (Fig. 7C, second panel from right). These results indicate that all AUF1 isoforms bind to the AREs located in fragment B of the human iNOS mRNA 3’-UTR (see Fig. 7A).

The in vivo relevance of AUF1 binding to the AU-rich sequences located in fragment B (see Fig. 7A) was confirmed in the TetON system. Therefore, a pcDNA4/TO-based luciferase expression vector with the iNOS 5’-UTR and an iNOS 3’-UTR lacking the first, the second, and the third AU-rich element (5’-UTR-Luc-3’-UTR without ARE 1–3) was generated. DLD1-TR7 cells were transiently transfected with the 5’-UTR-Luc-3’-UTR without ARE 1–3 vector or the 5’-UTR-Luc-3’-UTR plasmid (as a control) as described above.

As shown in Fig. 7D, the absence of the three AU-rich elements enhanced doxycycline-induced firefly luciferase activity (comparison of 5’-UTR-Luc-3’-UTR with 5’-UTR-Luc-3’-
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Regulation of mRNA stability is an important mechanism to control gene expression. The mRNAs of many genes whose expression has to be controlled precisely (e.g. cytokines, proto-oncogenes) contain AREs in their 3'-untranslated regions (9, 41, 42). These cis-acting elements are targets for different RNA-binding proteins (RNA-BP), which regulate mRNA degradation.

Therefore, these experiments clearly demonstrate that all AUF1 isoforms mediate their destabilizing effect on iNOS expression by binding to ARE 1–3 in the iNOS 3’-UTR sequence.

Cytokine Incubation Reduces Intracellular Binding of AUF1 to the Human iNOS mRNA—To elucidate the mechanism of AUF1-mediated destabilization of iNOS mRNA, we studied whether AUF1 binds to the human iNOS mRNA in intact cells and whether CM incubation modifies this interaction. For this reason we performed immunoprecipitation qRT-PCR analysis. Equal amounts of EGFP-AUF1-overexpressing cells were immunoprecipitated with a specific GFP antibody. The method was validated by immunoprecipitation qRT-PCR experiments using DLD-1 EGFP-PTB cells and the GFP antibody. As described for endogenous PTB (34), upon cytokine stimulation, also a nearly 5-fold enhanced intracellular binding of the EGFP-PTB fusion protein to the human iNOS mRNA was detected. In contrast, the intracellular binding of EGFP-AUF1 fusion proteins to the iNOS mRNA was significantly reduced after cytokine induction (Fig. 8). Because AUF1 destabilizes iNOS mRNA, these changes in RNA binding contribute to the stabilization of iNOS mRNA after cytokine induction.

DISCUSSION

The aim of the present study was to investigate the role of the RNA-BP AUF1 on human iNOS expression. AUF1 is a protein

FIGURE 7. Analysis of the AUF1 binding site in the human iNOS 3’-UTR RNA. Purified (GST) or GST-AUF1 fusion proteins were incubated with different radiolabeled RNAs generated by in vitro transcription using the different iNOS 3’-UTR fragments shown in panel A. After binding, proteins were UV-cross-linked to the RNA, and the complexes were digested with RNase. RNA-protein complexes were separated on SDS-polyacrylamide gels. DLD-1-TR7 cells (expressing a tetracycline repressor) were transfected with pCDNA4/TO-5’-UTR-Luc-3’-UTR-Luc-3’-UTR-Luc-3’-UTR without ARE 1–3 (***, ***, p < 0.001 versus Dox-induced pcDNA4/TO-5’-UTR-Luc-3’-UTR without ARE 1–3 transfected cells). Co, no doxycycline treatment. Panel D, depletion of ARE 1–3 abolishes destabilizing function of all AUF1 isoforms on the expression of an iNOS-luciferase-reporter construct. Columns (mean ± S.E.) represent the relative doxycycline-induced luciferase activities expressed as a percentage of the Dox-induced normalized luciferase activity in cells transfected with pcDNA4/TO-5’-UTR-Luc-3’-UTR and pcDNA4/TO-5’-UTR-Luc-3’-UTR without ARE 1–3 transfected cells). Co, no doxycycline treatment. Panel E, depletion of ARE 1–3 abolishes destabilizing function of all isoforms (pc37, p40, p42, p45). The renilla luciferase expression plasmid pEF-1α-renilla was cotransfected for normalization. After transfection cells were treated with 10 ng/ml Dox to induce firefly luciferase expression. After 24 h cells were lysed, and either firefly luciferase or renilla luciferase activities were determined. Panel A, structure of the human iNOS 3’-UTR mRNA and fragments used in RNA binding studies. Shown is a scheme of the human iNOS 3’-UTR (477 nucleotides) and the transcripts used in RNA binding studies. AU indicates the positions of the AUUUA and AUUUA repeats. The sequences of the wild-type and mutated subfragment B are shown. Panel B, all AUF1 isoforms bind to the AU fragment of the human iNOS 3’-UTR [32P]-radiolabeled RNA transcripts (3’-UTR, non-AU, AU; see panel A) were incubated with GST or the GST-AUF1 fusion proteins. The positions of RNA/AUF1 complexes are indicated. Panel C, all AUF1 isoforms bind to the fragments B and C of the human iNOS 3’-UTR [32P]-radiolabeled RNA transcripts (A, B, B mut, and C; see panel A) were incubated with GST or the GST-AUF1 fusion proteins. The positions of RNA/AUF1 complexes are indicated. Panel D, depletion of ARE 1–3 enhances the expression of an iNOS-luciferase-reporter construct. Columns (mean ± S.E.) represent the relative doxycycline-induced firefly luciferase activities expressed as a percentage of the Dox-induced normalized luciferase activity in cells transfected with pcDNA4/TO-5’-UTR-Luc-3’-UTR or pcDNA4/TO-5’-UTR-Luc-3’-UTR without ARE 1–3 (**, p < 0.001 versus Dox-induced pcDNA4/TO-5’-UTR-Luc-3’-UTR without ARE 1–3 transfected cells). Co, no doxycycline treatment. Panel E, depletion of ARE 1–3 abolishes destabilizing function of all AUF1 isoforms (ns, not significant versus Dox-induced pEGFP-C1 isoforms containing plasmids).
family of ARE binding factors consisting of four different isoforms generated by alternative splicing of a common transcript. In the literature conflicting results exist concerning the role of AUF1 as stabilizer or destabilizer of ARE-containing mRNAs (22, 23, 26, 43, 44). Also, opposite effects of the individual AUF1 isoforms on mRNA decay have been reported (25, 27). To analyze the effect of AUF1 on human iNOS expression in intact cells, we established four different DLD-1 cell lines constitutively expressing each AUF1 isoform as an EGFP fusion protein (Fig. 1B). Western blot experiments showed that overexpression of the different AUF1 isoforms did not affect endogenous AUF1 expression (see Fig. 1C). Therefore, the different AUF1 isoforms do not seem to interfere with the expression of the other isoforms. Incubation of the AUF1 isoform-overexpressing cells with a cytokine mixture needed to induce iNOS expression did not affect AUF1 protein expression; that is, neither the EGFP fusion proteins (Fig. 1B) nor endogenous AUF1 (Fig. 1C). Cytokine stimulation only slightly affected the intracellular localization of AUF1 as shown by fluorescent microscopy (see supplemental Fig. S1). Analysis of iNOS expression in these cells clearly demonstrated that all AUF1 isoforms reduce iNOS mRNA and iNOS protein levels as well as iNOS-mediated NO production to a similar extent (Fig. 2). Results using a RNA interference approach confirmed the negative regulatory effect of the AUF1 isoforms on iNOS mRNA and protein expression (Fig. 3). In addition to its role in mRNA decay AUF1 seems not to be an appropriate model to study general post-transcriptional mechanisms regulating also human iNOS expression.

In which way AUF1 decreases iNOS mRNA expression is still not clear. It seems not to be a direct effect, because AUF1 proteins lack decay-promoting activities (20). As demonstrated for other RNA-BPs involved in the post-transcriptional regulation of human iNOS expression, the endogenous AUF1 expression in DLD-1 cells is not affected by cytokines used to induce iNOS expression (Fig. 1C). Consequently, an altered AUF1 expression is not responsible for a cytokine-mediated increase of iNOS mRNA expression. It is known that the ARE-mRNA-stabilizing RNA-BP HuR translates from the nucleus to the cytoplasm to exert its stabilizing effect (49). In contrast in DLD-1 cells we have no clear evidence that cytokine incubation changes subcellular localization of AUF1 (supplemental Fig. 1).

Our data display clear evidence that the binding of AUF1 to the human iNOS mRNA is reduced upon cytokine stimulation. We demonstrated that the whole AUF1 protein family decreases iNOS expression by destabilization of the iNOS mRNA (Fig. 5). No differences between the individual isoforms could be detected, although they have different RNA binding affinities. This could be explained by the different expression levels of the isoforms; p37AUF1 and p42AUF1 isoforms have a high RNA binding affinity but are expressed in relatively low levels, whereas the most abundant p40AUF1 and p45AUF1 isoforms possess a low RNA binding affinity (24).

In accordance with our data that AUF1 mediates iNOS mRNA decay, we could demonstrate in intact cells that all AUF1 isoforms are able to decrease expression of a luciferase reporter mRNA containing the human iNOS 3'-UTR (Fig. 6, C and D). We characterized AUF1 binding sites in an AU-rich region of the iNOS 3'-UTR sequence and examines their biological relevance (Fig. 7). All data presented support our theory that all AUF1 isoforms reduce human iNOS expression by modulation of mRNA stability. We were not able to detect any stabilizing effect of AUF1 or any different effects of the individual isoforms. Data derived from an AUF1 knock-out mouse model support our findings that the destabilizing properties of AUF1 are very important in regulating the expression of proinflammatory cytokines. In these AUF1−/− animals the tumor necrosis factor-α and interleukin-1β levels are dramatically elevated after endotoxin challenge as a result of an impaired degradation of these mRNAs (46). The AUF1 knock-out mice also develop chronic inflammatory skin disease demonstrating the ability of AUF1 to modulate immune responses by regulation of mRNA stability of proinflammatory molecules (47).

Regulation of iNOS mRNA expression by AUF1 varies between mice and human. Lipopolysaccharide-stimulated iNOS mRNA expression in macrophages isolated from AUF1−/− mice was not significantly different compared with wild type mice macrophages (data not shown). This indicates that AUF1 does not modulate iNOS mRNA stability in mice. In human cells the RNA-BP PTB enhances iNOS mRNA stability (34), but in mice the same protein has been described to destabilize the iNOS mRNA by binding to the 3'-UTR (48). Overall, these data show that macrophages of AUF1-knock-out mice seem not to be an appropriate model to study general post-transcriptional mechanisms regulating also human iNOS expression.
This contributes to the increased iNOS expression under these conditions. It is very likely that post-translational modifications like phosphorylation of tyrosine, serine, or threonine residues might play a role in the cytokine-dependent alterations of AUF1 binding affinities. This idea is supported by data demonstrating that tyrosine phosphorylation of AUF1 may reduces binding of the protein to the sphingosine kinase 1 mRNA (50). Moreover, it has been described that phosphorylation of the p40AUF1 isoform changes the RNA binding affinity of the protein (51, 52).

Some publications demonstrate that AUF1 mediates mRNA degradation by recruiting the exosome to ARE-containing mRNAs (53, 54). Hence, we performed communoprecipitation experiments in DLD-1 cells to test whether AUF1 interacts with exosomal components. However, Western blot analyses provide no evidence for an AUF1 protein-protein interaction with a subunit of the exosome (data not shown). Although Chen et al. (18) detected an interaction between AUF1 and the exosome in in vitro experiments, recombinant AUF1 protein failed to promote exosome-mediated ARE-RNA decay. So it seems very unlikely that AUF1 contributes to iNOS mRNA degradation via this pathway in DLD-1 cells.

Another hypothesis claims that AUF1 facilitates mRNA degradation by a direct interaction with the poly(A)-binding protein. Both proteins build a stable complex thereby poly(A)-binding protein is removed from the poly(A) tail and, thus, promotes poly(A) tail shortening and mRNA decay (46, 55). In DLD-1 cells we tested this hypothesis by co-immunoprecipitation experiments. However, we detected no direct protein-protein interaction between AUF1 and poly(A)-binding protein (data not shown). Therefore, in DLD-1 cells another pathway is responsible for AUF1-mediated iNOS mRNA degradation. Further experiments will show if AUF1 mediates mRNA decay by recruiting components of the 5’ to 3’ degradation machinery to the mRNA.

Today it is established that stabilizing and destabilizing RNA-BPs, especially HuR and AUF1, can compete for binding to the same ARE or specific target mRNAs and thereby determine the rate of mRNA decay (50, 53). For human iNOS expression we could demonstrate that HuR competes with the destabilizing RNA-BP protein KSRP for the same ARE binding site. After cytokine stimulation, HuR replaces KSRP, and this contributes to iNOS mRNA stabilization (33). Thus, it could be possible that other iNOS mRNA-stabilizing proteins like HuR or PTB compete with AUF1 for binding to the iNOS 3’-UTR sequence after cytokine stimulation. So far, UV cross-linking experiments provide no evidence that support this hypothesis (data not shown). This may be explained by the fact that AUF1 binds to a distinct sequence in the iNOS 3’-UTR than HuR or PTB. It is conceivable that AUF1 competes with another yet unidentified stabilizing RNA-BP that binds to the same sequence.

In summary, the data presented in this paper indicate that all AUF1 isoforms reduce iNOS mRNA stability to a similar extent by binding to AU-rich sequences in the iNOS 3’-UTR. In the complex RNA-binding protein network regulating iNOS mRNA stability, AUF1 is a second important factor responsible for mRNA degradation in the absence of cytokines.

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AUF1 Regulates Human iNOS Expression

Similar Regulation of Human Inducible Nitric-oxide Synthase Expression by Different Isoforms of the RNA-binding Protein AUF1
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