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ATP Potentiates Interleukin-1β-induced MMP-9 Expression in Mesangial Cells via Recruitment of the ELAV Protein HuR*

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Renal mesangial cells express high levels of matrix metalloproteinase 9 (MMP-9) in response to inflammatory cytokines such as interleukin (IL)-1β. We demonstrate here that the stable ATP analog adenosine 5′-*O*-(thiotriphosphate) (ATPγS) potently amplifies the cytokine-induced gelatinolytic content of mesangial cells mainly by an increase in the MMP-9 steady-state mRNA level. A Luciferase reporter gene containing 1.3 kb of the MMP-9 5′-promoter region showed weak responses to ATPγS but confered a strong ATP-dependent increase in Luciferase activity when under the additional control of the 3′-untranslated region of MMP-9. By *in vitro* degradation assay and actinomycin D experiments we found that ATPγS potently delayed the decay of MMP-9 mRNA. Gel-shift and supershift assays demonstrated that three AU-rich elements (AREs) present in the 3′-untranslated region of MMP-9 are constitutively bound by complexes containing the mRNA stabilizing factor HuR. The RNA binding of these complexes was markedly increased by ATPγS. Mutation of each ARE element strongly impaired the RNA binding of the HuR containing complexes. Reporter gene assays revealed that mutation of one ARE did not affect the stimulatory effects by ATPγS, but mutation of all three ARE motifs caused a loss of ATP-dependent increase in luciferase activity without affecting IL-1β-inducibility. By confocal microscopy we demonstrate that ATPγS increased the nucleo cytoplasmic shuttling of HuR and caused an increase in the cytosolic HuR level as shown by cell fractionation experiments. Together, our results indicate that the amplification of MMP-9 expression by extracellular ATP is triggered through mechanisms that likely involve a HuR-dependent rise in MMP-9 mRNA stability.

The matrix metalloproteinases (MMPs)[1] are members of a family of zinc-dependent endopeptidases which specifically degrade components of the extracellular matrix (ECM). Therefore, MMPs have mainly been implicated in various diseases accompanied with an altered turnover of ECM. Besides the altered synthesis of single ECM components the increased expression and/or activity of MMPs seems of paramount importance for pathological remodeling processes within the kidney such as acute proliferative glomerulonephritis (1, 2). Mainly the altered expression of MMP-2 and MMP-9, which are also denoted as gelatinases, is crucially involved in the progression of glomerular inflammatory processes (2, 3). In addition to various inflammatory cytokines, the expression of MMP-9 can be activated by many other stimuli such as mitogens, growth factors, and activators of the Ras oncogene (for review, see Refs. 4 and 5). Although most of these stimuli can modulate gelatinolytic activity by influencing MMP-9 gene expression, the regulation of MMP-9 activity is also achieved by the processing of the inactive proenzyme by the action of other proteases and by an inhibition of the active enzyme by its endogenous inhibitors, the tissue inhibitors of MMPs (4, 5). Previously, we have demonstrated an additional mode of posttranscriptional regulation of MMP-9, which involves the reduction of cytokine-induced MMP-9 via reduction of mRNA stability exerted by exogenous and endogenouly produced NO (6). A variety of inducible genes including proto-oncogenes, transcription factors, cell cycle-regulating proteins, and cytokines have been demonstrated to be regulated by a modulation of mRNA turnover. Recent evidence has revealed that AU-rich sequences also denoted as AU-rich elements (AREs) located in the 3′-untranslated regions (UTRs) of these genes comprise specific cis-regulating elements which target mRNAs for rapid degradation (for review, see Refs. 7–10). Several studies have identified proteins specifically binding to AREs, among them RNA stabilizing factors of the embryonic lethal abnormal vision (ELAV) protein family especially the ELAV-like protein HuR (9–11). Overexpression and subsequent binding of HuR was shown to result in an efficient stabilization of ARE-containing mRNAs *in vivo* (9).

Besides the proinflammatory cytokines, MC are able to respond to a variety of other biological mediators including eicosanoids, growth factors, reactive oxygen species, NO, and to extracellular nucleotides such as ATP and UTP (12, 13). Previously, we have reported that in renal mesangial cells, extracellular ATP via the $P2Y_2$ receptor can cause a mobilization of intracellular calcium and subsequent activation of protein kinases C (14, 15). The cellular long term responses toward ATP and UTP include a variety of pathophysiological key functions

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[1] The abbreviations used are: MMP, matrix metalloproteinase; ARE, AU-rich element; ELAV, embryonic lethal abnormal vision; ECM, extracellular matrix; UTR, untranslated region; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MC, mesangial cells; EMSA, electromobility shift assay; ATPγS, adenosine 5′-*O*-(thiotriphosphate); PBS, phosphate-buffered saline; RLU, relative light unit; PKC, protein kinase C; MAPK, mitogen-activated protein kinase.

most importantly the inhibition of programmed cell death and an increase in cell proliferation (16–18). Both processes, cell proliferation and the excessive ECM degradation, are hallmarks of many chronic progressive glomerular diseases (1–3, 19). For this reason we tested whether extracellular nucleotides can influence the expression of MMP-9 in MC. Our data provide the first report that ATP and UTP can potentiate the cytokine-induced expression and activity of MMP-9. Furthermore, we implicate modulation of the nuclear-cytosolic shuttling of the RNA stabilizing factor HuR in the posttranscriptional regulation of MMP-9 by extracellular nucleotides.

EXPERIMENTAL PROCEDURES

Reagents—Human recombinant IL-1β was from Cell Concept (Umkirch, Germany). All nucleotides were obtained from Sigma (Deisenhofen, Germany). Actinomycin D (from *Streptomyces* species) was purchased from Alexis Biochemicals (Laeufelfingen, Switzerland). Ribonucleotides, restriction enzymes, and modifying enzymes were purchased from Roche Diagnostics GmbH (Mannheim, Germany). RNA oligonucleotides were synthesized from Whatman-Biometra (Göttingen, Germany).

Cell Culture—Rat glomerular MC were characterized as described previously (20) and grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 5 ng/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Serum-free preincubations were performed in Dulbecco's modified Eagle's medium supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin for 24 h before cytokine treatment. For experiments MC between passages 8 and 19 were used. All cell culture media and supplements were purchased from Invitrogen (Karlsruhe, Germany). A monoclonal anti-HuR and the anti-mouse horseradish peroxidase-linked antibodies were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

cDNA Clones and Plasmids—cDNA inserts for rat MMP-9 was generated as described recently (21). A cDNA insert from mouse 18 S rRNA was from Ambion (Austin, TX). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA clone from rats was generated as described previously (25).

Generation of Reporter Plasmids and Transient Transfection of MC—The MMP-9 reporter gene pGL3-MMP-9(1.3kb) encompassing a 1.3-kb fragment of the 5′-flanking region of the rat MMP-9 gene was cloned as described previously (22). The plasmid 3′-UTR-MMP-9 pGL3-MMP-9(1.3kb) was generated by cloning of a 662-bp fragment from the 3′-UTR of rat MMP-9 mRNA into the pGL3-MMP-9(1.3kb) promoter plasmid. The 3′-UTR sequence of the rat MMP-9 gene was generated by PCR from reverse transcriptase products using the XbaI-flanked (underlined) forward primer 5′-TATTCTAGACCAACCTTTACCAGCTACTCGAA-3′ and BamHI-flanked (underlined) reverse primer 5′-TATGGATCCATTCATTTATTTAAAAAAGAGTGT-3, corresponding to a region from nucleotides 2315–2338 and 2954–2977 of the rat MMP-9 cDNA (GenBank™ accession number U24441). The PCR products subsequently were digested with XbaI and BamHI restriction enzymes and cloned into the BamHI/XbaI cut pGL3-MMP-9(1.3kb) plasmid, thereby allowing a forced insertion of the 3′-UTR of rat MMP-9 mRNA at the 3′-end of the *luc*+ coding region of pGL3-MMP-9(1.3kb). Introduction of triple-point mutation into ARE sites (ATTTA to ACCCA) to generate ΔARE mutations were performed using the following (sense) primer: 5′-TACCGGCCCTTTTACCCATTATGTATGTGG-3′ (corresponding to a region from 2494 to 2523) to generate "3′-UTR-ΔARE1-pGL3-MMP-9(1.3 kb)" 5′-TTCACACACATGTACCCAACCTATAGAATG-3′ (corresponding to a region from 2529 to 2558) to generate "3′-UTR-ΔARE2-pGL3-MMP-9(1.3kb)" 5′-TTAGGGACAGAGGAACCCATTGGATGTTGG-3′ (corresponding to a region from 2735 to 2764) to generate "3′-UTR-ΔARE4-pGL3-MMP-9(1.3kb)." All mutants were generated by use of the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla CA). The plasmid "3′-UTR-ΔARE1-2-4-pGL3-MMP-9(1.3kb)" in which all three AREs have been mutated was generated by generation of a construct bearing double-mutated AREs ("3′-UTR-ΔARE1-2-pGL3-MMP-9(1.3kb)"), which then served as a template.

Transient transfections of MC were performed using the Effectene reagent (Quiagen, Hilden, Germany). Transfections were performed following the manufactorer's instructions. The transfections were done as triplicates and repeated at least three times to ensure reproducibility of the results. Transfection with pRL-CMV coding for *Renilla* luciferase was used to control for transfection efficiencies. Luciferase activities were measured with the dual reporter gene system (Promega) using an automated chemoluminescence detector (Berthold, Bad Wildbad, Germany).

Northern Blot Analysis—Total cellular RNA was extracted from MC using the Tri reagent (Sigma), and RNA was hybridized following standard procedures as described previously (21).

Preparation of Cytoplasmic Extracts—Cytoplasmic lysates were separated as described previously (22). Briefly, cytoplasmic lysates were prepared from rat MC by lysis in a hypotone extraction buffer containing 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μg/ml phenylmethanesulfonyl fluoride. Nuclei were removed by centrifugation (1200 × *g* for 15 min) and the supernatants used as cytoplasmic fractions.

RNA Electromobility Shift Assay (EMSA) and Supershift Analysis—Radiolabeled single-stranded RNA oligonucleotides were prepared by kinase reaction using T4 polynucleotide kinase. The labeled oligonucleotides were subsequently separated by Nick-Sephadex columns (Amersham Biosciences, Freiburg, Germany). The sequences for gene specific oligonucleotides are summerized in Table I. Approximately 30 fmol of the radiolabeled RNA oligonucleotide (~30,000 cpm/reaction) were incubated with 6 μg of cytoplasmic extract and incubated at room temperature for 15 min in a buffer containing 10 mM Hepes, pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 2 mM dithiothreitol, 5% glycerol, and 0.5% Nonidet P-40. To reduce nonspecific binding total yeast RNA (200 ng/ml final concentration) was added. The total volume of each reaction was 10 μl. RNA-protein complexes were separated in 6% nondenaturating polyacrylamide gels and run in Tris borate EDTA.

Supershift analysis was done by addition of 200 ng of a monoclonal anti-HuR-specific antibody (Santa Cruz Biotechnology) 15 min after the addition of the radioactive labeled RNA oligonucleotide and incubated for a further 15 min at room temperature.

In Vitro Degradation Assay—Degradation assays were performed as described previously with the following modifications (23). Instead of using *in vitro* transcribed radioactively labeled MMP-9 mRNA, we used preparations from cytokine-induced total RNAs, thereby yielding a high amount of endogenous MMP-9 mRNA. 20 μg of total RNA from one pool were aliquoted and subsequently incubated at room temperature with 130 μg of cytoplasmic extract derived from untreated or alternatively from ATPγS-treated MC for different time points. RNA from these incubations was isolated by standard procedures and assessed by Northern blot analysis using a ^{32}P-labeled cDNA insert specific for rat MMP-9 (21). The remaining (undegraded) MMP-9 hybridized signals were analyzed and quantified on an imaging system from Fujifilm (Raytest, Struabenhardt, Germany).

HuR Neutralization Experiments—The impact of HuR on the degradation of MMP-9 mRNA was tested by the addition of a neutralizing monoclonal anti-HuR antibody (Santa Cruz Biotechnology). 0.4 μg of the antibody was added to the cytoplasmic extracts from ATPγS-treated MC and preincubated at room temperature for 1 h before addition of the RNA samples. To exclude any unspecific inhibitory effects by the mouse serum, we tested in parallel normal mouse serum.

Western Blot Analysis—The total cellular level of HuR protein was analyzed by Western blot analysis using total cellular extracts (50 μg) using a monoclonal antibody specifically raised against full-length human HuR (Santa Cruz Biotechnology). A polyclonal antibody specific for human α-actin was purchased from (Chemicon, Hofheim, Germany). The secondary antibodies conjugated to horseradish peroxidase were incubated for 45 min at room temperature and blots were developed using the ECL system (Amersham Biosciences).

Immunocytochemistry of HuR—At ~60–80% confluence, mesangial cells were rendered serum-free for 24 h and thereafter stimulated with nucleotides for the indicated time periods. Subsequently, cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated for 30min at −20 °C with methanol containing 0.02% (w/v) EDTA. Cells were then washed twice with PBS, blocked for 1h in PBS containing 3% (w/v) bovine serum albumin and incubated for 2 h with monoclonal anti-HuR antibody. Cells were then washed several times with PBS and incubated for 1 h with an anti-mouse-Alexa 488-coupled secondary antibody and thereafter washed again with PBS. Cellular fluorescence was monitored using confocal microscopy (MicroRadiation, Bio-Rad, Hertfordshire, UK).

Statistical Analysis—Results are expressed as means ± S.E. The data are presented as *x*-fold induction compared with control conditions or compared with IL-1β-stimulated values. Statistical analysis was performed using Student's *t* test for significance. p values <0.05, <0.01, and <0.005 were considered significant.

RESULTS

Extracellular Nucleotides Potentiate IL-1β-induced Lytic Activity and mRNA Steady-state Levels of MMP-9—To evaluate

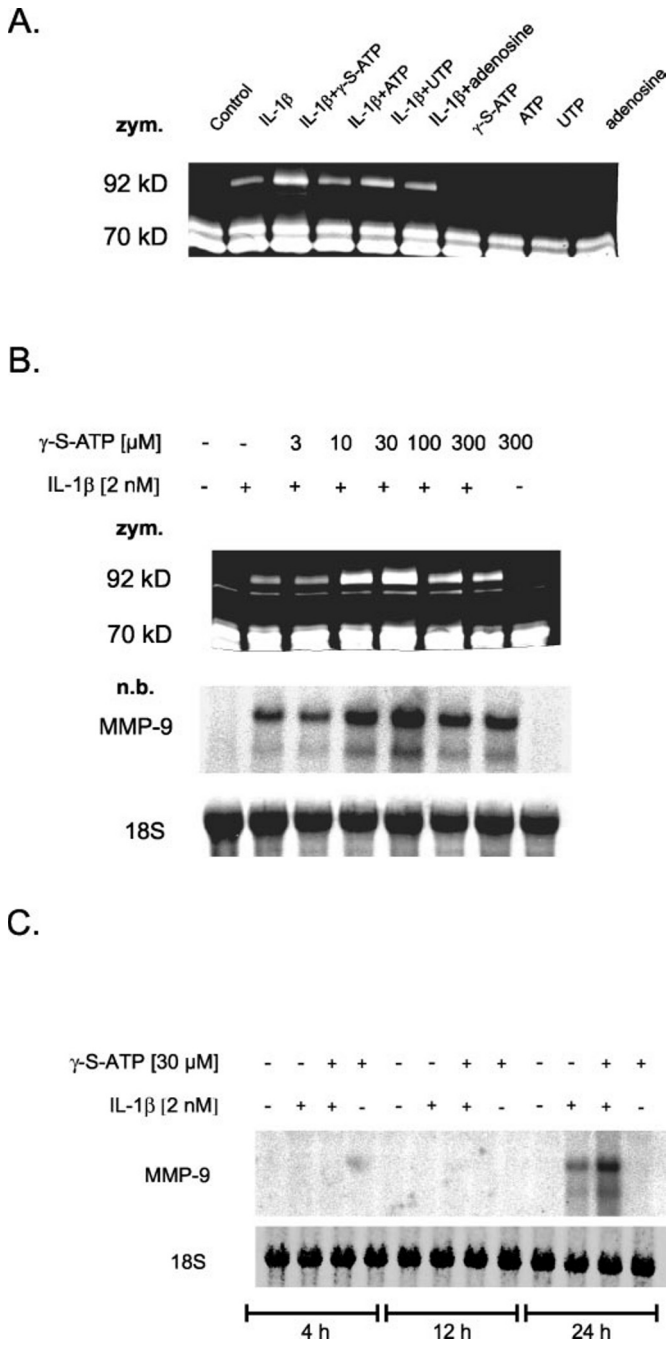

FIG. 1. *A*, effects of extracellular nucleotides on IL-1β-induced MMP-9 activity in mesangial cells. Quiescent mesangial cells were stimulated with either vehicle (*Control*) or IL-1β (2 nM) in the presence or absence of ATPγS, ATP, UTP, and adenosine (each at 30 μM). 24 h after stimulation, 10 μl of supernatants were subjected to SDS-PAGE zymography. The migration properties of lytic bands, corresponding to inactive pro-MMP-9 (92 kDa), and the inactive and active forms of MMP-2 (72 and 68 kDa), were determined using standard molecular mass markers. The data shown are representative for three similar experiments. *B*, dose-dependent amplification of IL-1β-induced MMP-9 activity and MMP-9 mRNA level by ATPγS. Quiescent mesangial cells were stimulated with either vehicle (−) or IL-1β (2 nM) (+) in the presence of the indicated concentrations (in μM) of ATPγS. After 24 h 10 μl of supernatants were collected for SDS-PAGE zymography (*zym*) and cell monolayers collected for mRNA isolation and Northern blot analysis (*n.b.*). Total cellular RNA (20 μg) was hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to a 18 S rRNA probe. Data are representative of three independent experiments giving similar results. *C*, time course of IL-1β-induced MMP-9 steady-state mRNA level in the presence or absence of ATPγS. Mesangial cells were stimulated with the IL-1β (2 nM) with or without ATPγS (30 μM) for the indicated time periods before mRNA was isolated for Northern blot analysis. Total cellular RNA (20 μg) was hybridized to a ^{32}P-labeled cDNA insert from KS-MMP-9. Equivalent loading of RNA was ascertained by rehybridization to a 18 S rRNA probe. Data are representative of two independent experiments giving similar results.

possible effects of extracellular nucleotides on the activity of MMP-9, MC were treated with IL-1β for 24 h in the presence or absence of either ATP (30 μM) or UTP (30 μM). Furthermore, we tested for possible effects of adenosine a degradation product of ATP, as well as ATPγS, a stable ATP analog. The gelatinolytic contents in the conditioned medium of MC were tested by zymography using gelatin as a substrate (21). We chose the 24-h treatment to allow accumulation of extracellular MMP-9, which is high enough to be detected by gelatin zymography (21). The cytokine-mediated gelatinolytic content of latent MMP-9, which is represented by one lytic band at 92 kDa, is strongly increased when MC were cotreated with ATPγS or UTP (Fig. 1*A*). No effect on the IL-1β-caused lytic content is seen with ATP and its degradation product adenosine (Fig. 1*A*). We suggest that under cell culture conditions the rapid degradation by ectonucleotidases removes ATP and therefore prevents sustained signaling by ATP. We observed that the levels of lytic bands migrating at 68 and 72 kDa, which corresponds to latent and active MMP-2, were not significantly changed, which is consistent with the constitutive expression of MMP-2 in rat MC (21). In the absence of IL-1β, none of the tested nucleotides or nucleoside was able to induce MMP-9 activity (Fig. 1*A*). Furthermore, the stimulatory effect of ATPγS occurred in a dose-dependent manner with a maximal effect seen with 30 μM of ATPγS (Fig. 1*B*). In contrast, the higher concentrations did not further augment the cytokine-induced gelatinolytic content in the conditioned media (Fig. 1*B*, *upper panel*).

To evaluate whether the increased level of gelatinolytic activity of MMP-9 is preceeded by an enhanced expression of MMP-9 mRNA, we performed Northern blot analyses using a cDNA from the rat MMP-9 gene. As shown in Fig. 1*B* (*lower panel*) similar to the changes in the gelatinolytic contents, ATPγS dose-dependently augmented the cytokine-induced MMP-9 mRNA level with a maximal effect seen at 30 μM ATPγS, whereas higher concentrations than 30 μM blunted the amplification of cytokine-induced MMP-9. No MMP-9 mRNA was detected in the absence of IL-1β when stimulating with the nucleotide alone (Fig. 1*B*, *lower panel*). To test the time-dependence of cytokine-induced MMP-9 by ATPγS we monitored the time course of MMP-9 induction by Northern blot analysis. As shown in Fig. 1*C* the indcution of MMP-9 mRNA as caused by the treatment of MC with either IL-1β or with IL-1β plus ATPγS did not occur at the early time points tested (4 and 12 h), thus indicating that ATPγS cannot induce MMP-9 mRNA expression by its own. Furthermore, these data indicate that the alterations of cytokine-induced gelatinolytic activity by ATPγS predominantly result from changes in the MMP-9 expression levels.

Involvement of the P2Y$_2$ Receptor in the Amplification Cascade of MMP-9 by ATP—Many of the physiologcial actions exerted by ATP involve the G-protein coupled P2Y$_2$ subtype of purinoceptors. We therefore tested by a pharmacological approach for the involvement of the P2Y$_2$ purinoreceptor in the amplification of cytokine-induced MMP-9 expression by use of suramin, a putative antagonist of P2Y$_2$ but not of P2Y$_4$ purinoceptors (24). As shown in Fig. 2, suramin dose-dependently inhibited the ATPγS-mediated potentiation of IL-1β gelatinolytic activity of MMP-9 without affecting the cytokine-caused gelatinolytic content of MMP-9. This suggests an involvement of the P2Y$_2$ subtype of purinoceptor in the ATP signaling of MMP-9 expression.

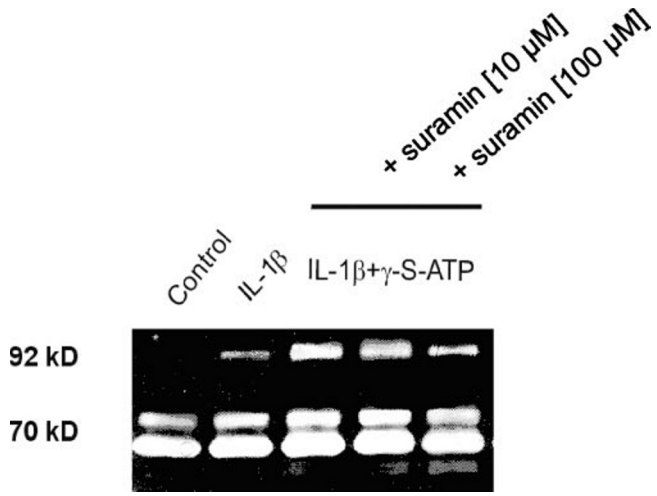

FIG. 2. **The ATPγS-triggered amplification of cytokine-induced gelatinolytic activity of MMP-9 is inhibited by suramin.** Quiescent mesangial cells were simultaneously treated for 24 h with either vehicle (*Control*) or IL-1β (2 nM) and IL-1β (2 nM) plus ATPγS (30 μM) in the presence of the indicated concentrations of suramin before 10 μl of cell supernatants were subjected to SDS-PAGE zymography. Migration properties of lytic bands were determined with molecular mass markers. The data are representative for two independent experiments giving similar results.

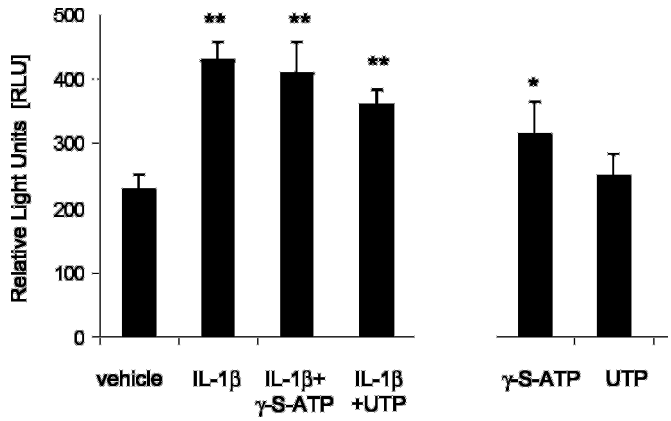

FIG. 3. **The extracellular nucleotides ATPγS and UTP do not affect cytokine-mediated promoter activity of a 1.3-kb portion of rat MMP-9 promoter.** Luciferase activities of a 1.3-kb promoter fragment of the rat MMP-9 5′-flanking region are shown. MC were transiently cotransfected with 0.4 μg of pGL-MMP-9(1.3kb) and with 0.1 μg of pRL-CMV coding for *Renilla* luciferase. After overnight transfection, MC were treated for 24 h with vehicle (control), IL-1β (2 nM), ATPγS (30 μM), UTP (30 μM), or a combination of IL-1β and nucleotide as indicated before being harvested for measurement of dual luciferase activities, as described under "Experimental Procedures." Values for beetle luciferase were related to values for *Renilla* luciferase and are depicted as RLU. Data are the means ± S.D. ($n = 6$). **, $p \leq 0.01$, compared with vehicle.

Nucleotides Have No Effects on Cytokine-induced MMP-9 Promoter Activity—To further evaluate whether the ATP-mediated amplification of IL-1β-induced MMP-9 expression resulted from an increase in MMP-9 gene transcription we assessed promoter activites derived from a 1.3-kb fragment of the rat MMP-9 promoter region (pGL-MMP-9(1.3 kb)) by luciferase reporter gene assays. This promoter region contains several functional elements necessary for cytokine-mediated regulation of MMP-9 expression most important one NF-κB binding site and one AP-1 response element which is close to a functional Ets-1 binding site (22). Transient transfection of MC with pGL-MMP-9(1.3 kb), comprising the 1.3-kb promoter fragment fused to the luciferase reporter gene, was followed by a 24-h treatment with either vehicle, IL-1β (2 nM), with or without either ATPγS (30 μM), or UTP (30 μM) or with each nucleotide alone and subsequently assayed for luciferase activity (Fig. 3). IL-1β significantly stimulated luciferase activity (1.9-fold, $p < 0.01$), but the promoter activity was only weakly enhanced by either nucleotide alone. These data demonstrate that the amplification of cytokine-induced mRNA steady-state levels by ATPγS cannot be located to the upstream 1.3-kb MMP-9 promoter context.

ATPγS Inhibits the Decay of Cytokine-induced MMP-9 mRNA—To test whether the ATP effects relay to some post-transcriptional events, we performed actinomycin D experiments. MC were stimulated for 20 h with IL-1β (2 nM) before transcription was blocked by actinomycin D (5 μg/ml). Subsequently, cells were either directly homogenized (vehicle, 0 h) or left untreated (vehicle, 12 h) or alternatively treated with the stable ATP analog ATPγS (30 μM). After 12 h cells were homogenized for the isolation of total RNA. The MMP-9 mRNA from untreated MC displayed a strong reduction by almost 80% ($p < 0.005$) in the mRNA steady-state level (Fig. 4*A*). Most interestingly, the decay of MMP-9 mRNA was completely blocked in the presence of ATPγS, thus indicating that ATPγS can stabilize the IL-1β-induced MMP-9 mRNA (Fig. 4*A*).

Furthermore, time course experiments revealed that the mRNA stabilizing effects on MMP-9 transcripts by ATPγS already occurred at 4 h and were maximal 12 h after the blockade of transcription by actinomycin D (Fig. 4*B*). Recently it has been reported that transcription blockage by actinomycin D can induce a redistribution of the mRNA stabilization factor HuR from the nucleus to the cytoplasm and therefore may cause an increase in the mRNA half-life (10). However, in our experiments all cells were equally treated with actinomycin D, and therefore, the net effect on MMP-9 decay exclusively depend on the presence or absence of extracellular ATPγS.

In contrast, IL-1β by itself had no effect on the stability of cytokine-induced MMP-9 mRNA, since the addition of IL-1β (2 nM) after transcriptional blockade did not cause any changes in the mRNA decay of MMP-9 (Fig. 4*C*).

In summary, these data indicate that ATPγS can augment the cytokine-induced MMP-9 expression by an increase of MMP-9 mRNA stability.

The Cytoplasmic Fractions of ATP-treated MC Stabilize MMP-9 mRNA in Vitro—The involvement of cytoplasmic factors exerting MMP-9 mRNA stabilizing properties was furthermore tested by an *in vitro* degradation assay. By this assay the time course of degradation of purified MMP-9 mRNA exhibited by any trans-acting factor accumulated in the cytoplasmic extracts from ATPγS-treated MC was compared with the degradation profile derived from untreated MC. Similar volumes of each cytoplasmic extract (with a total protein content of 130 μg) were incubated with 20 μg of total RNA isolated from a common pool of cytokine-treated MC containing a high level of MMP-9 mRNA. Subsequently, we isolated total RNA after the indicated time points and performed Northernblot analysis using a probe from the rat MMP-9 cDNA (Fig. 5). As shown before ATPγS alone is not able to induce endogenous MMP-9 mRNA levels (Fig. 1). Therefore, most of the MMP-9 mRNA detected by degradation assay is derived from the exogeneous pool of MMP-9 mRNA but not from endogenous MMP-9 transcripts within the cytoplasmic extracts. We observed that the primary undegraded MMP-9 transcripts, which had been exposed to the cytoplasmic fractions from ATP-treated MC, displayed a delayed degradation when compared with MMP-9 transcripts exposed to cytoplasmic extracts from untreated cells, which is indicated by the different time courses of decay of MMP-9 mRNA (Fig. 5). In contrast, the amount of the stable GAPDH mRNA was not affected by none of the extracts tested,

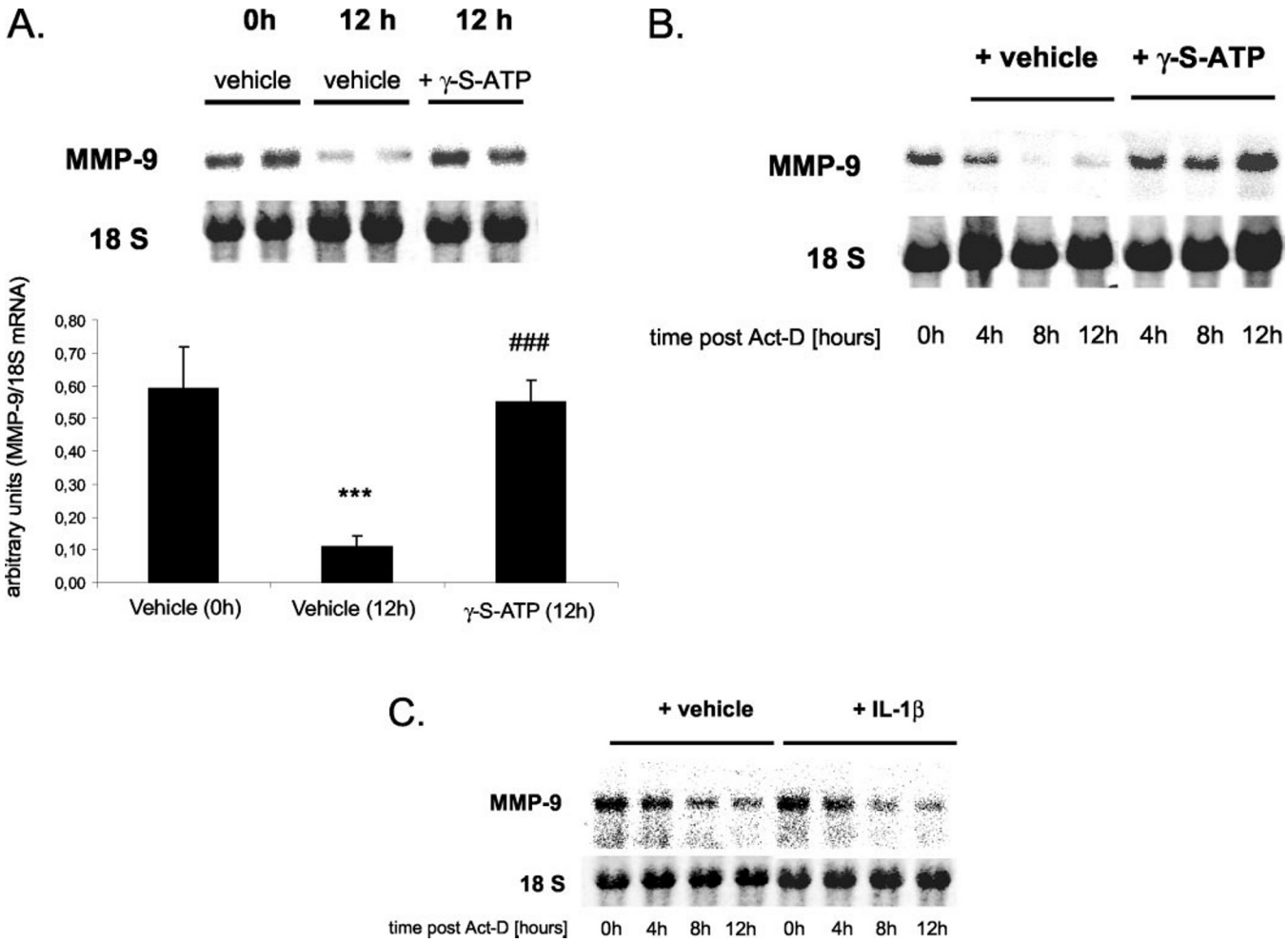

FIG. 4. **ATPγS, but not IL-1β, prevents the decay of cytokine-induced MMP-9 mRNA.** Quiescent MC were treated for 20 h with IL-1β (2 nM) to reach maximal level of MMP-9 transcripts. Thereafter cells were washed twice and incubated with actinomycin D (5 μg/ml) for 30 min. This time point was set as 0 h, and cells were additionally treated for either 12 h (*A*) or for the indicated time points (*B*) without (*vehicle*) or with 30 μM ATPγS before being harvested and extracted for total cellular RNA. 20 μg of total cellular RNA were hybridized to ^{32}P-labeled MMP-9 or 18 S cDNA probes, respectively. The *lower panel* of *A* shows a densitometric analysis of three independent experiments. *C*, quiescent MC were treated for 20 h with IL-1β (2 nM) before cells were washed twice and incubated with actinomycin D (5 μg/ml) for 30 min. This time point was set as 0 h, and cells were additionally treated for for the indicated time points without (+*vehicle*) or with 2 nM IL-1β (+*IL-1β*) before being harvested and extracted for total cellular RNA. 20 μg of total cellular RNA were hybridized successively to ^{32}P-labeled MMP-9 or 18 S cDNA probes, respectively. The blot is representative for two independent experiments.

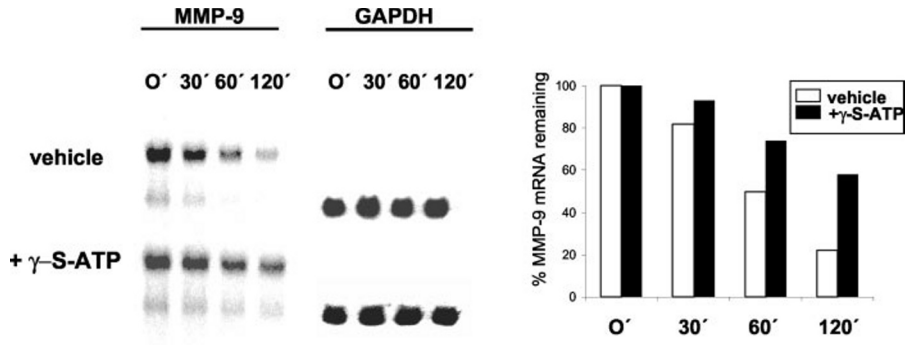

FIG. 5. **Cytosolic lysates of ATP-treated MC exert anti-degradative properties on MMP-9 mRNA in an *in vitro* RNA degradation assay.** Portions of 20 μg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 μg of cytoplasmic extract derived from either untreated (*vehicle*) or + ATPγS-treated MC (+*γ-S-ATP*). After the indicated time periods incubations were stopped by isolation of total RNA. RNA samples were collected and assessed for the amounts of remaining MMP-9 mRNA level by Northern blot analysis by use of a ^{32}P-labeled cDNA insert specific for rat MMP-9. To prove specificity of the effects, we furthermore hybridized with the stable mRNA species GAPDH. Similar results were obtained in three independent experiments.

thus suggesting that the effects on mRNA decay are specific for MMP-9 mRNA. These data indicate that the cytoplasmic extracts from ATPγS-treated MC contain RNA protective factors responsible for the delayed decay of MMP-9 mRNA.

The 3′-UTR of the MMP-9 Gene Specifically Confers an ATP-dependent Increase in Promoter Activity—Regulation of mRNA stability is in many cases determined by AREs within the 3′-UTR of genes (7, 8, 11). Since MMP-9 contains four copies of ARE motifs within its 3′-UTR (ARE-1, ARE-2, ARE-3, and ARE-4 in Table I), we tested whether the increase in MMP-9

TABLE I
Oligonucleotides used in EMSA

The sequence of oligonucleotides was derived from the region encompassing AUUUA-rich motifs within the 3′-UTR region of the rat MMP-9 gene. The position corresponding to the rat MMP-9 gene (GeneBank™ accession number U24441) is indicated by numbers. The consensus sequence is indicated by bold letters, and the nucleotides changed for mutation are underlined. nt, nucleotide.

Oligonucleotide	Starting nt		Ending nt
UTR-ARE-1	2500	5′-CCCUUUU**AUUUA**UUAUGUAUG-3′	2520
UTR-ΔARE-1		5′-CCCUUUU**ACCCA**UUAUGUAUG-3′	
UTR-ARE-2	2536	5′-ACAUGU**AUUUA**ACCUAUAGAA-3′	2556
UTR-ΔARE-2		5′-ACAUGU**ACCCA**ACCUAUAGAA-3′	
UTR-ARE-4	2742	5′-CAGAGGA**AUUUA**UUGGAUGUU-3′	2762
UTR-ΔARE-4		5′-CAGAGGA**ACCCA**UUGGAUGUU-3′	

mRNA stabilization by ATPγS depends on the presence of these ARE motifs downstream of the coding sequence of the gene. To this end the promoter activity of a luciferase reporter gene, which was driven by the pGL-MMP-9(1.3kb) upstream promoter region (Fig. 6*A*), was compared with that derived from transient transfection of a similar MMP-9 promoter construct containing an additional 662 bp from the 3′-UTR of the MMP-9 gene cloned downstream of the luciferase coding sequences (3′-UTR-pGL3-MMP-9(1.3kb) in Fig. 6*B*). Transient transfection of MC with both MMP-9 reporter genes was followed by a 20-h treatment with either vehicle (control), IL-1β (2 nM), ATPγS (30 μM), or both in combination. Stimulation of MC with IL-1β leads to a significant increase of MMP-9 promoter activity (3.4-fold, $p < 0.005$) (Fig. 6*A*). Addition of ATPγS did not further increase the IL-1β-induced pGL-3-MMP-9 luciferase activity (3.5-fold, $p < 0.005$) but caused a significant enhancement of cytokine-triggered promoter activity when the luciferase coding region was under the additional control of the 3′-UTR of MMP-9 (Fig. 6*B*). Similarly, stimulatory effects on basal luciferase activity by ATPγS alone were significantly increased when the luciferase gene was under the additional control of the 3′-UTR of MMP-9 (from 1.6 ± 0.02-fold ($p < 0.05$) to 2.3 ± 0.015-fold induction ($p < 0.005$); mean ± S.D., $n = 3$). Most probably this is due to the stabilization of the highly constitutively expressed luciferase mRNA by ATPγS via the inserted 3′-UTR of MMP-9. Accordingly, the high level of basal promoter activities measured with all pGL-MMP-9 reporter constructs to some extent may cover the strong stimulatory effects by IL-1β (in presence or absence of ATPγS) observed for the endogneous MMP-9 expression. In contrast, the low basal expression of endogenous MMP-9 may explain the lack of ATP effects on basal MMP-9 levels demonstrated by Northern blot and zymography, respectively (Fig. 1).

Mutagenesis Studies with MMP-9 Reporter Genes Bearing Mutated AREs—To investigate the impact of each single ARE motif on the ATP-dependent mRNA stabilization, we generated different point mutations by replacing the pentameric "AUUUA" motif of ARE-1, ARE-2, and ARE-4 to an inactive "ACCCA" sequence (depicted in Fig. 7), which impairs its RNA binding affinity. As shown in Fig. 7*B*, MC transiently transfected with 3′-UTR-MMP-9 promoter constructs bearing one mutated ARE motif displayed similar induction profiles by ATP and IL-1β as those derived with the wild-type 3′-UTR pGL MMP-9 promoter, although the absolute rates of luciferase activities differed between single constructs (compare relative light units (RLUs) in Fig. 7*B* with RLU in Fig. 6*B*). In contrast, mutation of all three putative ARE motifs (3′-UTR-ΔARE-1-2-4 pGL3-MMP-9) resulted in a loss of ATP-dependent effects on reporter gene activities (Fig. 7*B*) similar to those observed with the wild-type MMP-9 promoter construct lacking the 3′-UTR (Fig. 6*A*). These data indicate that none of the ARE motifs within the 3′-UTR of MMP-9 is indispensable for the ATP-dependent MMP-9 mRNA stabilization. However, mutation of all three ARE motifs results in a loss of ATP dependent increase in luciferase activities without affecting the stimulatory effects by IL-1β. This indicates that the ATP-dependent effects via 3′-UTR are mainly attributable to AREs but not to the presence of 3′-enhancer elements.

ATP Augments the RNA Binding to AREs within the 3′-UTR of MMP-9—The stability of many short-lived mRNAs is modulated through specific binding of proteins to the AREs present in their 3′-UTR. We therefore used EMSA to determine whether treatment of cells with ATPγS would cause an increase in the consitutive binding of HuR-containing complexes bound to different ARE motifs within the 3′-UTR of MMP-9 (25). The RNA binding to MMP-9-specific AREs was monitored by using ^{32}P-labeled RNA oligonucleotides comprising the different wild-type or mutant AREs of MMP-9 (denoted as UTR-ARE or UTR-ΔARE as depicted in Table I).

MC were either left untreated or treated for 4 h with different concentrations of ATPγS and subsequently lysed for cytosolic fractions. Using an RNA oligonucleotide containing the wild-type ARE-1 motif from the 3′-UTR of MMP-9, we observed the constitutive binding of three major constitutive complexes of high electrophoretic mobility, and interestingly, the binding of all complexes was dose-dependently increased when cells had been treated with ATPγS (Fig. 8*A*).

Similar to ARE-1, the constitutive RNA binding of two main complexes to ARE-2 and ARE-4 encompassing oligonucleotides was dose-dependenty increased by ATPγS and a maximal RNA binding capacity observed with 30 μM ATPγS (Fig. 8, *B* and *C*). As we have described previously, an ARE-3-containing RNA oligonucleotide displayed no *in vitro* RNA binding capacity most probably due to the different bases flanking this ARE motif (25).

Additionally, the binding affinity of RNA oligonucleotides bearing a mutated ARE motif (ACCCA in stead of AUUUA, Table I) was strongly reduced when compared with oligonucleotides bearing the wild-type ARE (Fig. 8, *A–C*) demonstrating the functionality of each ARE motif.

In contrast to the effects observed with ATPγS, the short term treatment of MC with IL-1β (4 h) did not modulate the constitutive RNA binding to any of the ARE motifs tested (Fig. 8*D*).

ATP-inducible Complexes Binding to MMP-9-specific AREs Contain the mRNA Stabilizing Factor HuR—We have described previously that the ELAV-like RNA-binding protein HuR is critically involved in the regulation of MMP-9 mRNA stability (25). To test whether the ATPγS-inducible complexes interacting with the ARE motifs of MMP-9 contain any HuR-like protein, we performed supershift analysis. We focused on HuR (HuA), which participates in the regulation of ARE-mediated RNA turnover (10) and which has been identified as a target of NO-dependent MMP-9 mRNA decay in rat MC (25). As shown in Fig. 8, *A–C* (*right panels*), the addition of anti-HuR antibody caused appearance of two supershifted bands (*arrowheads*) independent of which ARE oligonucleotide was used in the binding assay. Most interestingly, the addition of super-

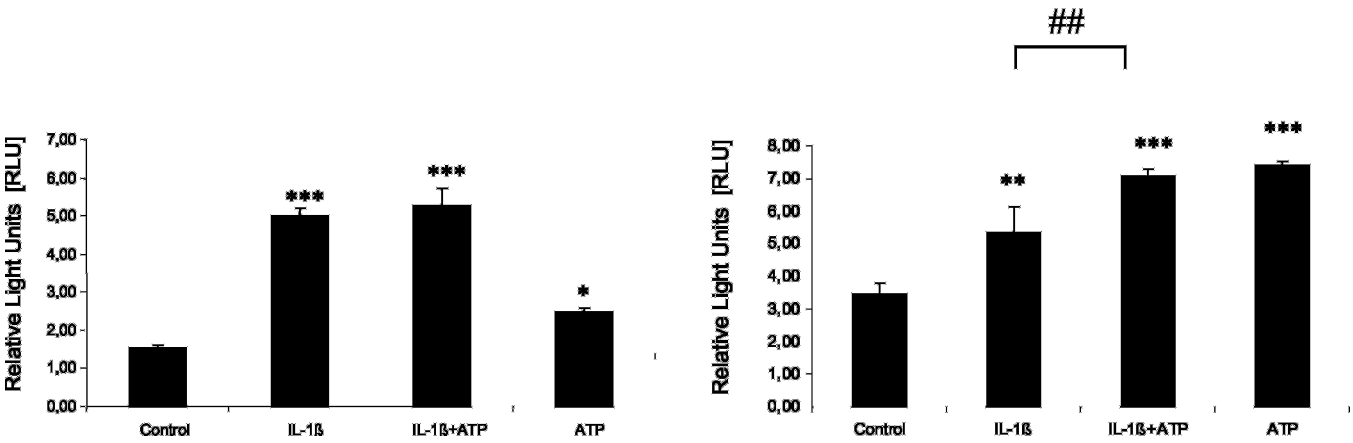

FIG. 6. **Influence of the MMP-9–3′-UTR on MMP-9 promoter driven luciferase activity.** *A*, subconfluent MC were transiently cotransfected with 0.4 μg of pGL-MMP-9(1.3kb) and 0.1 μg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 20 h with vehicle (*Control*) or with IL-1β (2 nM) or ATPγS (30 μM) or both in combination. *B*, to assess the influence of the 3′-UTR of the MMP-9 mRNA MC were alternatively transfected with 0.4 μg of 3′-UTR-MMP-9 pGL-MMP-9(1.3kb) containing additionally the 3′-UTR of MMP-9 downstream of the luciferase coding sequence before being treated with vehicle, IL-1β (2 nM), or ATP (30 μM) or both in combination. The values for beetle luciferase were related to values for Renilla luciferase and are depicted as RLU. Data (means ± S.E.) are the results of triplicates and are representative for three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$ compared with unstimulated control values and to IL-1β-stimulated values (##, $p \leq 0.01$).

shift antibody results in an almost complete shift of all complexes indicating that HuR is a main constituent of the ATP-regulated complexes. Similar to the EMSAs with cytoplasmic fractions, recombinant HuR protein displays a strong *in vitro* binding affinity to all three wild-type ARE motifs from rat MMP-9 mRNA (UTR-ARE-1, UTR-ARE-2, and UTR-ARE-4) (25). These data implicate that ATPγS, by increasing the constitutive HuR binding to AREs, stabilizes the cytokine-induced MMP-9 mRNA.

The mRNA Stabilization Factor HuR (HuA) Is Critically Involved in Nucleotide-induced MMP-9 mRNA Stabilization—In a previous study we have demonstrated that neutralization of HuR by addition of anti-HuR antisera results in the accelerated decay of MMP-9 mRNA, whereas addition of recombinant HuR has an opposite effect on the mRNA decay of MMP-9 (25). To further test the functional role of HuR in the ATPγS-mediated stabilization of MMP-9 mRNA, we examined the effect of the neutralizing HuR antibody by *in vitro* degradation assays. To this end, the cytoplasmic fractions from ATPγS-treated MC (which confer protective properties toward cytokine-induced MMP-9 mRNA) were preincubated for 1 h with a monoclonal anti-HuR antibody (a total amount of 400 ng of antiserum) before the decay of MMP-9 RNA was monitored by Northern blot analysis. Total cellular RNA was isolated after a further 2-h coincubation with total cellular RNA, a time point where the stabilizing effects by ATPγS are most obvious (Fig. 5). As a negative control the same volume of vehicle (mouse IgG) was preincubated. As shown in Fig. 9 the level of MMP-9 mRNA was reduced when HuR was neutralized by the anti-HuR antibody (Fig. 9). In contrast, mouse IgG on its own had no effects on the MMP-9 level (Fig. 9). Again, the steady-state mRNA level of GAPDH was not affected in the degradation assay, thus indicating that the modulation of mRNA stability by HuR is not observed with a non-ARE-containing mRNA.

The ATP-induced Nucleo-cytoplasmic Shuttling of HuR Is Inhibited by Suramin—We next investigated the subcellular localization of HuR by confocal microscopy. Several reports have documented a predominant nuclear localization of HuR (9, 10, 26), although fractions of HuR protein have also been found in the cytoplasm (27). Under basal conditions (0 min, Fig. 10*A*) we observed a strong fluorescence in the cell nuclei but almost no staining within the cytoplasm, thus indicating that HuR shows an almost exclusive nuclear distribution.

Treatment of MC with 30 μM ATPγS triggered a substantial increase in cytoplasmic HuR as is indicated by the appearance and increased fluorescence in punctated structures outside the nucleus (Fig. 10*A*, *upper panel*) A maximal density of immunopositive grains in the cytoplasm was observed after 2 h of treatment with ATPγS. A granular distribution of HuR within the cytoplasm has also been shown for the ELAV homolog proteins Hel-N1 and Hel-N2 (28). Incubations of longer than 2 h reduced the degree of cytosolic staining (Fig. 10*A*). These results indicate that the rise in RNA binding to the 3′-UTR of MMP-9 is paralleled by an increased nuclear-cytoplasmic shuttling of the RNA stabilizing protein HuR.

To investigate whether the $P2Y_2$ purinoreceptor subtype is involved in the ATPγS-dependent increase in cytoplasmic staining of HuR, we tested the effects of the putative $P2Y_2$ antagonist suramin (24). Interestingly, the ATPγS-mediated effects on HuR translocation were inhibited at all time points tested (Fig. 10*A*, *lower panel*), which again indicates the involvement of $P2Y_2$-dependent signaling in the regulation of HuR by extracellular ATP.

The subcellular localization of endogenous HuR was also monitored by Western blot analysis. MC were treated for 4 h with different concentrations of ATPγS (3, 10, and 30 μM) and subsequently fractionated into nuclear and cytoplasmic fractions. We observed that untreated MC contain low HuR protein levels in the cytoplasm (Fig. 10*B*), which is consistent with the weak fluorescence in the perinuclear region observed by confocal microscopy (Fig. 10*A*).

Stimulation with ATPγS caused a dose-dependent increase in the level of cytosolic HuR with a maximal effect seen at 30 μM ATPγS (Fig. 10*B*, *left panel*). The addition of suramin to ATP (30 μM) resulted in a strong reduction of HuR accumulation again demonstrating a $P2Y_2$-dependent mechanism (Fig. 10*B*, *left panel*).

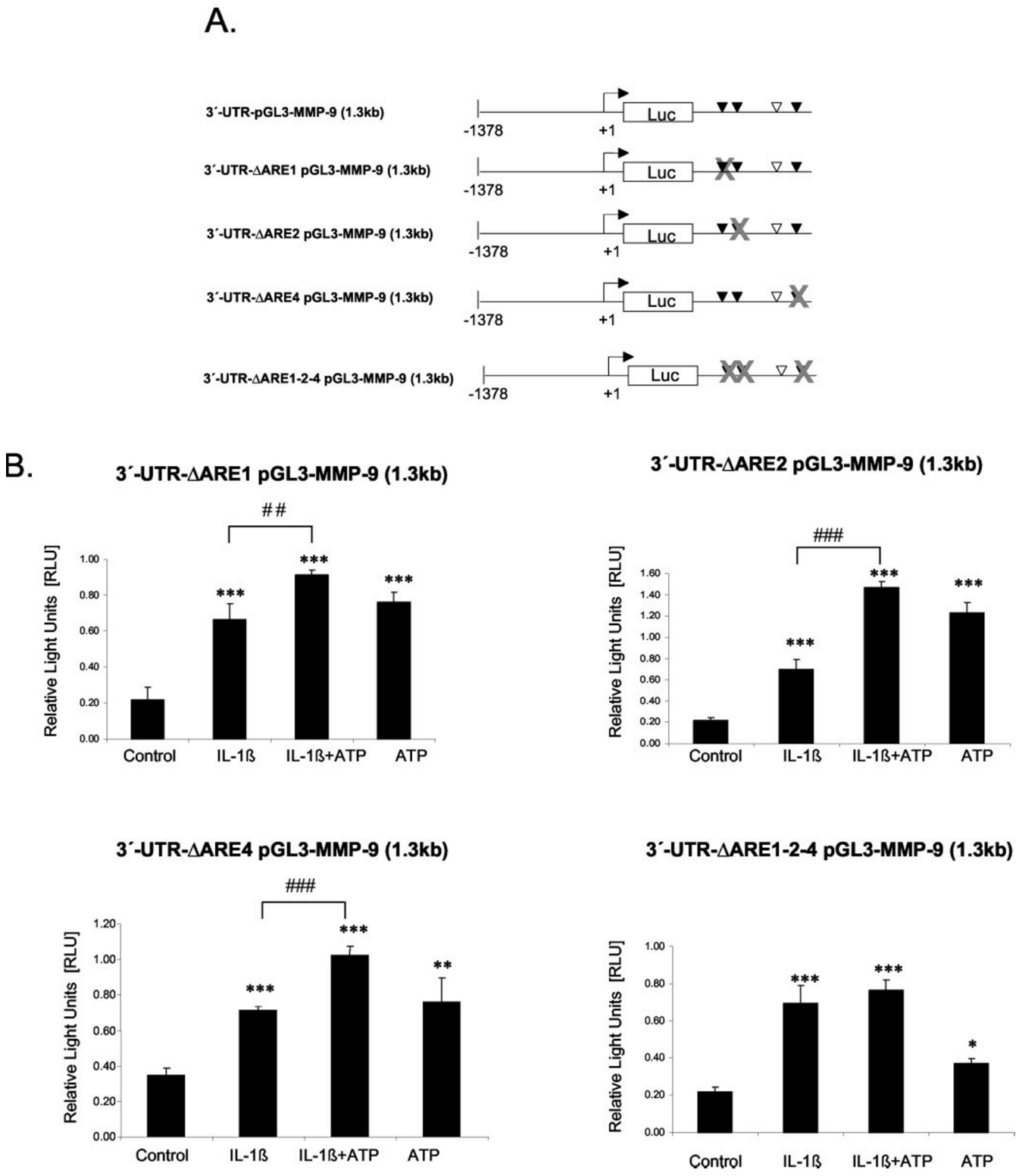

FIG. 7. **Analysis of mRNA-stabilizing effects exerted by MMP-9-mutated 3′-UTR constructs.** *A*, schematic representation of the wild-type (3′-UTR-pGL-MMP-9(1.3kb)) bearing the 3′-UTR of MMP-9 downstream of the luciferase coding region (*Luc*) and positions of corresponding single-mutated (3′-UTR-ΔARE1-, ΔARE2-, and ΔARE-4-pGL-MMP-9(1.3kb)) or triple-mutated (3′-UTR-ΔARE1-2-4-pGL-MMP-9(1.3kb)) AU-rich elements. *B*, relative luciferase activities of mutated 3′-UTR-MMP-9 promoter constructs. Subconfluent MC were transiently cotransfected with 0.4 μg of either 3′-UTR-pGL-MMP-9(1.3kb) or the indicated point-mutated MMP-9 promoter constructs and 0.1 μg of pRL-CMV coding for *Renilla* luciferase. After an overnight transfection MC were treated for 20 h with vehicle (*Control*) or with IL-1β (2 nM) or ATPγS (30 μM) or both in combination as indicated. Values for beetle luciferase are related to values for *Renilla* luciferase and are depicted as RLU. Data (means ± S.E.) are the results of triplicates and are representative for three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$ compared with unstimulated control values and to IL-1β-stimulated values (##, $p \leq 0.01$; ###, $p \leq 0.005$).

Finally, we tested for possible ATPγS-induced changes in the nuclear HuR content. We observed that the nuclear amount of HuR is dramatically higher than that of cytoplasmic HuR (Fig. 10*C*). Therefore, no significant change in the nuclear HuR level was detectable when cells were treated with ATPγS (Fig. 10*B*, *right panel*) despite the marked changes in cytoplasmic HuR.

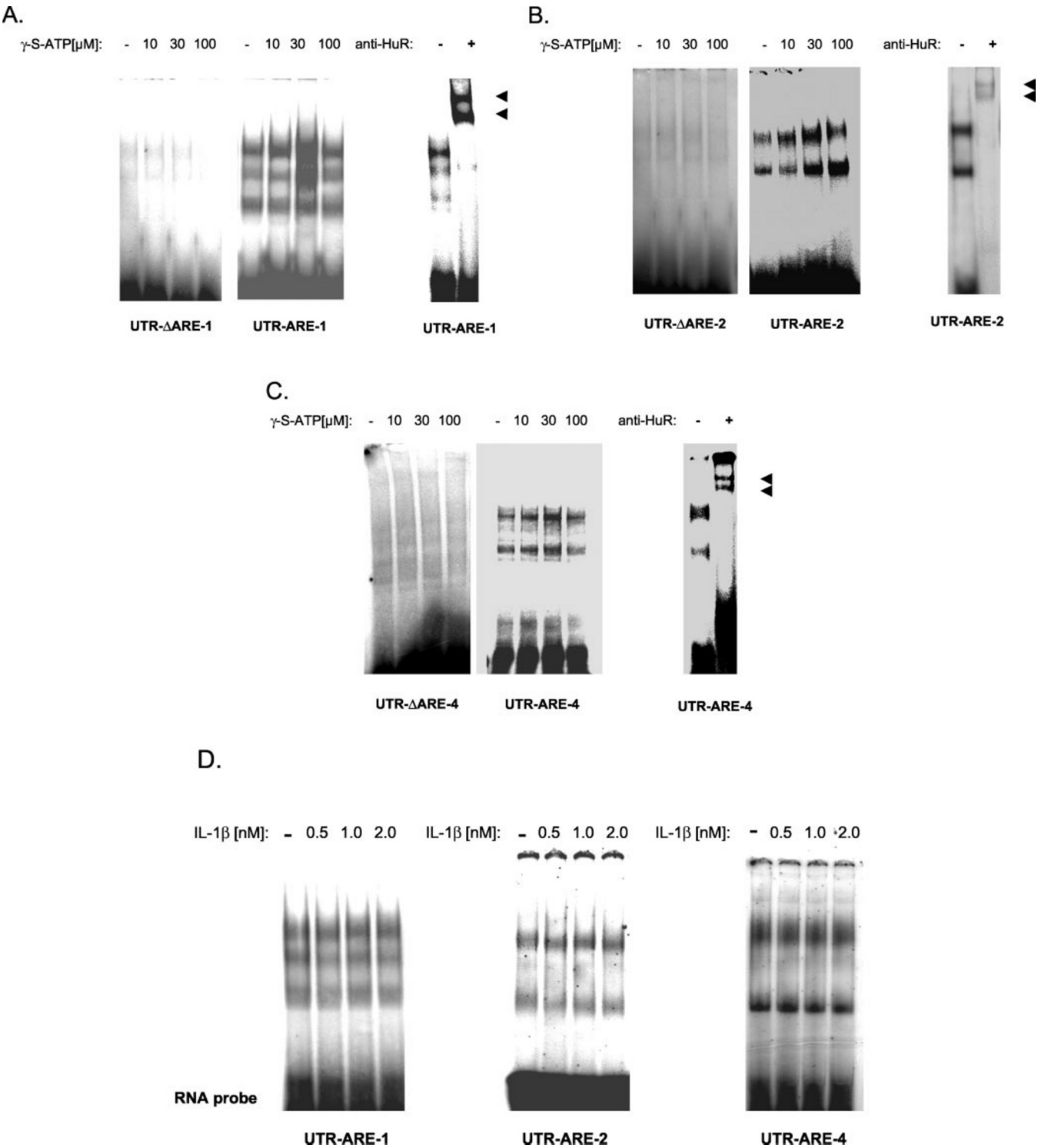

FIG. 8. **ATPγS causes an increase in the constitutive RNA binding of HuR to AU-rich motifs within the 3′-UTR of MMP-9.** RNA binding was analyzed by EMSA using gene specific wild-type oligonucleotides (*UTR-ARE*) or oligonucleotides bearing mutations of three bases within the AU-rich element (*UTR-ΔARE*) as depicted in Table I. MC were either left untreated (−) or stimulated for 4 h with the indicated concentrations of ATPγS before cells were lysed for preparation of cytoplasmic extracts. 4 μg of cytoplasmic extracts were incubated with a ^{32}P-labeled RNA probe derived from the corresponding AU-rich region of the 3′-UTR of rat MMP-9. *A–C*, UTR-ARE-1 (*A*), UTR-ARE-2 (*B*), and UTR-ARE-4 (*C*) and RNA binding assessed in 6.0% native PAGE gels. The results shown in each panel are representative for three independent experiments giving similar results. Supershift analysis was done by incubating the ^{32}P-radiolabeled oligonucleotides with 6 μg of cytoplasmic extract derived from ATPγS-treated MC (30 μM) in the presence of 1 μl (200 ng) of a monoclonal anti-HuR antibody (*anti-HuR*). The antibody was added 15 min after addition of the radiolabeled oligonucleotide and incubated for a further 15 min at room temperature. *Arrowheads* indicate the supershifted complexes. Similar results were obtained in three independent experiments. *D*, short term stimulation with IL-1β does not affect the consitutive RNA binding to AREs within the 3′-UTR of MMP-9 MC were either left untreated (−) or stimulated for 4 h with the indicated concentrations of IL-1β before cells were lysed for preparation of cytoplasmic extracts. The procedures for EMSA were similar to those described in the legend to *A*. The different RNA probes (UTR-ARE-1, UTR-ARE-2, and UTR-ARE-4) were derived from the corresponding AU-rich region of the 3′-UTR of rat MMP-9. The EMSAs shown are representative for two independent experiments giving similar results.

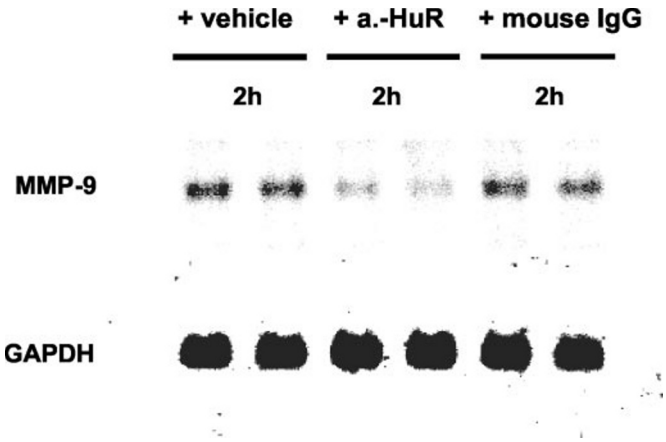

FIG. 9. **Neutralization of HuR reduces the stability of MMP-9 mRNA.** Portions of 20 μg of total RNA from a common pool of total RNA isolated from cytokine-stimulated MC were mixed with 130 μg of cytoplasmic extract derived from MC treated for 4 h with ATPγS. The cytoplasmic extracts were kept either untreated (*vehicle*) or, alternatively, pretreated for 1 h with 0.4 μg of a monoclonal anti-HuR antibody (+*anti-HuR*) or with the same volume of mouse IgG (+*mouse IgG*) before the cytoplasmic extracts were incubated with the total RNA portions. Incubation with the RNA was stopped after additional 60 min before RNA was extracted for Northern blot analysis. Equal transfer and loading of total RNA were ascertained by hybridization to a GAPDH probe. Samples derived from one cytoplasmic extract were subjected to RNA in duplicates. Similar results were obtained in two independent experiments.

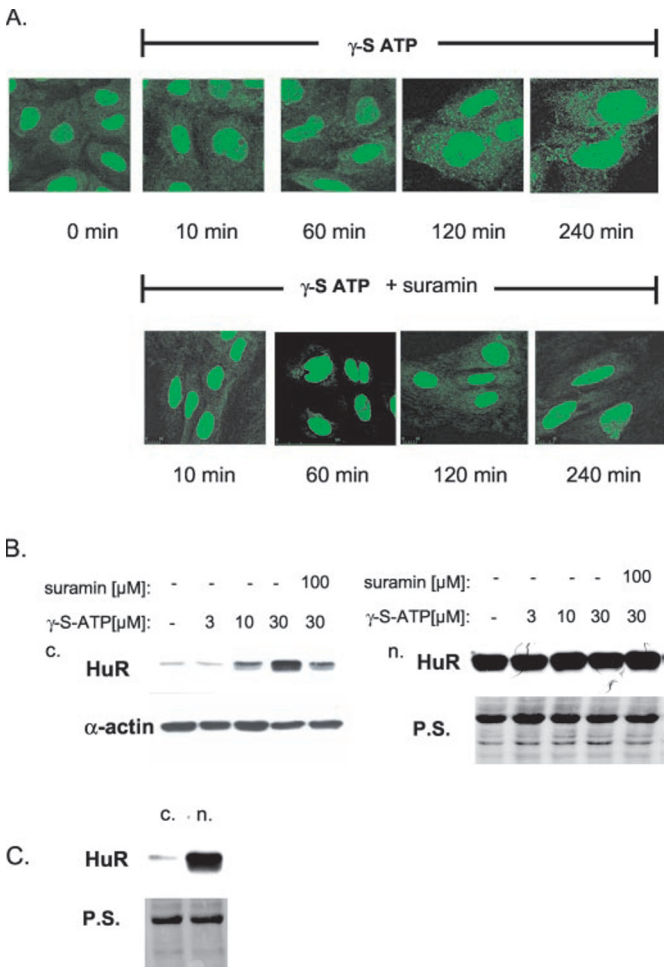

FIG. 10. **Suramin inhibits the ATPγS-triggered nucleo-cytoplasmic shuttling of the ELAV protein HuR.** *A*, indirect immunofluorescence showing the changes in the localization of HuR during stimulation of cells with ATPγS. Quiescent mesangial cells were stimulated for the indicated time periods with either vehicle (0 min) or ATPγS (30 μM) in the absence (*upper panels*) or presence of 100 μM suramin (*lower panels*). Thereafter cells were fixed and stained with an anti-HuR antibody and anti-mouse-Alexa 488 as a secondary antibody. *B*, Western blot analysis showing ATP-induced redistribution of HuR from the nucleus to the cytoplasm. MC were stimulated for 4 h with different concentrations of ATPγS as indicated and then lysed for cellular fractionation. Protein lysates (100 μg) from cytoplasmic (*left panel*) or nuclear (*right panel*) fractions were subjected to SDS-PAGE and probed with a HuR-specific monclonal antibody. To correct for variations, the blot was either stripped and incubated with an α-actin antibody (*left panel*) or loading of equal amounts of total protein was proven by Ponceau-S staining (*P.S.*) (*right panel*). Data are representative of two independent experiments giving similar results. *C*, relationship between the cytoplasmic and nuclear levels of HuR. Equal amounts of total protein extracts (100 μg) from nuclear (*n.*) or cytoplasmic (*c.*) fractions from untreated MC were subjected on SDS-PAGE and probed with a HuR-specific antibody. Loading of equal amounts of total protein was proven by Ponceau-S staining (*P.S.*).

In summary, our data indicate that the ATPγS-dependent rise in the IL-1β-induced MMP-9 expression in MC is functionally linked to mechanisms, which involve increased RNA binding of HuR. Additionally, the increase in RNA binding by ATP is paralleled by an increase in the nucleo-cytoplasmic shuttling of HuR.

DISCUSSION

MMP-9 is a protease that is strongly induced by inflammatory cytokines such as IL-1β and tumor necrosis factor α in many cell types including glomerular MC. Here we have focused on possible modulatory effects on MMP-9 by extracellular ATP and UTP, since both nucleotides can mediate a variety of cell responses in MC including cell growth and inhibition of apoptosis (17, 18). Moreover, extracellular nucleotides have also been reported to modulate experimental forms of glomerulonephritis *in vivo* (29).

We demonstrate that the stable ATP analog ATPγS as well as UTP potently amplify the IL-1β-mediated expression of MMP-9. The stimulatory effects of ATP on MMP-9 expression could be potently inhibited by the addition of suramin, a potent, although nonselective, antagonist of $P2Y_2$ purinoceptors. The occupation of $P2Y_2$ receptors in rat MC can activate different isoforms of protein kinase C (PKC) (15, 24) but also different MAPK pathways including the extracellular signal-regulated kinases (18), the stress-activated protein kinase, and p38-MAPK cascade (30, 31). Interestingly, several studies have implicated MAPK pathways as well as PKC-dependent signaling cascades in the up-regulation of MMP-9 expression (32–35), mainly via the activation of NF-κB and AP-1 transcription factors (6, 33–35). In rat MC none of the nucleotides was able to induce MMP-9 expression on its own. Obviously, in MC none of the signaling pathways affected by extracellular nucleotides is sufficient to transcriptionally activate MMP-9 and an additional cytokine-triggered signal is essentially required. The assumption that cytokines and extracellular nucleotides can activate two independent signaling pathways is further underlined by the observation that both ATP and UTP have only marginal effects on the cytokine-evoked activity of a 1.3-kb fragment of the rat MMP-9 promoter, although this promoter region is sufficient to mediate a strong activation by IL-1β in rat MC (22). In line with these suggestions we found a substantial contribution of posttranscriptional regulation to the ATP-mediated amplification of cytokine-induced MMP-9 expression. However, we cannot exclude the additional involvement of transcriptional regulation by ATPγS attributable to some enhancer elements upstream from the proximal 1.3 kb of MMP-9. Therefore, a possible contribution of transcriptional regulation exerted by extracellular nucleotides has to be checked by additional reporter gene experiments using larger fragments of the rat MMP-9 promoter.

By use of actinomycin D experiments and *in vitro* RNA degradation assay, we further demonstrate that treatment of cells with ATPγS significantly increased the stability of MMP-9 mRNA. The 3′-UTR of rat MMP-9 contains several copies of AU-rich elements, which are considered as important determinants of RNA turnover (7, 8, 36, 37). Concomitantly, we demonstrate that the 3′-UTR of MMP-9 confers an ATP-dependent increase of basal and cytokine-induced MMP-9-driven luciferase activity when fused downstream of the luciferase reporter gene. Focusing on the responsible cis regulatory regions we found four copies of AUUUA pentameric motifs within the 3′-UTR of the rat MMP-9 mRNA potentially involved in the regulation mRNA decay of MMP-9. Interestingly, these motifs have been implicated in the rapid turnover of many mRNA species. Proteins functionally targeting these AREs include destabilizing factors such as AUF 1 (38) as well as mRNA stabilizing factors, most prominently members of the ELAV protein family (10, 23, 39). By gel-shift analysis we demonstrate binding of complexes to three different ARE motifs of the 3′-UTR of MMP-9 ("MMP-9-ARE-1," "MMP-9-ARE-2," and "MMP-9-ARE-4"), the binding of these complexes being substantially increased in cells treated with ATPγS but not with IL-1β. We focused on the ELAV-like RNA-binding protein HuR, which is known to bind AREs with a high affinity thereby increasing the stability of respective mRNAs (10, 23, 39). The binding affinity to the MMP-9-specific AREs was strongly impaired when the uracil base within the AUUUA pentameric motif was replaced by cytosine, which confirms the functionality of the AU-rich elements within the 3′-UTR of MMP-9. Concomitantly, the presence of HuR in the cytoplasmic extracts from ATP-treated MC was confirmed by supershift analysis. In addition, we have previously demonstrated that the MMP-9-specific ARE displays a high binding affinity to recombinant HuR protein, thus demonstrating a functional binding capacity of HuR to these ARE motifs within the 3′-UTR of MMP-9 (25). The RNA binding of HuR has been shown to parallel the *in vivo* ability of ARE sequences to direct mRNA degradation (40). Consistent with these findings we demonstrate the functional importance of HuR by *in vitro* degradation assays. The addition of a neutralizing HuR antibody to the lysates of ATP-treated MC results in a significant acceleration of the decay of MMP-9 mRNA but not of GAPDH mRNA, which lacks AU-rich elements within its 3′-UTR (Fig. 9). Our findings suggest that ATP triggers an increase in HuR RNA-binding to the 3′-UTR of MMP-9 mRNA and thus conveys protection of MMP-9 transcripts from rapid degradation. It is interesting to note that in the *in vitro* degradation assays we did not observe degradation fragments of MMP-9 mRNA. The gradual shortening of the poly(A) tail of mRNA followed by a rapid exonuclease-dependent 3′- to 5′-digestion is thought of as a major mechanism of mRNA decay within eukaryotic cells (41). Moreover, the rate of deadenylation seems an important control point for the rate of mRNA degradation. The disappearance of the MMP-9 positive band without detectable intermediates observed in our experiments indicates a rapid 3′- to 5′-degradation, which is consistent to the findings of Chen *et al.* (42). Since the cDNA insert used for Northern blot analysis in this study covers a 3′-region of MMP-9 mRNA (1227–1939), this probe is not optimal for monitoring of mRNA intermediates.

So far little is known about the exact mechanisms by which HuR regulates mRNA stability. Unlike the other members of the ELAV family (HuB, HuC, or HuD), which all show a tight tissue-specific regulation, HuR is ubiquitously expressed and is predominantly located in the nucleus (10, 11, 39, 40, 43–45). Mechanisms of HuR dependent mRNA decay can involve a nuclear-cytoplasmic movement (26, 46, 47) but also changes in the HuR expression level (25, 49). Using confocal fluorescence microscopy we demonstrate that HuR is predominantly nuclear but partially distributes in distinct punctuate structures in the cytoplasm following treatment with ATPγS. This is in agreement with a recent study that has shown that ATP in higher concentrations (1 mM) inhibits the activity of AMP-activated kinase and thereby elevates the HuR level in the cytoplasm of colorectal carcinoma RKO cells (50). AMP-activated kinase is an enzyme that is considered to act as a cellular defense mechanism to protect cells from ATP depletion (51). Most intriguingly, the expression of HuR was shown to be down-regulated in quiescent cells but increased following serum stimulation. Therefore, ATP as a proliferative stimulus may affect a default pathway of mRNA decay, thereby regulating the expression of a variety of growth regulatory proteins. Besides AMP-activated kinase the activation of PKC plays a cardinal role in the enhanced stability of mRNAs coding for p21 and IL-1 (52, 53). Mapping of the protein sequence has revealed that HuR contains a multitude of putative PKC phosphorylation sites, which makes it tempting to speculate that ATP might regulate HuR via PKC. Other studies have implicated the involvement of MAPK pathways in the regulation of the nucleo-cytoplasmic shuttling of HuR (48, 54). Whether changes in HuR phosphorylation, either by different PKCs or by the MAPK pathway, account for the ATP-induced HuR redistribution in MC is currently being investigated in our laboratory. In this context it is noteworthy that ATP has been shown to activate different PKC isoenzymes (14, 15) as well as the three major MAPK cascades (18, 30, 31).

The excessive degradation of extracellular matrix is an important feature in the progression of many acute inflammatory diseases accompanied by a cellular hyperproliferation. Our present findings present a molecular mechanism that may explain how extracellular nucleotides transiently potentiate the cytokine-mediated cellular capacities to degrade extracellular matrix without having any effects on MMP-9 expression in resting cells. To the best of our knowledge this is the first time that the stable ATP analog ATPγS was shown to be able to regulate the expression of MMPs by posttranscriptional mechanisms. Furthermore, our data emphasize the importance of modulation of mRNA stability in the tight regulation of MMP-9.

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