FEBS Letters 589 (2015) 1943–1950

FEBS Letters

journal homepage: www.FEBSLetters.org

Lymphotoxin α, a novel target of posttranscriptional gene regulation by HuR in HepG2 cells

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ARTICLE INFO

Article history:
Received 3 March 2015
Revised 24 April 2015
Accepted 6 May 2015
Available online 14 May 2015

Edited by Beat Imhof

Keywords:
AU-rich element
Hepatocellular carcinoma
Lymphotoxin α
mRNA-stability

ABSTRACT

The role of the RNA-binding protein human antigen R (HuR) in hepatocarcinogenesis is still elusive. By employing short hairpin (sh)RNA-dependent knockdown approach, we demonstrate that lymphotoxin α (LTα) is a target of posttranscriptional gene regulation by HuR in hepatocellular carcinoma (HepG2) cells. Consequently, the increased mRNA decay upon HuR depletion significantly affects lymphotoxin expression at both, the mRNA and protein level. Biotin-pulldown assay showed that HuR specifically interacts with the 3′-untranslated region (3′-UTR) of the LTα mRNA. Furthermore, electrophoretic mobility shift assay (EMSA) implicates that the RNA-binding critically depends on the RNA recognition motif 2 (RRM2) and the hinge region of HuR.

1. Introduction

The RNA-binding protein HuR controls many aspects of post-transcriptional processing of adenylate- and uridylate-rich element (ARE)-bearing mRNA subsets including splicing, polyadenylation, mRNA-stabilization and translation [1,2]. The modulation of these mRNA functions is mediated by an increased binding of HuR to AREs residing in most cases within the 3′-UTR of otherwise labile mRNAs. Members of the Hu-protein family are characterized by a unique structure of three highly conserved RNA recognition motifs (RRMs) and a less conserved Hinge region between the RRM2 and RRM3, which is highly relevant for the intracellular HuR localization and nucleo-cytoplasmic shuttling [2]. The two N-terminal RRMs (RRM1 and RRM2) which represent the most conserved RRMs among different Hu family members are proposed as main RNA-binding domains whereas the carboxy (C)-terminal RRM3 is mainly involved in protein–protein interactions and relevant for stabilization of RNA–protein complexes. However, a crucial role of this RRM for RNA binding has also been demonstrated [2,3]. Since many HuR target mRNAs encode proteins implicated in cancer-related cell functions [4–6], HuR has emerged as an important posttranscriptional regulator of carcinogenesis. In line with this concept, high HuR expression levels have been reported in many human tumors concomitant with a high-grade malignancy and a poor outcome [7–9]. In the liver HuR is implicated in the regulation of hepatocyte proliferation, differentiation and hepatocellular carcinoma (HCC) transformation [10,11].

Lymphotoxin α (LTα) is a member of the tumor necrosis factor (TNF) superfamily which can either exist as a membrane-bound heterodimer ($LT\alpha_1\beta_2$), or as soluble homotrimer ($LT\alpha_3$) [12]. The latter one signals mainly through binding to the TNF receptors TNFRI and II and shares some biological activities with TNFα most importantly, the activation of canonical and non-canonical NFκB signaling cascades [13]. Although LTα is mainly secreted by activated immune cells and lymphoid tissue inducer cells sustained LTα signaling is involved in chronic inflammation-induced liver

Abbreviations: ARE, AU-rich element; EMSA, electrophoretic mobility shift assay; HCC, hepatocellular carcinoma; HuR, human antigen R; LTα, lymphotoxin α; shRNA, short hairpin RNA; 3′-UTR, 3′-untranslated region; RRM, RNA recognition motif

Author contributions: NS, AD, AB, TB, TS have performed the experiments for the original version of the manuscript AD and AB have performed the experiments needed for the revision NS, AD, TS, JP and WE have designed the study and analyzed the data WE has written the manuscript.

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http://dx.doi.org/10.1016/j.febslet.2015.05.015
0014-5793/

carcinogenesis [13]. Physiologically, LTα-mediated signaling controls hepatic stellate cell function and wound healing processes which are critical for liver regeneration [14]. Given the tumorigenic properties of LTα and HuR, we demonstrate that LTα is a direct target of posttranscriptional regulation by HuR. Furthermore, we characterized the HuR-LTα mRNA interaction by different RNA binding assays using various truncated HuR constructs.

2. Materials and methods

2.1. Materials

Actinomycin D (from *Streptomyces* species), and anti-β-tubulin antibodies were purchased from Sigma–Aldrich (Deisenhofen, Germany). Ribonucleotides and modifying enzymes were from Life Technologies (Karlsruhe, Germany). Antibodies against tristetraprolin (TTP) were from Abcam (Cambridge, UK). All the other antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Protein G Sepharose, the ECL system and Hyperfilm were from GE Healthcare (Freiburg, Germany). All cell culture media and supplements were purchased from Life Technologies.

2.2. Cell culture

HepG2 human hepatocellular carcinoma cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. 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 constant selection shRNA transfected cells were grown in medium supplemented with 2 μg/mL puromycin.

2.3. Stable knockdown of HuR

HepG2 cells stably expressing a HuR shRNA vector ("HuR k/d cells") and HepG2 cells expressing a non-target shRNA (ctr cells) were generated by lentiviral transduction as described previously [15].

2.4. Western blot analysis

Cells were grown on 6 cm dishes, harvested in 100 μl lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) and lyzed by repeated cycles of freezing–thawing. 10–20 μg of protein per sample were separated on SDS–PAGE and transferred onto Hybond nitrocellulose membrane (GE Healthcare). After blocking in 2% BSA the membranes were probed with specific antibodies and appropriate peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with chemiluminescence using an ECL system (GE Healthcare). Nuclear and cytoplasmic extracts from HepG2 cells were prepared by using a protocol from Schreiber et al. [16].

2.5. Plasmid constructs

Expression plasmids for purification of GST-tagged wild-type HuR (HuR-wt) and different HuR truncations were a kind gift from Prof. Dr. Chemnitz (University of Göttingen, Germany) and were cloned as described previously [17].

2.6. Generation of plasmids bearing the 3′-UTR of LTα for in vitro transcription

The plasmid "pcDNA 3.1 3′-UTRLTα" containing 584 bp of a region from the wild-type 3′-UTR of human LTα mRNA was generated by using *BamHI* and *Not I* restriction sites (underlined) as followed: *BamHI*-flanked (underlined) forward primer 5′-ATA GGA TCC GTC TTC TTT GGA GCC TTC GCT CTG-3′ and *NotI*-flanked (underlined) reverse primer 5′-ATA GCG GCC GCG GCT TTC AGA GCC TTT CCC TGC-3′, corresponding to a region from nucleotide 805 to 1389 of the human LTα mRNA (GenBankTM accession No. NM_001159740.2) directly into *BamHI*/*Not I* cut pcDNA.3.1 vector. The plasmid "pcDNA 3.1. 3′-UTR-LTα mut1/2" bearing point mutations in two adjacent ARE sites was generated by site-directed mutagenesis using the following (sense) primers: LTα mut1: 5′-GGC AGG GAG GGG ACT ACC CAT GAA GGC AAA AA-3′, (corresponding to a region from 1175 to 1207) and LTα mut2: 5′-GAA GGC AAA AAA ACC AAA GGA CCT ATC CAT GGA GGA TGG AGA GAG-3′, corresponding to a region from 1196 to 1240 of the human LTα mRNA. All mutants were generated with the Quick Change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing.

2.7. Generation reporter plasmids and transient transfection of HepG2 cells

The plasmid psiCHECK2-LTα-3′UTR was generated by cloning a 584 bp fragment from the 3′UTR of human LTα mRNA into the psiCHECK2 vector (Promega, Madison, WI). Using the *XhoI*-flanked (underlined) forward primer 5′-ATA CTC GAG GTC TTC TTT GGA GCC TTC GCT CTG-3′ and *NotI*-flanked (underlined) reverse primer 5′-ATA GCG GCC GCG GCT TTC AGA GCC TTT CCC TGC-3′ corresponding to a region from nucleotide 805 to 1389 of the human LTα cDNA (GeneBank accession No. NM_001159740.2), the 3′-UTR sequence of human LTα gene was generated by PCR from reverse transcriptase products and subsequently cloned into the *XhoI*/*NotI* cut psiCHECK2 vector, thereby allowing a forced insertion at the 3′end of the *Renilla* luciferase coding region.

For transient transfection, cells were grown on six-well plates and transfected with 400 ng of plasmid DNA using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Cells were lysed for luciferase assay 24 h after transfection and Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol and *Firefly* luciferase was used for normalization.

2.8. Expression and purification of HuR proteins

Expression and purification of wild-type or different GST-HuR deletion mutations was done as described previously [18].

2.9. RNA isolation and quantitative real-time PCR experiments

Total RNA was extracted from whole cells by using the Tri reagent (Sigma–Aldrich) and first strand cDNA was synthesized using random hexamer primer and SuperScript reverse transcriptase (Fermentas, St. Leon-Rot, Germany). Levels of specific cDNAs were determined by semiquantitative PCR or two-step real-time PCR as described previously [3]. For quantitative PCR, the C(T) values of mRNA levels were normalized to the C(T)values of GAPDH mRNA within the same sample. The following oligonucleotides were used for PCR: HuR forward (5′-CAC AGC TTG GGC TAC GGC TTT GTG-3′), and HuR reverse (5′-AGG ACC CGC GAG TTG ATG ATC CG-3′), LTα forward (5′-ATG ACA CCA CCT GAA CGT CTC-3′) and LTα reverse (5′-GGT CTC CAA TGA GGT GAG CA-3′), COX-2 forward (5′-TTC AAA TGA GAT TGT GGG AAA ATT GCT-3′), and COX-2 reverse (5′-AGA TCA TCT CTG CCT GAG TAT CTT-3′), cyclin A forward (5′-ATT AGT TTA CCT GGA CCC AG-3′), and cyclin A reverse (5′-CAC AAA CTC TGC TAC TTC TG-3′), GAPDH forward (5′-CAC CAT CTT CCA GGA GCG AG-3′), and GAPDH reverse (5′-GCA GGA GGC ATT CCT GAT-3′).

2.10. RNP-IP RT-PCR analysis

Immunoprecipitation of HuR was performed as described previously [3]. After washing the beads several times with binding/wash buffer (140 mM NaCl, 8 mM, K_2HPO_4, 2 mM Na_3PO_4, 10 mM KCl), the bound mRNA was extracted by addition of 1 mL Tri-reagent and reverse transcribed using SuperScript reverse transcriptase (Fermentas), and subsequently subjected to semiquantitative PCR by using gene-specific primers described before. Normalization of input RNA was confirmed by RT reaction of total cellular RNA isolated from 10% of the cell extract used for the IP.

2.11. Biotin pull-down assay

To generate biotinylated RNA sense probes for the biotin pull-down assay, 20 μg of either linearized plasmid pcDNA 3.1 3′-UTR-LTα or pcDNA 3.1 3′-UTR-LTα mut1/2 were in vitro transcribed and biotinylated as described previously [19].

2.12. Separation of polysomes from ribonucleoprotein particles (RNPs)

Separation of polysomes from RNPs was performed as described previously [20]. The purity of both fractions was confirmed by the detection of the translation initiation factor eIF4A which is exclusively found in polysomes but not in the fractions of translational inactive RNPs [21].

2.13. RNA electromobility shift assay (EMSA)

RNA gel shift assays for the assessment of RNA-binding capacities of different HuR truncations were accomplished as described previously [18]. The sequence of a RNA oligonucleotide corresponded to a region from the 3′-UTR of the human LTα mRNA from nucleotide 1182 to 1231 (GenBankTM accession No. NM-001159740.2) encompassing two adjacent ARE sites (underlined) as followed: 5′-AGG GGA CUA UUU AUG AAG GCA AAA AAA UUA AAU UAU UUA UUU AUG GGA-3. Supershift analysis was performed as described previously [18].

2.14. Statistical analysis

Results are expressed as means ± S.D. Unless indicated otherwise, experiments were performed at least three times. Statistical analysis was performed using Student's *t*-test.

3. Results

3.1. Reduced expression of LTα in HuR knockdown HepG2 cells

The mRNA coding for the precursor of lymphotoxin α (LTα) appeared among a previously published list of mRNAs being significantly downregulated in HuR deficient thymocytes [22]. Since upregulation of LTα is functionally relevant for HCC [13], we first tested LTα expression in HepG2 cells stably depleted for HuR by a specific shRNA (HuRk/d). Knockdown efficiency was confirmed by a robust reduction of HuR at the mRNA (Fig. 1A) and protein level, respectively (Fig. 1B) when compared to HepG2 cells expressing a non-target shRNA (ctr.). Similarly, HuR k/d cells revealed a strong and significantly reduction in steady-state LTα mRNA (Fig. 1A) and protein (Fig. 1B) contents. Subcellular cell fractionation furthermore showed that LTα in contrast to HuR protein, is mainly localized to the cytoplasm (Fig. 1C).

3.2. Posttranscriptional regulation of LTα mRNA

To analyze whether the HuR depletion-dependent changes in LTα expression are attributed to a reduced mRNA-stability ctr cells and HuR k/d cells were treated with actinomycin D (5 μg/mL). A comparison of the time-dependent mRNA decay in both cell types by qRT-PCR revealed that the half-life of LTα mRNA was clearly reduced in HuR deficient cells when compared with control cells (Fig. 2A, left panel). In contrast, both cell lines displayed a similar decay of Jun-N-terminal kinase (JNK) encoding mRNA which contains no prototypical HuR binding elements in its 3′-UTR and which is not a target of HuR-dependent mRNA-stabilization [21].

3.3. HuR depleted cells exert a clear shift from polysomes to ribonucleoprotein particles (RNPs)

As a direct consequence of HuR-dependent stabilization the HuR-bound mRNA cargo in many cases is increasingly transported to the translation apparatus and therefore we tested whether the relative LTα mRNA distribution between inactive RNPs and polysomes would be changed upon genetic HuR depletion. Previously, we could demonstrate that in HepG2 cells inhibitors of the actin-myosin dependent cytoskeleton can induce a strong change in polysomal mRNA loading concomitant with a change in the polysomal HuR allocation [21]. Similar to the changes in intracellular distribution of some prototypical HuR-target mRNAs [21], the stable knockdown of HuR caused a clear shift in LTα mRNA abundance from polysomal to RNP fractions (Fig. 2A, right panel). These data implicate that shRNA-mediated HuR depletion can affect LTα expression by different posttranscriptional events including mRNA-stability, mRNA translocation and/or translation.

3.4. Constitutive HuR binding to the 3′-UTR of LTα mRNA

Since the 3′-UTR of LTα contains two regions with typical HuR binding signatures (Fig. 2B), we tested for a direct HuR binding to LTα mRNA in HepG2 cells. Indeed, a strong and constitutive binding of HuR to LTα mRNA was confirmed by pull-down RT-PCR assay. Similarly, cyclooxygenase-2 (COX-2) and cyclin A encoding mRNAs, two well-described HuR targets, showed a constitutive binding with HuR (left panel of Fig. 2B). The specific HuR binding to the LTα-3′-UTR is proven by biotin pull-down assay using an in vitro transcribed RNA encompassing the 3′-UTR of LTα (Fig. 2C). In contrast, TTP another prominent ARE-binding protein highly abundant in HepG2 cells did not attach to LTα mRNA (Fig. 2C). The fact that HuR binding was strongly impaired if these AREs had been mutated strongly implicates that HuR binding to LTα mRNA is committed by these *cis*-regulatory elements (Fig. 2C).

3.5. The 3′UTR of LTα mRNA confers reporter gene activation by HuR

To further test whether HuR depletion would affect posttranscriptional gene expression driven by the 3′UTR of LTα, cells were transiently transfected with a luciferase reporter gene containing the complete 3′UTR of LTα downstream of the luciferase coding sequence and under the control of a constitutively active human simian virus promoter. Correspondingly, cells were transfected with the empty reporter gene. Importantly, luciferase activity was significantly lower in HuR depleted HepG2 cells than in control cells if the luciferase gene is under the control of the 3′UTR of LTα (Fig. 3A). In contrast, the knockdown of HuR did not affect luciferase activity driven by the empty reporter vector (Fig. 3A) demonstrating that the 3′UTR of the LTα mRNA confers HuR responsiveness.

Accordingly, using EMSA with an RNA oligonucleotide encompassing both AREs confirmed a strong binding of a fast migrating

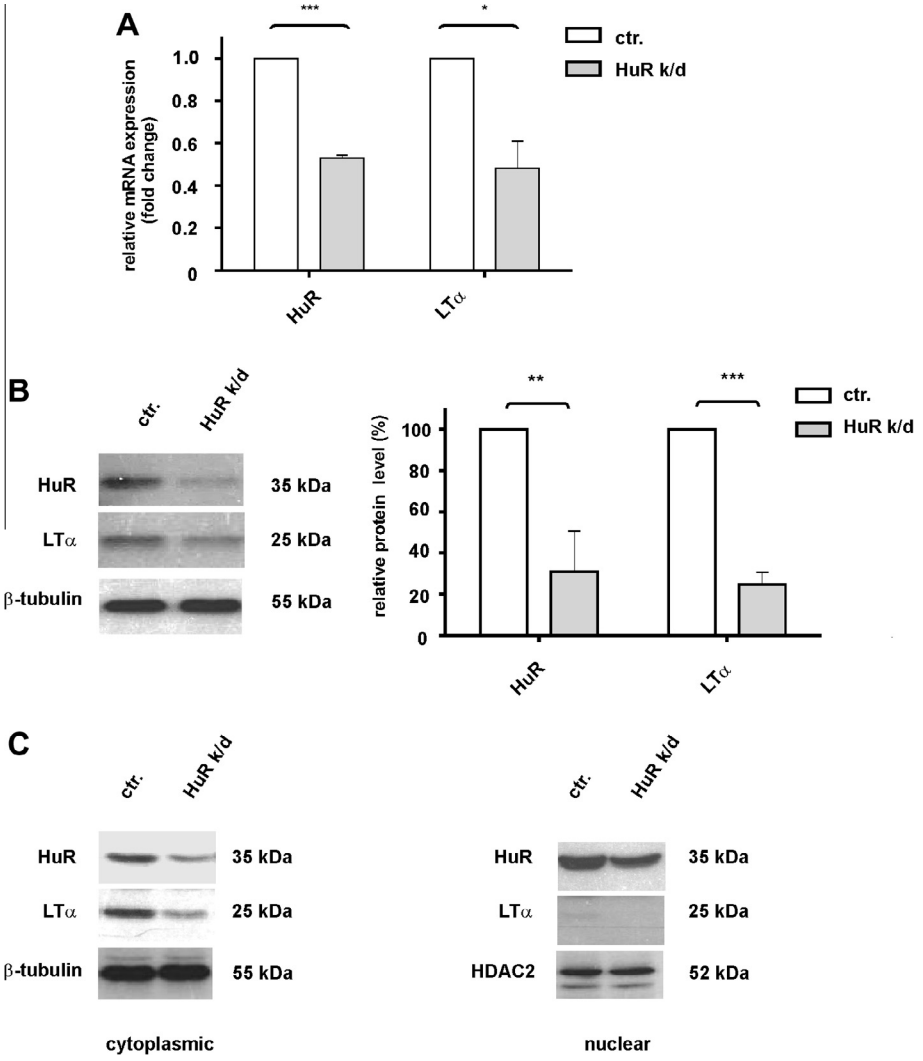

Fig. 1. (A) Downregulation of LTα in HuR depleted HepG2 cells. Control HepG2 cells (ctr.) or HuR depleted HepG2 cells (HuRk/d) were serum-starved for 16 h before the content of HuR and LTα encoding mRNAs was assessed by quantitative real-time PCR using GAPDH mRNA as a normalization control. Values represent means ± S.D. ($n = 4$) and are depicted as – fold induction ($^{***}P \leqslant 0.005$, $^{*}P \leqslant 0.05$) compared to control cell levels. (B, C) Cells were treated as described in (A), harvested for either total cell lysates (B) or nuclear and cytoplasmic cell fractions, respectively (C). Equal amounts of protein (10 μg) from specific cell fraction were subjected to SDS–PAGE and probed with the indicated antibodies using β-tubulin (B and C) or HDAC2 (C) as loading control. Data in (B) show densitometric analysis of HuR and LTα relative to β-tubulin in HuR k/d (grey bars) and control cells (open bars) from three independent experiments and show relative protein levels (%) ($^{***}P \leqslant 0.005$, $^{**}P \leqslant 0.05$) compared with the levels of the corresponding protein in control cells.

complex (Fig. 3B) in the cell lysates from HepG2 cells (ctr.) which was clearly reduced in the lysates isolated from HuR k/d cells (Fig. 3B). In a similar manner, GST-tagged HuR displayed the binding of a major complex which completely disappeared after the addition of HuR-specific supershift antibodies (Fig. 3C). In contrast, GST alone displayed no RNA binding affinity (Fig. 3C).

3.6. Identification of HuR domains critical for LTα mRNA-binding

Next, we compared the binding affinity of different GST-HuR fusion proteins including full-length HuR and various HuR truncations by EMSA (Fig. 4A). The purity and size of the indicated HuR constructs was initially confirmed by SDS–PAGE (Fig. 4B, upper panel). Testing the RNA-binding capacity of these HuR proteins by EMSA revealed that truncation of the N-terminal RRM1 did not affect the affinity of HuR to the LTα-specific RNA oligonucleotide (construct 5) and conversely, RRM1 alone (construct 4) failed to interact with the same RNA probe (Fig. 4B, lower panel). Similarly, RRM2 alone (construct 6) and a GST-fusion protein containing the hinge region plus RRM3 (construct 7) exerted no RNA affinity (Fig. 4B, lower panel). Interestingly, deletion of the C-terminal RRM3 (construct 3) abrogated binding of a fast migrating complex but did substantially increase the binding of a slow migrating complex which probably represents a HuR dimer. These data demonstrate that RRM1 and RRM3 are dispensable for LTα mRNA-binding. Notably, when loading construct 5, we observed that, similar to full-length HuR, a main portion of radioactivity accumulated in the pockets of the EMSA gel. We suggest that the C-terminal RRM3 is not relevant for LTα mRNA-binding but presumably involved in the formation of larger RNA-bound HuR complexes which are unable to migrate through the gel.

4. Discussion

In this study, we have identified LTα mRNA as a novel target of HuR-dependent mRNA-stabilization. Functionally, the increase in LTα mRNA decay observed in stable HuR knockdown cells results in a significant reduction in LTα mRNA levels and is concomitant with a strongly diminished content of LTα protein (Fig. 1A). We

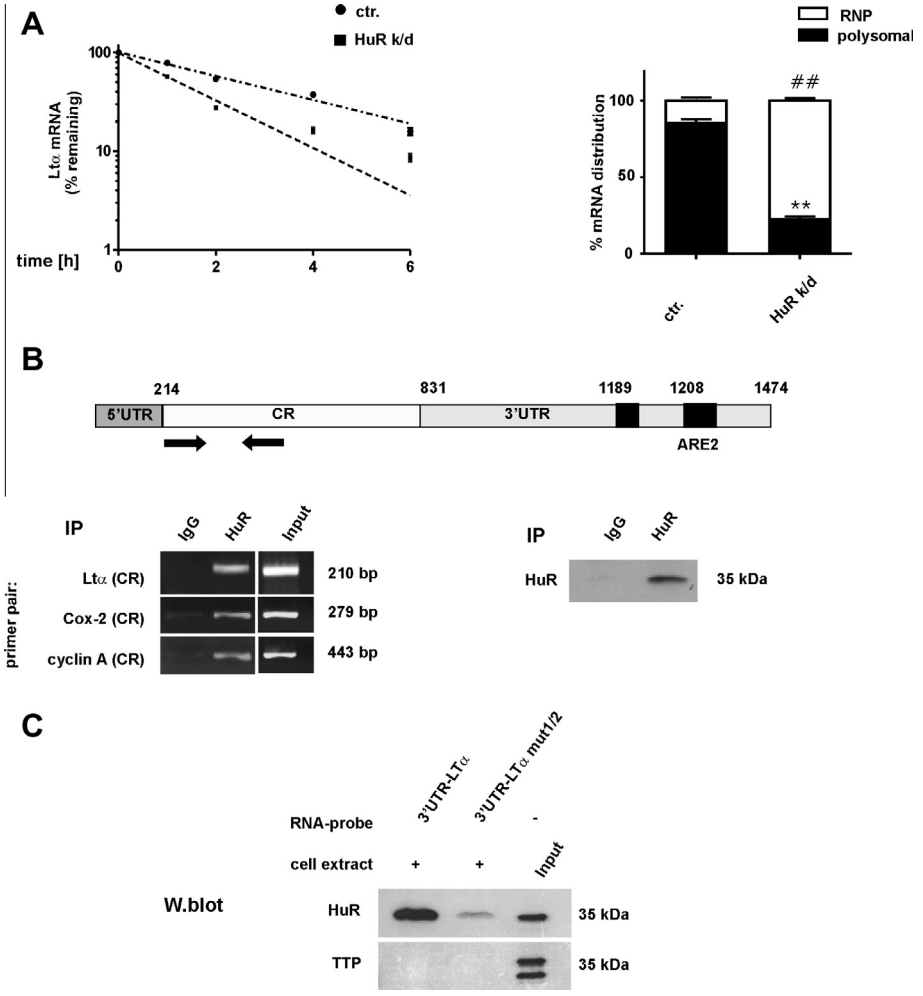

Fig. 2. LTα mRNA is a target of different HuR-triggered mRNA functions. (A, left panel) Comparison of LTα-mRNA decay after administration of actinomycin D (5 μg/mL) in ctr. cells (filled circles) and HuR k/d cells (filled squares). mRNA levels were quantified by qRT-PCR using GAPDH as a normalization control. Data show means of a triplicate experiment as compared to the levels of mRNA measured before the addition of actinomycin D (set as 100%) (A, right panel) The total RNA from polysomal (black bars) and RNP fractions (white bars) was isolated and the content of the HuR target LTα mRNA was measured by qRT-PCR as described in Materials and Methods. The LTα mRNA levels within the indicated fractions are depicted in a relationship to the amount of mRNA pooled from both fractions. Values represent means ± S.D. ($n = 3$). $^{**}P \leqslant 0.01$ versus polysomal fractions (black bars) from control cells; $^{\#\#}P \leqslant 0.01$ versus vehicles from RNP fractions (open bars) from control cells and are furthermore depicted as % relative mRNA distribution. (B) Constitutive binding of HuR to the 3′-UTR of LTα mRNA in control HepG2 cells. A schematic representation of the LTα-3′-UTR and AREs (black boxes) is shown in the upper panel. (left panel) For the assessment of endogenous HuR-mRNA interactions, HuR was pulled down out of total cell lysates from control HepG2 cells and HuR-bound mRNAs were subsequently analyzed by semiquantitative RT-PCR using gene-specific primers from the coding regions (CR). Normalization of similar amounts of input RNAs added to the IPs was confirmed by RT-PCR. The specific immunoprecipitation (IP) of HuR was confirmed by western blot analysis shown in the right part of the panel (C). For biotin pull-down assay a biotinylated transcript encompassing 584 nucleotides of the 3′-UTR of human LTα (3′-UTR-LTα) or, alternatively, a corresponding transcript with mutated AREs (3′-UTR-LTα mut1/2) was incubated with total cell lysates from control HepG2 cells. Specific binding of HuR was confirmed by Western blotting (W. blot).

further demonstrate that HuR specifically binds to the 3′-UTR of LTα mRNA with two adjacent ARE motifs being critical for the protein–RNA interaction (Fig. 2C).

In an attempt to further delineate which HuR domain is involved in LTα mRNA-binding, we demonstrate that HuR binding is structurally related to a minimal region encompassing RRM2 and the hinge domain since deletion of neither the C-terminal RRM3 nor of the N-terminal RRM1 abrogated the affinity of HuR to the 3′-UTR of LTα mRNA (Fig. 4B). Furthermore, EMSA results suggest that RRM3 is involved in the formation of higher molecular weight HuR complexes which were unable to migrate through the non-denaturating EMSA gel (Fig. 4B). This observation is in a full agreement with a previously published study demonstrating that RRM3 in HuR is mainly required for the cooperative assembly of HuR oligomers on RNA [23]. In addition, RRM3 was found to be involved in poly A-binding [24] and relevant for the interaction with other protein ligands [25].

Furthermore, our data indicate that the posttranscriptional regulation of LTα by HuR is not restricted to T cells but is also relevant for non-immune cells. Similar to LTα, TNFα another member of the TNF superfamily is a well described target gene of the RNA-binding protein HuR [26,27]. Interestingly, in a contrast to HuR, TTP an ARE-binding protein which is critically involved in posttranscriptional control of TNFα expression [28], could not be detected in the LTα-biotin pull-down assay (Fig. 2C). Notably, in addition to TTP a complex network of different RNA binding proteins like AUF1 and KSRP were shown to compete with HuR for binding to a common target mRNA [29]. Therefore, it is tempting to speculate that posttranscriptional regulation of LTα is not solely by HuR but instead of this determined by the relative abundance and activity of a set of functionally antagonising and/or cooperating ARE-RNA binding proteins. Owing to the known preference of some miRNAs seeds to reside in a close context of AREs and to the described ability of HuR to modulate miRNA binding, LTα mRNA

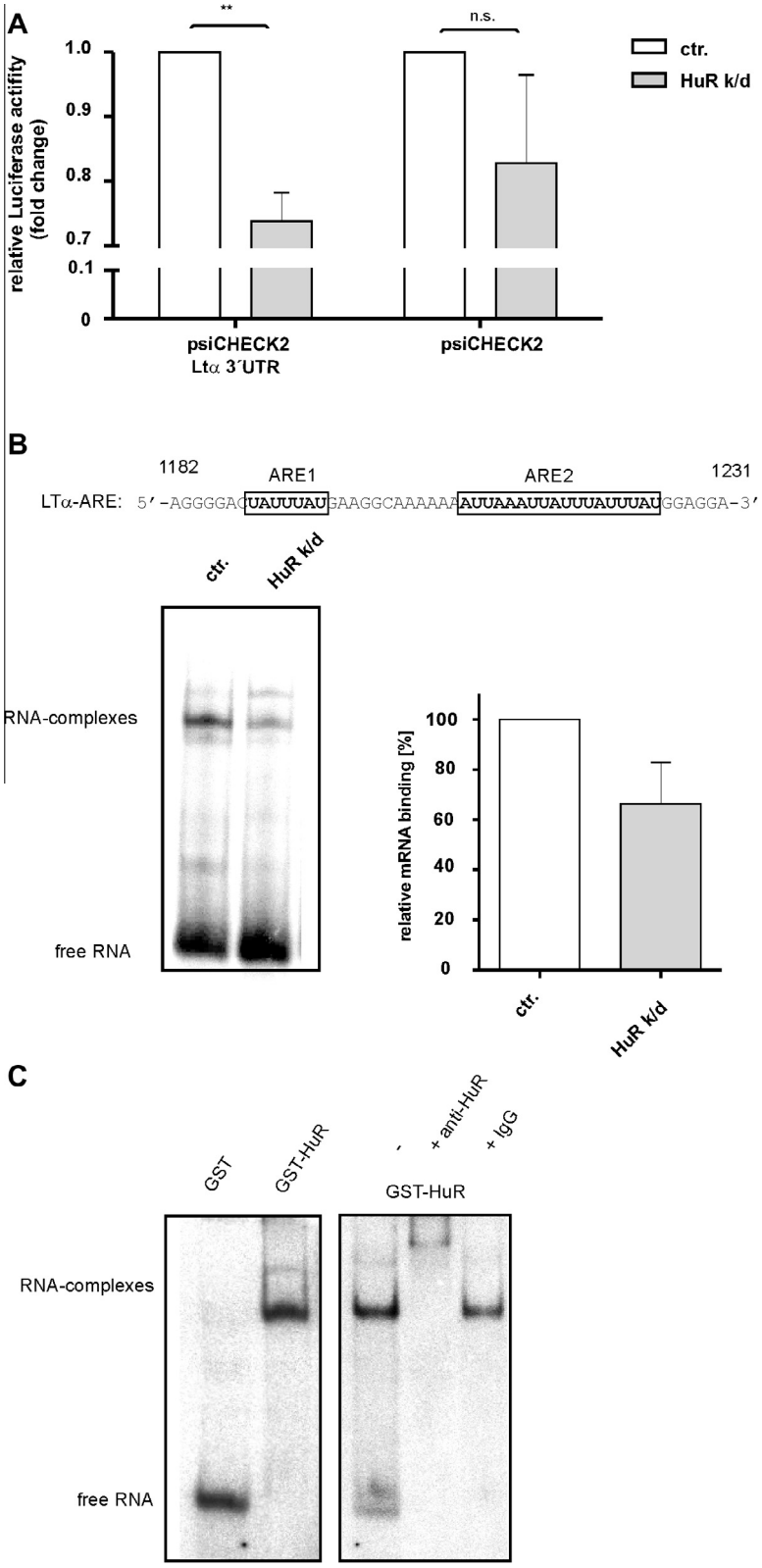

Fig. 3. (A) Luciferase assays from control cells (white bars) or HuR k/d cells (grey bars) transfected with either an empty psiCHECK2 vector or with a construct carrying the complete 3′-UTR sequence of human LTα mRNA (psiCHECK2-LTα-3′UTR). *Renilla* luciferase activities for each construct was normalized with *Firefly* luciferase activities and are depicted as relative luciferase activities. Data (means ± S.D.) represent the results of three independent experiments. $^{**}P \leqslant 0.01$; n.s. not significant (B). RNA binding of total cell lysates (5 μg) from control HepG2 cells (ctr.) or HuR k/d cells was analyzed by EMSA using a ^{32}P-labeled RNA probe encompassing two AREs from the 3′-UTR of LTα mRNA (upper panel). A denistometrical analysis of mRNA binding relative to ctr. cells (100%). (C) Similarly, recombinant GST-HuR exerts a high RNA-binding affinity to the 3′-UTR of LTα. For EMSA, 200 ng of bacterially expressed GST-HuR or GST was incubated with the LTα-3′-UTR-specific oligonucleotide shown in (B). For supershift analysis, 200 ng of anti-HuR antibody or mouse IgG were added to the binding reaction. Gel pictures are representative for three independent experiments.

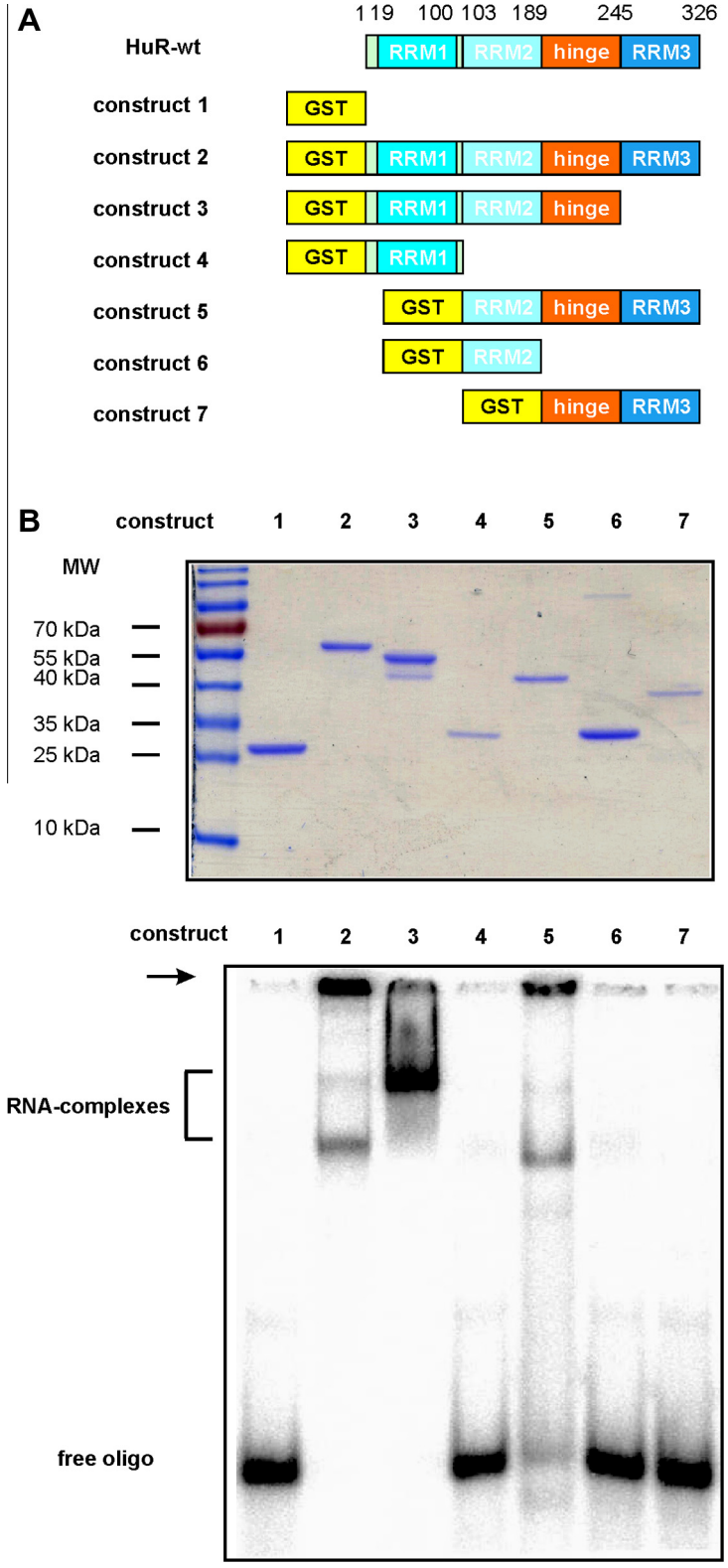

Fig. 4. Identification of HuR domains critical for LTα mRNA binding. (A) Schematic representation of constructs coding for different GST-HuR fusion proteins (B). In each lane 1 μg of recombinant protein was loaded on SDS–PAGE and proteins subsequently stained by Coomassie-blue. ^{32}P-labeled RNA probe depicted in panel A was incubated with the indicated fusion proteins and RNA-binding assessed by EMSA. Positions of the gel pockets are indicated by an arrow.

levels may additionally result from combinatorial posttranscriptional gene regulation by HuR and miRNAs [30].

Mechanistically, LTα via binding and signalling through the TNF receptors type I and II exhibits overlapping biological activities with TNFα [13,31]. In the liver, LTα mediated signalling which involves an activation of the classical and alternative NFκB pathway, was previously identified as a crucial step in hepatitis-induced HCC and is therefore considered as a promising target for novel HCC preventive pharmacotherapies [31]. Pathologically, several lines of evidence gathered from clinical

studies, animal models and cell culture models implicate that HuR is overexpressed in many tumors. In the liver, HuR seems essential for the differentiation, proliferation and survival of HCC [13]. For this reason, besides LTα, HuR emerges as a promising target for novel anti-tumor therapies. Further studies are needed to confirm a regulatory role of HuR in the control of LTα expression and its pathological impact on HCC.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

We gratefully acknowledge Roswitha Müller and Silke Kusch for their technical assistance. We thank Prof. Jan Chemnitz, (Leibniz Institute for Experimental Virology, Hamburg, Germany) for kindly providing the GST-HuR constructs. This work was supported by the State of Hesse [Onkogene Signaltransduktion L-4-518/55.004] and by the Deutsche Forschungsgemeinschaft [GRK 1172 and EB 257/6-1].

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