### Manuscript Number:
EBJO-D-11-00227R1

### Full Title:
HuR Thermal Stability is dependent on Domain Binding and upon Phosphorylation

### Article Type:
Original Paper

### Keywords:
HuR; Phosphorylation; Post-translational Modifications; RNA Binding Protein; RNA Recognition Motif; Protein Thermal Stability.

### Corresponding Author:
Irene Díaz-Moreno, Ph.D.
University of Seville - CSIC
Seville, SPAIN

### Corresponding Author Secondary Information:

### Corresponding Author's Institution:
University of Seville - CSIC

### Corresponding Author's Secondary Institution:

### First Author:
Rafael M. Scheiba, Ph.D. Student

### First Author Secondary Information:

### Order of Authors:
Rafael M. Scheiba, Ph.D. Student
Ángeles Aroca, Ph.D.
Irene Díaz-Moreno, Ph.D.

### Order of Authors Secondary Information:

### Abstract:
Human antigen R (HuR) is a multitasking RNA binding protein involved in post-transcriptional regulation by recognizing Adenine and uracile Rich Elements (AREs) placed at the 3’ untranslated regions of mRNAs. The modular architecture of the protein, which consists of two N-terminal RNA recognition motifs (RRMs) in tandem spaced from a third one by a nuclear-cytoplasmic shuttling sequence, controls stability of many mRNA targets, as well as their translation rates. A higher level of regulation comes from the fact that both localization and function of HuR is strictly regulated by phosphorylation. Here, we report how the thermal stability of RRM2 is decreased by the presence of RRM1, indicating that both domains are interacting in solution. In addition, even though no significant structural changes are observed among mutants of HuR RRM12 mimicking phosphorylated species, slight differences in stability are appreciable, which may explain the RNA binding activity of HuR.

### Response to Reviewers:
See attachment.
HuR Thermal Stability is dependent on Domain Binding and upon Phosphorylation

Rafael Manfred Scheiba, Ángeles Aroca and Irene Díaz-Moreno*

Instituto de Bioquímica Vegetal y Fotosíntesis, cicCartuja, Universidad de Sevilla-CSIC, Americo Vespucio 49, Sevilla 41092, Spain

Running head: Thermal Stability of HuR

Address correspondence to:

Irene Díaz-Moreno

Telephone: +34 954489513; Fax: +34 954460065; Email: idiazmoreno@us.es
Abstract

Human antigen R (HuR) is a multitasking RNA binding protein involved in post-transcriptional regulation by recognizing Adenine and uracile Rich Elements (AREs) placed at the 3′ untranslated regions of mRNAs. The modular architecture of the protein, which consists of two N-terminal RNA recognition motifs (RRMs) in tandem spaced from a third one by a nuclear-cytoplasmic shuttling sequence, controls stability of many mRNA targets, as well as their translation rates. A higher level of regulation comes from the fact that both localization and function of HuR is strictly regulated by phosphorylation. Here, we report how the thermal stability of RRM2 is decreased by the presence of RRM1, indicating that both domains are interacting in solution. In addition, even though no significant structural changes are observed among mutants of HuR RRM12 mimicking phosphorylated species, slight differences in stability are appreciable, which may explain the RNA binding activity of HuR.

Keywords:

HuR; Phosphorylation; Post-translational Modifications; RNA Binding Protein; RNA Recognition Motif; Protein Thermal Stability.
Abbreviations:

AREs: Adenine and uracile Rich Elements

CARM1: Coactivator-associated ARginine Methyltransferase 1 protein

CD: Circular Dichroism

Chk2: Checkpoint 2 kinase

Cdk: Cycline dependent kinase 1

DSF: Differential Scanning Fluorimetry

DTT: DiThioThreitol

ELAV: Embryonic Lethal and Abnormal Vision

HNS: Human Novel Shuttling

HuR: Human antigen R

HuR FL: HuR full-length

$K_D$: Dissociation affinity constant

PKCα: Protein Kinase C α

PKCδ: Protein Kinase C δ

RBP: RNA Binding Protein

RMSD: Root Mean Square Deviation

RRM: RNA Recognition Motif

RRM12 WT: RRM12 wild-type

RT-PCR: Real Time Polymerase Chain Reaction

$T_m$: Midpoint Melting Temperature

UTRs: UnTranslated Regions
Introduction

Human antigen R (HuR) is a ubiquitous 36-kDa RNA Binding Protein (RBP) consisting of three RNA Recognition Motifs (RRMs; Birney at al. 1993; Ma et al. 1996). HuR (also known as ELAV-like protein 1) plays a key role in cell cycle, stress stimuli, inflammation and cancer. HuR controls such functions by recognizing the Adenine and uracile Rich Elements (AREs) placed at the 3’-Untranslated Regions (UTRs) of certain RNA (Abdelmohsen et al. 2007a; Brennan et al. 2001; Dixon et al. 2001; Gorospe 2003; Sengupta et al. 2003). As a consequence, the expression level of these RNA targets is affected, so dependent processes in the cell are regulated. In fact, HuR has been characterized as an anti-apoptotic switch tightly regulated by a post-transcriptional orchestration (Abdelmohsen et al. 2007a). However, it has been recently reported that pro-apoptotic reactions can also be supported, which depend on the caspase-mediated cleavage of HuR (Mazroui et al. 2008).

It is worth to mention that there are many studies concerning the behavior of HuR in the cellular environment, although little is known about the structure and the related molecular mechanisms of this RBP. HuR is a multidomain protein whose three RRM domains – named RRM1 and RRM2 - are in tandem only separated by a 3_{10}-helix turn, whereas the C-terminal RRM3 motif is spaced by a 60-residue linker spanning the hinge called Human Novel Shuttling (HNS) sequence (Figure 1a; Fan et al. 1998). Actually, HNS is known to determine the cellular localization of HuR either in the nucleus or the cytoplasm. Recently, the crystal structure of the first N-terminal RRM domain has been solved (Benoit et al. 2010), although the global protein structure remains unknown.

Post-translational modifications play an essential role in the cellular function of HuR. Recent research has revealed several phosphorylation sites in HuR which
influence the interaction with its RNA targets, with other proteins and even in its cellular localization (Abdelmohsen et al. 2007b; Doller et al. 2008; Kim et al. 2008a-c). Such phosphorylations can be performed by different kinases as Checkpoint 2 kinase (Chk2), Cycline-dependent kinase 1 (Cdk1) and Protein Kinases C α or δ (PKCα or PKCδ). Upon HuR phosphorylation, different cellular responses have been described (Abdelmohsen, 2007 a,b; Doller et al. 2008; Kim 2008a-c). Whereas the HuR capability for binding to RNA targets increases or decreases when Chk2 phosphorylates HuR at Ser88 or Ser100 residues, respectively (Abdelmohsen et al. 2007b), the addition of a phosphate group to Ser158, Ser221 and Ser318 by PKC favors the cytoplasmic localization of HuR instead of the preferred nuclear localization of the protein (Doller et al. 2008, 2009), along with an enhancement in the mRNA binding (Doller et al. 2007). In addition to Ser221 at HNS, HuR also becomes phosphorylated at Ser242, which is also involved in the nucleocytoplasmic shuttling (Kim et al. 2008c). The HuR shuttling can provide information about the cell state. Indeed, an increase of cytoplasmic HuR levels is an indicator for the stress response of the cell (Gorospe, 2003) or different kind of cancer diseases (Denkert et al. 2004; Heinonen et al. 2005).

An additional HuR post-translational modification consists on a methylation at Arg117 by CARM1 (Coactivator-associated ARginine Methyltransferase 1) protein (Li et al. 2002).

Given that both functionality and localization of HuR are strictly regulated by phosphorylation, exploring the stability of its N-terminal RRM domains after being post-translationally modified would be highly valuable to understand the pleiotropic role of HuR in mRNA metabolism. Within this frame, this work suggests that the domains RRM1 and RRM2 as a cooperative assembly remains unchanged upon phosphorylation events of three Ser residues localized inside RRM motifs (the non-conserved Ser88 and Ser158 at RRM1 and RRM2, respectively) and at the interdomain
linker (Ser100 highly conserved among the family members HuB, HuC and HuD, as well as the ELAV - Embryonic Lethal and Abnormal Vision - *Drosophila* homologue).
Materials and Methods

Site-Directed Mutagenesis of HuR RRM Constructs

pGEX 5X2 vectors containing the sequences coding for HuR full-length (HuR FL) as well as individual N-terminal RRM domains – RRM1 and RRM2 - and the two-domain construct RRM12, have been kindly provided by Dr. M. Gorospe (National Institute of Health, Baltimore, USA) and Dr. J.A. Steitz (Yale University, New Haven, USA). These genes were further cloned into the pGEX-4T2 vector, which was modified for RRM12 and HuR FL as follows: The GST sequence was substituted by a 6xHis-tag using the following primers: 5´ CATCATCACCACCATCACctggttcgcgtggatccccagg 3´ (forward primer) and 5´ GTGATGGTGGTGATGATGcatgaatactgttccctgtg 3´ (reverse primer) to facilitate the purification. Both GST and 6xHis tags were cleaved with thrombine, resulting in a short additional aminoacid sequence for all constructs “GSPGIPSNYEDH”, with a negligible effect on the secondary structure analysis. Serines at positions 88, 100 and 158 of the RRM12 construct were replaced by alanines or aspartates by site-directed mutagenesis (Mutagenex, Piscataway, USA).

Protein Expression and Purification of HuR Constructs

Recombinant proteins were expressed in E. coli BL21-T1R (SIGMA, St. Louis, USA) cells as follows. Competent cells were transformed with plasmid DNA and were grown at 30 ºC for HuR FL and at 37 ºC for RRM1, RRM2 and RRM12 constructs, both in LB medium supplemented with ampicillin (50 µg/ml). Protein expression was induced by the addition of 1 mM IPTG once the culture reached an O.D.600 of 0.6–0.8. After 5 h expression in LB medium at 30 ºC for HuR FL and at 37 ºC for the other constructs, cells were harvested by centrifugation at 7000 g and further resuspended in 50 mM Tris Buffer (pH 8.0) for its storage at -80 ºC. The HuR FL protein was resuspended in the same buffer but supplemented with 800 mM NaCl.
GST fusion proteins were purified using a Glutathione Sepharose High Performance Matrix (GE Healthcare, Piscataway, USA), whereas His-tagged constructs were purified by nickel affinity chromatography (Ni Sepharose™ Fast Flow Matrix, GE Healthcare, Piscataway, USA). All constructs were expressed with thrombine-cleavable GST or His tags (GE Healthcare, Piscataway, USA). To separate HuR RRM single domains from the cleaved GST protein, a gel filtration chromatography (sephadex G-75 matrix; SIGMA, St. Louis, USA) was performed.

Samples were concentrated to 80 µM in 10 mM sodium phosphate (pH 7.3) with 0.5 mM DTT. HuR FL was supplemented with 800 mM of NaCl and 0.1% of Sarkosyl detergent to increase its solubility during all purification steps. Protein concentration was determined using spectrophotometry with predicted extinction coefficients. All molecular weights of the HuR constructs used in this work were verified by MALDI-TOF spectroscopy.

**Circular Dichroism Spectroscopy**

All Circular Dichroism (CD) spectra were recorded in the far-UV range (190–250 nm) at 298 K on a Jasco J-815 spectropolarimeter, equipped with a Peltier temperature-control system, using a 1-mm quartz cuvette. Protein concentration was 12 µM in 10 mM sodium phosphate buffer (pH 7.3) supplemented with 0.5 mM DTT. For each sample, 20 scans were averaged for further secondary structure analysis using CDPRO software (Sreerama et al. 2000), which includes the algorithms CONTIN, SELCON and CDSSTR, as well as the CLSTR option to compare with a set of proteins with similar folds.

Thermal unfolding experiments were carried out in a range of temperatures from 298 K to 371 K. For all these assays, the HuR species at 12 µM final concentration were dissolved in 10 mM sodium phosphate (pH 7.3) with 0.5 mM DTT. Temperature was
increased at a rate of 1 K per min with an error within ± 0.1 K. Spectra were recorded at
the scan rate, band width and sensitivity of 200 nm min⁻¹, 1.0 nm and 0.2 deg,
respectively. Protein unfolding was monitored by recording the CD signal at 195, 208
and 235 nm. The experimental data were fitted to a two-state native-denatured model
(Privalov 1979).

RNA binding was monitored by adding increasing amounts of protein to 4 µM
AU-11mer (AUUUUAUUUU) RNA in 10 mM sodium phosphate pH 7.3, 0.5 mM
DTT solution. A temperature of 298 K was chosen to optimize the signal change upon
protein binding. Each CD spectrum was the average of 10 scans. The integral of this
averaged signal between 260 and 275 nm was fitted against the protein concentration
according to Santoro and Bolen (Santoro and Bolen, 1988).

**Differential Scanning Fluorimetry**

Thermal unfolding of HuR constructs was monitored by Differential Scanning
Fluorimetry (DSF), in the presence of the fluorescent SYPRO Orange dye (Invitrogen,
Carlsbad, CA, USA), by using an IQ5 Multicolor Real-Time PCR Detection Instrument
(BioRad; Niesen et al. 2007). The commercial dye (5000× concentrate in DMSO) was
at least ten-fold diluted in 10 mM sodium phosphate buffer (pH 7.3), supplemented with
0.5 mM DTT, and the HuR samples (10-40 µg protein) were added at 25 µL final
volume. The thermal unfolding process was monitored between 293 K and 369 K,
increasing the temperature at a rate of 1 K per min. The values for the midpoint melting
temperature (T_m) were calculated from the first derivative in Origin 8.0 (Microcal Inc.)
and a non linear curve fitting function was used (Privalov, 1979).
Results

HuR RRM Domains Adopt a Canonical Topology with Negligible Changes in their Secondary Structure upon Phosphorylation

The crystallographic structure of HuR RRM1 – recently published by Benoit et al. 2010 - shows the canonical RRM folding adopting the βαβαβ topology.

We have obtained a homology model of HuR RRM12 construct (Figure 1b and 1c) using the crystallographic structure of its homologue HuD RRM12 as a template (PDB entry 1FXL; Wang et al. 2001). Sequence identity to the target was 75.4 % and the model was built with the SWISS-MODEL server (Arnold et al. 2006; Kiefer et al. 2009) and graphically represented using Chimera software (Pettersen et al. 2004). Figure 1c shows the superposition of both HuR structures: the homology model of RRM12 and the crystallographic structure of RRM1.

Our homology model is in a good agreement with the secondary structure contents for HuR constructs. Figure 2 shows the normalized far-UV CD spectra of isolated RRM1 and RRM2 domains, the tandem RRM12 and the HuR FL protein. Notably, all HuR species show similar global secondary structures with minor differences, as summarized in Table 1. Whereas all constructs share similar β-strand and turns contents, RRM2 differs from RRM1 and RRM12 in its higher α-helix content.

RRM12 mutants, in which Ser88, Ser100 and Ser158 have been substituted by aspartic acid residues to mimic phosphorylation events, exhibit secondary structure as that of RRM12 wild-type (RRM12 WT). In addition, Ser-by-Ala control mutations show similar CD spectra (Figure 3 and Table 1).

For further thermal stability on RRM12 WT and its mutants, the impact of Cys13 in the homodimer formation needs to be evaluated (Meisner et al., 2007; Benoit et al., 2010). Figure 4 shows an SDS-page gel of RRM12 WT in absence and in presence of DiThioThreitol (DTT) at 0.5 and 5 mM, as reducing agent. RRM12 WT is
clearly a monomer upon DTT addition, albeit the monomer-dimer equilibrium appears in samples devoid of DTT. This data are recently confirmed by analytical ultracentrifugation on RRM12 WT samples containing 0.5 mM DTT (data not shown). Thus, RRM12 WT construct, which includes Cys13, behaves as a monomer, at least in the experimental conditions used in this work.

**Thermal stability of HuR RRM2 is decreased by the presence of RRM1**

Recently, it has been demonstrated that the thermal stability of RNA binding domains reveals interactions between neighboring modules (Aroca et al. 2011; Díaz-Moreno et al. 2010). Thermal unfolding studies on the single N-terminal RRM segments and the two-domain construct from HuR were performed in order to confirm the assembly between RRM1 and RRM2, as inferred from the homology model of HuR RRM12 and the crystal structure of HuD RRM12 (Wang et al. 2001). CD spectroscopy shows that the $T_m$ for isolated RRM1 (335 ± 3 K) is lower than the one for RRM2 (341 ± 2 K; Table 2). Interestingly, RRM12 is as stable as RRM1 (335 ± 2 K) suggesting that inter-domain interactions are taking place. Such interaction lowers the $T_m$ of RRM2 in ca. 6 K, as previously reported for other RNA binding proteins (Aroca et al. 2011; Díaz-Moreno et al. 2010). In addition, the denaturation curve of RRM12 is not the sum of the denaturation curves of the two individual RRM1 and RRM2 domains, revealing that only one transition state is observed (not two). Indeed, the cooperativity of the RRM12 denaturation is strongly reduced as compared with that of the individual domains.

These changes in stability between isolated RRM2 and RRM2 in RRM12 construct are confirmed by DSF although $\Delta T_m$ is somewhat slightly higher (7 K; Table 2 and Figure 5a). Intriguingly, $T_m$ values calculated by DSF for HuR species are always
equal or lower than those estimated by CD, although $\Delta T_m$ is quite independent of the technique used (see Table 2).

**Stability of HuR RRM12 is Regulated by Phosphorylation**

To analyze the phosphorylation effect of serine residues on the stability of HuR RRM12 construct, this post-translational modification has been mimicked by Ser-to-Asp substitutions. Even though the use of Ser/Asp mutations simulates a constitutively phosphorylated protein with only one negative charge, it is herein extensively recommended since two out of three serine residues of RRM12 WT – those at positions 88 and 100 - become phosphorylated by the same kinase, Chk2, being *in vitro* kinase assays undesirable.

The non-conserved serine residues, which are localized inside the RRM core, play an essential role in the stability of HuR RRM12. It is worth to mention that phosphorylation at Ser88 in RRM1 mimicked by the S88D mutant makes the RRM12 construct slightly more stable than its control mutant (S88A) and RRM12 WT. Indeed, $T_m$ of RRM12 S88D is increased in more than 5 K, using both CD and DSF approaches (Table 2 and Figure 5b). In contrast, the addition of a negatively-charged group at position 158 (mutation S158D) slightly destabilizes HuR RRM12 with regard to the S158A mutant and RRM12 WT, despite the discrepancies on $\Delta T_m$ between CD and DSF. The well-conserved Ser100, which takes part of the short linker between RRM1 and RRM2, displays no significant contributions in thermal stability of HuR RRM12 upon mutations ($\Delta T_m < 2.0$ K). As expected, the non-phosphorylatable Ser-to-Ala RRM12 mutants behave as RRM12 WT in terms of thermal stability, suggesting that HuR phosphorylation has functional consequences rather than structural effects.
RNA binding of HuR RRM12 is Regulated by Phosphorylation

To understand how the interaction of HuR-RRM12 with c-fos AU-11 mer RNA may be regulated upon phosphorylation, we assessed the affinity of RRM12 WT and its phosphomimetic mutants for the RNA target and explored whether the phosphorylation could modulate recognition in vitro, similarly than in vivo. We used CD to obtain quantitative data over affinities which lie in the µM range. Our CD data show that the affinity of the two RRM1 and RRM2 domains for the RNA is in the low micromolar range (2.6 ± 0.2 µM; Table 3 and Figure 6). Next, we investigated the effect of phosphorylation at RRM1 and at the RRM12 linker by RRM12 S88D and RRM12 S100D mutants, respectively, which show $K_D$ values comparable with that of RRM12 WT (2.7 ± 0.2 µM for S88D and 2.0 ± 0.1 µM for S100D; Table 3). In contrast, RRM12 S158D favors RNA binding (0.6 ± 0.3 µM; Table 3) in agreement what has been previously published in vivo (Doller et al., 2007).
Discussion

HuR consists of three RRM domains, whose function in RNA binding is well-characterized, despite the global function and working mechanisms of HuR FL protein are still not fully understood. The interaction between RRM1 and RRM2 as a tandem construct shows the meaning of the modules and the role of binding to each other. The combination of the individual RRM domains with additional post-translational modification sites enables a high variety of regulation of HuR. With the possibility of being phosphorylated (Kim et al. 2008a-c), methylated (Li et al. 2002), ubiquitinated (Abdelmohsen et al. 2009), submitted to protease cleavage mechanism (Mazroui et al. 2008) and recently neddylated (Emroupe et al. 2011), HuR has a huge probability of changing its cellular localization, the binding to other proteins and RNA processing.

Thermal stability studies on HuR species indicate the importance of the cooperation between the the two N-terminal RRM domains of HuR, which work as a functional unit. The comparison of $T_m$ values for isolated RRM1 or RRM2 and the two-domain construct RRM12 reveals that RRM12 shows the same thermal stability as RRM1, while RRM2 is substantially more stable. In addition, the fact that the denaturation curve of HuR RRM12 is not the sum of those from the two individual RRM1 and RRM2 domains suggest cooperativity between both modules.

It is tempting to speculate that the RRM12 modular interaction is essential for RNA recognition activity, similarly to what previously observed for RRM1-RRM2 motifs of the homologous HuD protein upon c-fos RNA binding (Wang et al. 2001). Indeed, the preferred orientation between RNA binding domains helps to establish a high-affinity RNA-binding platform (Vitali et al. 2006; Li et al. 2010) and/or to stabilize a suitable conformation that can adapt to the changes in the direction of the RNA chain inside the highly structured 3' UTRs, as previously suggested (Díaz-Moreno et al. 2010).
To study changes in structure and stability of HuR induced by serine phosphorylation, we have designed three Ser-by-Asp mutations. Two of them are localized at the RRM cores, while the third one is in the inter-domain linker. For none of these phosphomimetic mutants significant changes in secondary structure were observed, unlike what has been recently published for other RNA binding domains (Díaz-Moreno et al. 2009). Therefore, phosphorylation effects on HuR seem to be essentially related to RNA binding properties and/or intermolecular protein interactions than to changes on the HuR structure, as confirmed our CD RNA binding titrations (Figure 6).

Nevertheless the thermal stability of HuR constructs is regulated by phosphorylation. The phosphomimetic mutant S88D slightly stabilizes RRM1 in the RRM12 context, which can be explained by the addition of a negative charge into the protein loop mainly governed by two positively charged residues (Benoit et al. 2010). Thus, Asp88 could minimize the electrostatic repulsion between Arg85 and Lys89, which would restrict the loop mobility. In terms of RNA binding, it has been previously reported that in vivo HuR phosphorylation at Ser88 increases the docking of RNA targets to the RNA binding sites (Abdelmohsen et al. 2007b). Also it is proposed that the phosphoserine at position 88 makes a Mg^{2+}-ion-mediated interaction with a phosphate group from RNA (Benoit et al. 2010). However, no substantial differences in binding affinities were observed between RRM12 WT and the phosphomimetic RRM12 S88D mutant by performing in vitro CD titrations using c-fos-RNA.

Slightly destabilizing phosphorylation of Ser158 could be explained based on electrostatic repulsion with another nearby negative residue Glu162, although the negatively-charged Asp158 is added at the N-end of helix α2 of HuR RRM2. Post-translational modification of Ser158 at RRM2 domain – mimicked by the RRM12 S158D mutation – tightly regulates the binding of HuR RRM12 with c-fos RNA in...
vitro. Actually, the RNA binding affinity of RRM12 S158D is four times larger than the one of RRM12 WT, in agreement with previous data *in vivo* (Doller et al. 2007). Phosphorylation at the level on the RRM12 linker region – Ser100 – has also a negligible effect on HuR stability. A plausible explanation is that this solvent-exposed residue does not make many contacts with neighbors. Intriguingly, phosphorylation at Ser100 increases RNA binding *in vivo* (Abdelmohsen et al. 2007b), although the equivalent serine in the homologous HuD – Ser126 - is facing away from the RNA in the HuD/c-fos mRNA crystal structure (Wang et al. 2001). *In vitro* CD titrations reveal no effect of the S100D mutation on RNA recognition with respect to RRM12 WT. *in vitro* Therefore, phosphorylation at this site would influence RRM2–interdomain linker interactions and the rearrangement between RRM domains, rather than directly repulsing RNA (Benoit et al. 2010).

Perturbations in stability of HuR upon post-translational modifications such as phosphorylation may explain the HuR behavior in binding RNA molecules, as well as in determining their lifetime and translation rate.
Acknowledgements

The authors wish to thank Dr. M. Gorospe (NIH, Baltimore, USA) and Dr. J.A. Steitz (Yale University, New Haven, USA) for providing the HuR vectors. We are grateful to Prof. Miguel A. De la Rosa and Dr. Antonio Díaz-Quintana for critical reading of the manuscript. For financial support we thank the Andalusian Government (P07-CVI-02896) and the Spanish Scientific Council Fellowship (JAEpre_08_00375).
References


Figure Legends

Figure 1. HuR protein. (a) Schematic domain organization of HuR and constructs used in this study. (b) Sequence alignment of HuR and its homologous HuD protein. Green, red and blue boxes highlight RRM1, RRM2 and RRM3 domains, respectively. HNS is also represented. Secondary structure elements are marked by blue arrows for β-strands and red coil symbols for α-helices based on the prediction using PSIPRED server. Phosphorylation sites of serines, which have been mutated in this study, are framed in yellow boxes. (c) Superposition between the crystal structure of HuR RRM1 (PDB entry 3HI9; Benoit et al. 2010) and the homology model of HuR RRM12 built as described in Materials and Methods. The RMSD for backbone atoms of HuR RRM1 domain in both structures is 0.583 Å. Side-chains of serine residues to be phosphorylated are included.

Figure 2. Far-UV (190-250 nm) CD spectra of different HuR domain constructs. RRM domains are represented as follows: RRM1 in solid line (—), RRM2 in dashed line (− −), RRM12 in dotted line (····) and HuR FL protein in dash dotted line (− · −).

Figure 3. Far-UV (190-250 nm) CD spectra of RRM12 WT and its phosphomimetic mutants. RRM12 WT is shown in blue solid line (—); RRM12-S88A in green solid line (—at) and RRM12-S88D in green dashed line (− −); RRM12-S100A in black solid line (—) and RRM12-S100D in black dash line (− −); RRM12-S158A in red solid line (—) and RRM12-S158D in red dashed line (− −).

Figure 4. SDS-PAGE electrophoresis of HuR RRM12 WT. Line 1 stands for a HuR RRM12 WT sample devoid of DTT, whereas lines 2 and 3 correspond to protein samples previously incubated with 5 and 0.5 mM of DTT,
respectively, for 90 min before loading into the gel. In each line, 4 µg of HuR RRM12 WT was loaded onto an 18% SDS-PAGE gel. M: Pro-stain protein molecular weight marker (Intron Technologies Inc.).

Figure 5. Effect of Phosphomimetic Mutations on the Thermal Stability of HuR.
Unfolding thermal denaturation of HuR RRM species and their mutants was determined by DSF by following the fluorescent changes of SYPRO Orange. (a) RRM1 is represented in filled squares (■), RRM2 in filled circles (●) and RRM12 WT in open triangles (△). Ser-by-Asp substitutions are represented as follows: (b) RRM12 S88D (■); (c) RRM12 S158D (●); (d) RRM12 S100D (▲). Fitting unfolding curves are represented by solid lines, and they are superimposed on experimental data. The melting points ($T_m$) of the transitions are marked by dashed lines.

Figure 6. Changes in the CD signal in the range of 260-275 nm region of the c-fos 11-mer RNA (5´AUUUUAUUUU 3´) spectrum during a titration with HuR RRM12 WT. Dissociation constant is also shown.
Table 1. Percentage of secondary structure for the different constructs of HuR RRM domains and mutant species.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>α-helix (%)</th>
<th>β-strand (%)</th>
<th>Turn (%)</th>
<th>Unstructured (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM1</td>
<td>6.01 ± 0.57</td>
<td>36.21 ± 1.25</td>
<td>19.49 ± 2.05</td>
<td>37.74 ± 3.95</td>
</tr>
<tr>
<td>RRM2</td>
<td>10.84 ± 0.22</td>
<td>34.11 ± 0.65</td>
<td>19.13 ± 0.90</td>
<td>30.81 ± 1.61</td>
</tr>
<tr>
<td>RRM12 WT</td>
<td>5.72 ± 0.77</td>
<td>39.67 ± 4.39</td>
<td>21.22 ± 1.80</td>
<td>33.03 ± 3.10</td>
</tr>
<tr>
<td>RRM12 S88D</td>
<td>11.03 ± 0.56</td>
<td>33.26 ± 2.05</td>
<td>19.64 ± 2.06</td>
<td>35.76 ± 4.75</td>
</tr>
<tr>
<td>RRM12 S88A</td>
<td>5.87 ± 0.44</td>
<td>39.00 ± 1.68</td>
<td>20.42 ± 1.41</td>
<td>34.25 ± 2.40</td>
</tr>
<tr>
<td>RRM12 S100D</td>
<td>5.34 ± 1.25</td>
<td>42.71 ± 4.30</td>
<td>19.25 ± 2.01</td>
<td>32.60 ± 3.81</td>
</tr>
<tr>
<td>RRM12 S100A</td>
<td>3.18 ± 0.39</td>
<td>40.88 ± 1.34</td>
<td>21.23 ± 1.15</td>
<td>34.78 ± 2.90</td>
</tr>
<tr>
<td>RRM12 S158D</td>
<td>5.14 ± 0.50</td>
<td>40.28 ± 1.58</td>
<td>20.51 ± 1.12</td>
<td>33.81 ± 3.17</td>
</tr>
<tr>
<td>RRM12 S158A</td>
<td>5.16 ± 0.34</td>
<td>39.79 ± 1.40</td>
<td>21.04 ± 1.65</td>
<td>33.75 ± 3.29</td>
</tr>
<tr>
<td>HuR FL</td>
<td>9.18 ± 1.79</td>
<td>34.95 ± 0.83</td>
<td>20.01 ± 2.39</td>
<td>35.23 ± 4.74</td>
</tr>
</tbody>
</table>

*This makes reference to both disordered and flexible and ordered but non-regular structured parts of the protein.
Table 2. $T_m$ values of HuR RRM domains and their phosphomimetic mutants, as calculated by CD and DSF

<table>
<thead>
<tr>
<th>Constructs</th>
<th>$T_m$ (K) by CD</th>
<th>$T_m$ (K) by DSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM1</td>
<td>335 ± 3</td>
<td>333 ± 1</td>
</tr>
<tr>
<td>RRM2</td>
<td>341 ± 2</td>
<td>339 ± 1</td>
</tr>
<tr>
<td>RRM12 WT</td>
<td>335 ± 2</td>
<td>332 ± 1</td>
</tr>
<tr>
<td>RRM12 S88D</td>
<td>336 ± 1</td>
<td>336 ± 2</td>
</tr>
<tr>
<td>RRM12 S88A</td>
<td>331 ± 1</td>
<td>330 ± 2</td>
</tr>
<tr>
<td>RRM12 S100D</td>
<td>334 ± 2</td>
<td>333 ± 1</td>
</tr>
<tr>
<td>RRM12 S100A</td>
<td>333 ± 1</td>
<td>330 ± 3</td>
</tr>
<tr>
<td>RRM12 S158D</td>
<td>330 ± 2</td>
<td>328 ± 3</td>
</tr>
<tr>
<td>RRM12 S158A</td>
<td>335 ± 1</td>
<td>330 ± 2</td>
</tr>
</tbody>
</table>
Table 3. $K_D$ values of the HuR RRM12 construct and its phosphomimetic mutants, as calculated by CD titration experiments with c-fos 11-mer RNA (5’AUUUUAUUUU 3’)

<table>
<thead>
<tr>
<th>Constructs</th>
<th>$K_D$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRM12 WT</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>RRM12 S88D</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>RRM12 S100D</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>RRM12 S158D</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 6

**RRM12 WT**

\[ K_D = 2.6 \pm 0.2 \mu \text{M} \]

\[ \Delta \varepsilon_M (\text{M}^{-1} \text{cm}^{-1}) \]

\[ [\text{HuR RRM12 WT}] / [\text{RNA-AUUUUUAUUUU}] \]