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Post-transcriptional regulation of the inflammatory marker C-reactive protein by the RNA-binding protein HuR and miR-637

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Abstract

C-reactive protein (CRP), an acute phase plasma protein, is a major component of inflammatory reactions functioning as a mediator of innate immunity. It has been widely used as a validated clinical biomarker of the inflammatory state in trauma, infection, and age-associated chronic diseases including cancer and cardiovascular disease (CVD). Despite this, the molecular mechanisms that regulate CRP expression are not well understood. Given that the *CRP* 3'-untranslated region (UTR) is long and AU-rich, we hypothesized that CRP may be regulated post-transcriptionally by RNA-binding proteins (RBP) and by microRNAs. Here, we found that the RBP HuR bound directly to the *CRP* 3'UTR and affected *CRP* mRNA levels. Through this interaction, HuR selectively increased *CRP* mRNA stability and promoted CRP translation. Interestingly, treatment with the age-associated inflammatory cytokine IL-6 increased binding of HuR to *CRP* mRNA, and conversely, HuR was required for IL-6-mediated up-regulation of CRP expression. In addition, we identified miR-637 as a microRNA that potently inhibited CRP expression in competition with HuR. Taken together, we have uncovered an important post-transcriptional mechanism that modulates expression of the inflammatory marker CRP, which may be utilized in the development of treatments for inflammatory processes that cause CVD and age-related diseases.

Introduction

Inflammatory processes and their inherent regulatory controls are critical for the immune response to injury and pathogens throughout the lifespan. However, inflammation has now been identified as an important underlying factor in many chronic diseases including cardiovascular disease (CVD), diabetes mellitus, cancer and metabolic disorders. Age itself is a critical factor in the development of the inflammatory state and risk for these conditions. This age-associated inflammatory state, known as inflammaging, is defined as a low-grade, sterile inflammation state that occurs with age and characterized by serum elevations in pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) as well as the acute-phase reactant C-reactive protein (CRP) (1). Given the incidence, morbidity, and mortality of inflammation-based chronic disease, pro-inflammatory molecules are avidly studied, including CRP.

CRP, a pentraxin protein, is an established marker of acute phase reactions (2). It is an important inflammatory biomarker that is influenced by the action of numerous activated cytokines such as IL-6, IL-1β and TNFα (3, 4). It is well-established that CRP and IL-6 circulating levels correlate in humans (5, 6). It has been widely used as a validated clinical biomarker of the inflammatory state and an independent predictor of cardiovascular disease. There is some evidence that CRP is not only a biomarker of cardiovascular and metabolic disease, but also a specific risk factor for disease with some data supporting the idea that CRP is an active participant in atherogenesis and events at the endothelium (7). In diabetes, CRP contributes to the development of insulin resistance and may thus be an etiologic factor in diabetes mellitus type 2, especially in the elderly (8). In cancer, CRP may play any of three possible roles: as a marker of cancer susceptibility in the setting of chronic inflammation, as a marker of occult cancer, or as a causal factor (9). Currently, anti-inflammatory clinical trials in the setting of cardiovascular disease and other inflammatory conditions focus on modulating CRP production by inhibiting TNFα and IL-6, thereby reducing hepatic production of the protein (10). Even though CRP plays a central role in aging and age-related disease, most of the molecular mechanisms that regulate CRP expression are not known.

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Several studies have focused on the transcriptional regulation of CRP. The CRP promotor contains consensus sequences for the transcription factors STAT3 and C/EBP*β,* which bind and activate CRP transcription downstream of IL-6 signaling (11-14). In addition, it has been shown that NF-κB p50 and Oct-1 bind to the CRP gene promoter via a nonconsensus κB site, which also overlaps with the proximal C/EBP site (15, 16). IL-1*β*, which alone does not induce CRP expression in human hepatoma Hep3B cells, synergistically enhances the effects of IL-6 by activating the transcription factor NF-κB (17, 18). The HNF-1 and HNF-3 transcription factors are also involved in regulating CRP expression via IL-6 (12, 19). Although the transcriptional modulation of CRP has been explored, we have limited knowledge of the post-transcriptional mechanisms that regulate CRP expression.

Mammalian cells regulate gene expression robustly via post-transcriptional mechanisms controlled by RNA-binding proteins and microRNAs (miRNAs), two types of major etiologic factors in disease (20, 21). In addition, these factors are being evaluated for the diagnosis and management of disease. The human antigen R (HuR) is a ubiquitously expressed RBP belonging to the Hu/Elav family that modulates the stability, translation and localization of subsets of mRNAs by interacting with uridylate (U)-rich or adenylate-uridylate (AU)-rich elements in their 3'-untranslated regions (UTRs) (22, 23). By regulating the expression of specific sets of proteins, HuR critically influences a variety of processes such as cell proliferation and survival, as well as the immune and stress responses (24, 25). HuR modulates inflammatory responses by promoting the expression of pro-inflammatory proteins such as COX-2, IL-8, TGF-β and TNF-α and by inhibiting the expression of anti-inflammatory proteins such as IL-10 (26, 27). HuR is implicated in inflammatory diseases including rheumatoid arthritis, asthma, and inflammatory bowel disease as well as in cardiovascular disease, cancer, and neurodegenerative diseases. HuR also plays a role in cellular senescence and vascular aging by controlling the turnover and/or translation of mRNAs encoding SIRT1, TNF-α, ICAM1, and VCAM1 (28-30).

miRNAs are small non-coding RNAs that modulate gene expression post-transcriptionally by binding to target mRNAs bearing regions of partial complementarity with the miRNA (31). By affecting the expression of target mRNAs, miRNAs regulate a variety of important processes including cell

proliferation, cellular senescence and inflammation (32, 33) and various age-related diseases such as CVD, neurodegeneration, and metabolic diseases (34). Given the fact that the *CRP* 3'UTR contains AU-rich elements, we hypothesized that post-transcriptional regulatory factors bind and regulate CRP expression. Here, we present evidence that CRP expression is regulated via a competitive interaction between the RBP HuR and miR-637 with the *CRP* 3'UTR.

Materials and Methods

Cell Culture and small RNA transfection. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS). HepG2 cells were maintained in MEM containing 10% FBS. Where indicated, cells were treated with 50 ng/ml IL-6 (R & D Systems), 2 μg/ml actinomycin D (EMD Millipore), or vehicle control (PBS or DMSO, respectively). Control small interfering RNA (Ctrl siRNA) and HuR siRNA (5'-AAGAGGCAATTACCAGTTTCA-3') were obtained from Qiagen. Pre-miRNA precursor control and miR-637 and anti-miR miRNA inhibitor control and miR-637 were purchased from Ambion. All siRNA, miRNA and plasmids were transfected using either Lipofectamine-2000 (Invitrogen) or Lipofectamine-RNAiMAX (Invitrogen). RNA and protein were isolated from the cells forty eight hours after transfection.

Ribonucleoprotein immunoprecipitation assay. For ribonucleoprotein immunoprecipitation (RIP), HepG2 cells or HeLa cells (24 hrs after transfection with miR-637; Fig. 5B) were lysed in 20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$ and 0.5 % NP-40 for 10 min on ice and centrifuged at 10,000 *g* for 15 min at 4°C. The supernatants were incubated with mouse IgG agarose beads (Sigma) and coated with anti-HuR (Santa Cruz Biotechnology) or with normal mouse IgG (Santa Cruz Biotechnology) antibodies for 1 h at 4°C. After repeated washing with ice-cold NT2 buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40), the complexes were incubated with RNase-free DNase I for 10

min at 30°C and subsequently with 0.1% SDS– 0.5 mg/ml proteinase K for 15 min at 55°C. The RNA from the IP samples was extracted using acidic phenol, precipitated in ethanol, and analyzed by RT-qPCR.

RNA isolation and RT-qPCR analysis. Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. After reverse transcription (RT) using random hexamers (Invitrogen) and SSII reverse transcriptase (Invitrogen), the abundance of transcripts were assessed by quantitative PCR (qPCR) analysis using the 2x SYBR Green Master Mix (Applied Biosystems). QuantiMir RT kit (System Biosciences) was used for cDNA synthesis for miRNAs, U6 snRNA and snoRNAs. RT-qPCR analysis was performed on Applied Biosystems model 7500 Real-Time PCR machine. The following primer pairs were used (forward and reverse, respectively, in each case): AGACATGTCGAGGAAGGCTTTT and TCGAGGACAGTTCCGTGTAGAA for *CRP* GTGACATCGGGAGAACGAAT and GCGGTCACGTAGTTCACAAA for *ELAVL1* (HuR), GCTCCTCCTGTTCGACAGTCA and ACCTTCCCCATGGTGTCTGA for *GAPDH*, CCCTATCAACTTTCGATGGTAGTCG and CCAATGGATCCTCGTTAAAGGATTT for *18S*, AGATGGTCAAGGTCGCAAGCT and GGGCATATCCTACAACAAACTTGTC for *HPRT1*, ATTTGGGTCGCGGTTCTTG and TGCCTTGACATTCTCGATGGT for *UBC*, TACAAGTACCTCACCGCTTGGT and TGATCTTGTCTTGGTGCTCGTA for Renilla (*RL*), CTAAGAAGGGCCTGCAGAAGAT and AAGCCCTGGTAGTCGGTCTTAG for Firefly (*FL*), To measure the abundance of miRNAs, the following forward primers were used: GCGATAACTGACGAAGACTAC for RNU49, TTAAACCACCAAGATCGCTGA for RNU24, GATATCACTGTAAAACCGTTCC for U47, ACTGGGGGCTTTCGGGCTCTGCGT for miR-637, CACCACGTTTATACGCCGGTG for U6. The universal primer supplied by QuantMir RT kit was used as reverse primer. For PBMCs analysis, HuR expression was normalized to the average of *HPRT* and *UBC* expression using gene specific primers. miR-637 expression was normalized to the average of snoRNAs RNU24, RNU49 and U47.

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Biotin pull-down analysis. The *CRP* 3'UTR fragments a,b,c and d were amplified from the psiCHECK2 luciferase vectors containing the respective 3'UTRs using primers that contained the T7 RNA polymerase promoter sequence (T7, 5'-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3') (35). After purification of the template PCR products, biotinylated transcripts were synthesized using the MaxiScript T7 kit (Ambion). Whole-cell lysates were incubated with 1 μg of purified biotinylated transcripts for 1 hr at room temperature and then complexes were isolated with streptavidin-coupled Dynabeads M-280 Streptavidin (Invitrogen). The proteins present in the pulldown material were analyzed by immunoblotting with anti-HuR antibodies (Santa Cruz Biotech). The biotinylated *GAPDH* 3'UTR was used as a negative control. The following primers pairs were used:
(T7)AGCTGTGGGTCCTGAAGGTA and AAGTAAACAGGGGCTTTATT for fragment (a),
(T7)AGCTGTGGGTCCTGAAGGTA and AGAAATTATCTCCAAGATCT for fragment (b),
(T7)GATAATTTCTTACCTCACAT and ATTTATACCTAGTGCTTCAT for fragment (c),
(T7)ATGAAGCACTAGGTATAAAT and AAGTAAACAGGGGCTTTATT for fragment (d),
(T7)CCTCAACGACCACTTTGTCA and GGTTGAGCACAGGGTACTTTATT for fragment *GAPDH* 3'UTR.

3'UTR luciferase reporter assays. The cDNA fragments corresponding to the entire 3'UTR and partial 3'UTR of human *CRP* mRNAs were amplified by PCR using specific primers. After XhoI and NotI digestion, the PCR product was cloned downstream of the Renilla open reading frame of the psiCHECK2 reporter plasmid. The psiCHECK2-*CRP* (3'UTR-Mut) construct containing specific point mutations (CC to GG) in the miR-637 binding site was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. For Fig. 1E, 24 hrs after transfection with 200 ng of the reporter constructs, RIP assays were performed as described above with the exception that complexes were incubated with RNase-free DNase I for 1 hr at 30°C. HuR binding to the ectopic luciferase transcripts was determined by RT-qPCR analysis using primers specific for Renilla luciferase (*RL*) and Firefly luciferase (*FL*) mRNAs. HeLa cells were transfected with 100 ng of the indicated

3'UTR luciferase reporter constructs and transfected again 24 hrs later with the siRNA of Ctrl and HuR or miR-637. Twenty-four hrs after that, RL and FL activities were measured using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's instructions, or *RL* mRNA and *FL* mRNA levels were determined by RT-qPCR analysis.

Polysome analysis. For polysome analysis, 48 h after transfection of HeLa cells with Ctrl siRNA or HuR siRNA, cells were incubated with 100 μg/ml cycloheximide for 10 min and lysed with PEB (polysome extraction buffer) containing 20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$ and 0.5% NP-40. Cytoplasmic lysates were fractionated by centrifugation through 10-50 % linear sucrose gradients and divided into 12 fractions. The total RNA in each fraction was extracted with TRIzol (Invitrogen) and analyzed by reverse transcription followed by RT-qPCR analysis.

Biotinylated miR-637 pulldown assays

Twenty-four hr after transfection of HeLa cells with biotinylated miR-637 or with biotinylated control miRNA from *Caenorhabditis elegans* cel-miR-67 (biot-miR-637, biot-Ctrl-miR, both from GE Healthcare Dharmacon), HeLa cells were lysed in 20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.3% NP-40, 50 U of RNAse Out (Invitrogen), and protease cocktail inhibitor (Roche Applied Science) for 10 min on ice and centrifuged at 10,000 *g* for 10 min at 4°C. Streptavidin Dynabeads (Invitrogen) were preincubated in lysis buffer with yeast tRNA (1 mg/ml) and BSA (1 mg/ml) overnight at 4°C. Cytoplasmic lysates were added to the beads and incubated for 4 h at 4°C. After the beads were washed with lysis buffer, the RNA bound to the beads and the RNA in cytoplasmic extracts were isolated using TRIzol (Invitrogen) as described above and analyzed by RT-qPCR.

Western blot analysis. Cells were washed twice with 1× cold PBS and lysed using 2× Laemmli sample buffer. The cell lysates were fractionated by SDS-PAGE, transferred onto membranes, and analyzed

using primary antibodies that recognized HuR (Santa Cruz Biotechnology), CRP (Millipore) or loading control β-actin (Santa Cruz Biotechnology). Following incubation with appropriate secondary antibodies, signals were detected by using enhanced chemiluminescence (ECL).

Clinical Study Participants. A sub-cohort of women with either low (≤3 mg/L) or high (≥20 mg/L) hsCRP levels (n=15/group) were chosen from the Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) study of the National Institute on Aging Intramural Research Program (NIA IRP), National Institutes of Health (NIH). This study has been approved by the NIEHS Institutional Review Board (IRB). All study participants signed informed consent documents approved by the IRB. Previously, we examined CRP levels in a larger cohort containing these women (36) and here we analyzed a subset of these participants from whom we had also obtained and stored PBMCs. Nineteen white and 20 African American females with an average age of 49.7+8.1 years were used for this study. RNA was isolated from PBMCs using TRIzol (Invitrogen) according to the manufacturer's instructions. Serum IL-6 levels were quantified using Searchlight protein arrays from Aushon Biosystems (Billeria, MA).

Results

The RNA-binding protein HuR interacts with the 3'UTR of *CRP*. To investigate whether *CRP* mRNA associated with HuR, we performed ribonucleoprotein (RNP) immunoprecipitation (RIP) assays using anti-HuR antibodies in parallel with control IgG antibodies using the human hepatoma cell line HepG2, chosen because hepatic cells are a major source of circulating CRP *in vivo*. The interaction of HuR with *CRP* mRNA was assessed by isolation of RNA in the IP material (Fig. 1A) followed by reverse transcription (RT) and quantitative real-time PCR (qPCR) analysis to measure the levels of *CRP* mRNA in the HuR IP relative to the control IgG IP. As shown in Fig. 1B, *CRP* mRNA was highly enriched (more than ~7-fold) in HuR IP samples compared with IgG IP samples. To identify the area(s) of interaction of endogenous HuR with ectopic *CRP* mRNA, partial *in vitro*-transcribed biotinylated RNAs

spanning the *CRP* 3'UTR (Fig. 1C, schematic) were incubated with HeLa cell lysates. After pulldown using streptavidin-coated beads, the association of HuR with each biotinylated transcript was assessed by immunoblotting. As shown, HuR associated with *CRP* 3'UTR fragments a, b and d, and most strongly with fragment b. HuR did not bind to the negative control transcript, biotinylated *GAPDH* 3'UTR (Fig. 1C).

To confirm that HuR binds to the CRP 3'UTR *in vivo*, we used heterologous luciferase reporter plasmids (Fig. 1D) that expresses Renilla luciferase (RL) from constructs lacking or containing the *CRP* 3'UTR (psiCHECK2 or psiCHECK2-*CRP* 3'UTR). These plasmids also contain Firefly luciferase (FL), which served as an internal transfection control. We used RIP assays to test binding of HuR to the ectopically expressed luciferase transcripts. As shown, HuR selectively associated with *RL* mRNAs containing the *CRP* 3'UTR fragments a, b and d (Fig. 1E), further confirming our *in vitro* binding data.

To investigate if HuR regulates *CRP* mRNA stability and translation via the specific regions where it binds preferentially within the *CRP* 3'UTR (region b,d; Fig 1C), we first analyzed the luciferase reporter constructs. The ratio of RL/FL activity from each transfected reporter plasmid indicated that silencing HuR significantly decreased the levels of psiCHECK2-*CRP* 3'-a, b and d luciferase reporters while it did not affect the activity of the psiCHECK2-*CRP* 3'-c reporter (Fig. 2F). Consistent with the strong HuR binding in the biotin precipitation experiments (Fig. 1C), the most robust regulation of luciferase activity by HuR was mapped to *CRP* segment 3'-b (Fig. 1F). To verify if the individual HuR binding sites (a,b and d) regulate the stability and/or translation of the *CRP* mRNA, we examined the ratio of the reporter mRNA levels after silencing HuR. HuR silencing decreased the levels of *RL* mRNA expressed from psiCHECK2-*CRP* 3'-a, b and d, indicating that HuR primarily effects mRNA stability of these individual binding sites (Fig. 1G). Taken together, these data strongly suggest that HuR enhances CRP expression by associating with specific positive regulatory elements in the *CRP* 3'UTR to regulate *CRP* mRNA stability.

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HuR promotes CRP protein expression by regulating *CRP* mRNA stability and translation. We tested whether HuR directly affected CRP expression by silencing HuR using specific HuR-directed small interfering (si) RNA. Silencing HuR decreased *CRP* mRNA and protein levels (Fig. 2A), indicating that HuR positively regulates CRP expression. Because HuR was previously shown to stabilize several target mRNAs (27) and the *CRP* 3'UTR reporter constructs in Fig. 1G, we investigated if HuR regulates endogenous *CRP* mRNA turnover. After silencing HuR in HeLa cells, actinomycin D was used to inhibit *de novo* transcription and the time needed for *CRP* mRNA to be reduced to 50% of its initial abundance (its half-life [$t_{1/2}$]) was then calculated by measuring *CRP* mRNA levels using RT-qPCR, using *18S* rRNA levels for normalization. We found that silencing HuR selectively lowered the half-life ($t_{1/2}$) of *CRP* mRNA to ~6 h, compared to its half-life in Ctrl siRNA-transfected cells, which was far greater than 6 h. The half-life of *GAPDH* mRNA, encoding a housekeeping protein, was unaffected by HuR silencing (Fig. 2B). These data suggest that HuR enhances CRP expression at least in part by stabilizing *CRP* mRNA.

In addition to stabilizing some target mRNAs, HuR also modulates the translation of several mRNAs (22). To investigate if HuR also affects CRP translation, we performed polysome analysis in HeLa cells expressing normal HuR levels or HuR levels reduced by silencing. The cytosolic fractions were centrifuged through sucrose gradients in order to separate ribosome components (40S and 60S, fractions 2 and 3), monosomes (single ribosomes, 80S, fraction 4), as well as low-molecular-weight (LMW, fractions 5-7) and high-molecular-weight (HMW, fractions 8-10) polysomes. From each of the twelve fractions obtained, RNA was extracted and the levels of *CRP* and *GAPDH* mRNAs were quantified by RT-qPCR analysis. The results showed that in control cells, *CRP* mRNA levels peaked at fraction 7 while HuR silencing showed a distinct leftward shift peaking at fraction 5, indicating that *CRP* mRNA formed on average smaller polysomes after silencing HuR (Fig. 2C). The distribution of the housekeeping *GAPDH* mRNA was the same between the two groups, indicating that silencing HuR specifically affected *CRP* mRNA translation. In sum, HuR increases CRP expression levels by both stabilizing *CRP* mRNA and enhancing its translation.

IL-6 upregulation of CRP is dependent on HuR. IL-6 is a potent mediator of inflammatory processes and it has been proposed that the age-associated increase in IL-6 accounts for some of the phenotypic changes observed with advancing age. IL-6 and CRP levels increase with age and are highly correlated (37). In addition, previous data have shown that IL-6 can upregulate CRP levels (14). Given this relationship, we hypothesized that HuR may contribute to the increased *CRP* mRNA expression in response to IL-6. To test this idea, we treated HepG2 cells with IL-6 and then performed HuR RIP assay. This analysis revealed that more HuR associated with *CRP* mRNA after treatment with IL-6 (Fig. 3A). To address whether HuR is required for IL-6-mediated upregulation of CRP, we silenced HuR and treated cells with IL-6 (Fig. 3B and 3C). HuR silencing significantly decreased *CRP* mRNA and protein levels in the presence of IL-6. In contrast, HuR mRNA and protein levels were not affected by IL-6 treatment. These findings indicate that IL-6-mediated upregulation of CRP is dependent on HuR.

miR-637 interacts with the *CRP* mRNA. Recently, several studies showed that RBPs and miRNAs bind and functionally regulate shared target mRNAs (21, 25, 35). Therefore in addition to HuR, it is likely that miRNAs also regulate CRP. Using TargetScan to test this possibility, we found that the *CRP* mRNA contains one predicted miR-637 site in its 3'UTR. To investigate whether miR-637 regulates CRP expression, we overexpressed miR-637 by transfecting the miR-637 precursor and found that this intervention decreased *CRP* mRNA and protein abundance (Fig. 4A and 4B), indicating that CRP levels can be modulated by miR-637. In contrast, decreasing the levels of endogenous miR-637 by transfection with a miR-637 antagomir (anti-miR-637) increased both *CRP* mRNA and CRP protein expression levels (Fig.4C and 4D). Similar results were also observed using a locked nucleic acid ((LNA)-antimir-637) (data not shown). For further analysis of the effects of miR-637 on CRP expression, we performed luciferase assays using miR-637 target reporter constructs bearing *CRP* 3'UTRs (Fig. 4E). We observed that miR-637 repressed luciferase activity from the psiCHECK2-*CRP* 3'UTR and fragment *CRP* 3'-c, which contains the predicted miR-637 site (Fig. 4E,F), but this effect is abolished when the miR-637 site

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299 was mutated (Fig. 4F). These results suggest that miR-637 reduces the expression levels of CRP by
300 interacting with the *CRP* 3'UTR.

301 To confirm that miR-637 binds to *CRP* mRNA, we performed precipitations with a biotin-labeled
302 miR-637 followed by RNA isolation and RT-qPCR analysis. Indeed, *CRP* mRNA was enriched in the
303 biotin miR-637 pulldowns (Fig. 5A). Since RBPs can cooperate or compete with miRNAs to control
304 gene expression either cooperatively or antagonistically (38, 39), and that both miR-637 and HuR interact
305 with the *CRP* 3'UTR, we tested whether miR-637 and HuR might compete or work cooperatively to
306 modulate expression of CRP. First, we examined whether silencing HuR affects miR-637 binding to *CRP*
307 mRNA. Decreasing HuR levels significantly increased binding of biotin miR-637 to the *CRP* transcript
308 (Fig. 5A). Second, we performed the reverse experiment, overexpressing miR-637 and examining HuR
309 binding to *CRP* mRNA by RIP analysis. miR-637 overexpression significantly decreased HuR binding to
310 *CRP* mRNA (Fig. 5B), indicating that HuR and miR-637 bind competitively to *CRP* mRNA.

311 Next, we examined the effect of HuR silencing on miR-637-induced repression of CRP
312 expression. Silencing HuR additively repressed the miR-637-mediated repression of CRP expression as
313 indicated by a decrease in the levels of *CRP* mRNA and protein in miR-637 overexpressed cells (Fig. 5C).
314 In contrast, ectopic HuR overexpression inhibited the miR-637-mediated repression of CRP expression
315 (Fig. 5D). In addition, we investigated if HuR and miR-637 competed to regulate CRP expression in
316 response to IL-6 treatment. As shown in Fig.5E, overexpression of miR-637 or silencing HuR decreased
317 *CRP* levels following IL-6 treatment. Simultaneous miR-637 overexpression and HuR silencing in cells
318 had an additive effect on decreasing CRP abundance in response to IL-6. These findings suggest that
319 HuR and miR-637 competitively regulate *CRP* mRNA.
320
321 **Individuals with high CRP have high levels of HuR and low levels of miR-637.** In order to test
322 whether HuR and miR-637 may affect CRP levels *in vivo*, we obtained peripheral blood mononuclear
323 cells from participants from the HANDLS study that have either low ($\leq$3 mg/L) or high ($\geq$20 mg/L)
324 circulating protein levels of hsCRP. HuR mRNA levels were higher and miR-637 levels were lower in

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individuals with high hsCRP, consistent with our *in vitro* data that HuR positively and miR-637 negatively regulated CRP expression (Fig. 6A and 6B). As previously reported, we found a positive relationship between circulating IL-6 levels and hsCRP levels (Fig. 6C).

Discussion

CRP has been well studied as an acute phase marker during infection and inflammation, and it is now also well known to have clinical and pathological significance as a marker of age-associated disease, most prominently for CVD. Although some mechanisms that regulate CRP transcription have been explored, whether CRP expression is regulated by post-transcriptional mechanisms has not been reported. Here, we found that HuR and miR-637 play key roles in the post-transcriptional regulation of CRP through the AU-rich or U-rich elements in the *CRP* 3'UTR.

The RBP HuR, a well-established RNA-binding protein and post-transcriptional regulator, affects the expression of its target mRNAs by binding to specific AU- or U-rich elements in their 3'UTRs (22). Our results indicate that HuR interacts with *CRP* mRNA via the *CRP* 3'UTR and regulates *CRP* mRNA stability and translation. Notably, HuR binds to *CRP* mRNA after IL-6 treatment, indicating the importance of HuR in mediating IL-6-induced CRP expression. The fact that HuR regulates other proinflammatory proteins, such as COX-2, IL-8, TGF-β and TNF-α, suggests that HuR can serve as a master upstream modulator of inflammatory conditions (26, 27). These data indicate that HuR might be valuable as a therapeutic target for acute or chronic inflammatory diseases. Currently, anti-inflammatory agents under investigation are directed at the IL-1, TNFα and IL-6 pathways. Canakinumab and low-dose methotrexate lead to reductions in IL-6 and CRP (10); however, our findings that HuR is essential for IL-6-mediated upregulation of CRP suggest that HuR may be a promising target for design of therapeutic agents to reduce CRP levels in CVD, diabetes mellitus, and other inflammatory conditions. Nonetheless, given the untoward and unintended serious side-effects (heart failure and vascular complications)

observed with anti-inflammatory agents that inhibit TNF-α and cyclooxygenase-2, it will be important to take into account the potential adverse events that may accompany modulating HuR function.

We also found that the miRNA miR-637 is able to inhibit the expression of CRP. miRNAs play a significant role in post-transcriptional gene regulation predominately by acting as repressors (40). miR-637, a primate-specific miRNA, was first identified from colorectal tumor tissue (41). While the biological function of miR-637 is still not fully understood, several targets such as osterix and collagen, type IV, alpha 1 (COL4A1) have been reported (42, 43). Decreased miR-637 expression was reported in four hepatocellular carcinoma cell lines; overexpression of miR-637 inhibited growth and enhanced apoptosis by suppressing expression of the autocrine leukemia inhibitory factor (LIF) (44), suggesting a tumor suppressor function for miR-637 in hepatocytes. Importantly, we found that miR-637 levels were lower in individuals with high circulating levels of hsCRP. These data indicate that miR-637 may have a direct role in modulating CRP levels *in vivo*, and that miR-637 may have an anti-inflammatory effect by decreasing circulating CRP levels. Future research is needed in order to fully investigate whether miR-637 targets other inflammatory pathways. It is possible that miR-637 can be used to lower levels of CRP in individuals at risk.

Here we report the binding of HuR and miR-637 to the *CRP* 3'UTR. Through the use of ectopic reporters, we found that the predominant sites where HuR interacted with and regulated *CRP* mRNA were the 780-1080 and 1441-2103 regions of the *CRP* 3'UTR, while the target site of miR-637 was located at the sequence spanning 1182-1204, directly in between these two segments. These findings suggest that HuR and miR-637 interact with different binding sites of the *CRP* 3'UTR. HuR and miR-637 modulated *CRP* mRNA in a functionally competitive manner: HuR promoted while miR-637 inhibited CRP expression. This result is consistent with other reports that HuR and miRNAs operate in opposite directions (23). For example, HuR inhibits miR-331-3p-mediated repression of ERBB2 expression (45) and miR-494-mediated repression of NCL expression (35). Also, HuR competes with miR-195 to regulate *STIM1* mRNA (39) and suppresses miR-1192 to modulate *HMGB1* mRNA (46). Although the specific mechanisms whereby HuR and miRNAs compete to regulate joint target mRNAs are not well

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understood, it is interesting to note that several competing miRNAs have binding sites near the HuR sites (47, 48), suggesting that HuR and miRNAs may compete via steric hindrance or local conformation changes of the mRNA.

IL-6 and CRP levels increase with age and are highly correlated (5, 6). Consistent with these findings, we found that IL-6 levels were higher in individuals with high hsCRP, regardless of age. Most significantly, in the small human cohort tested here, individuals with high levels of hsCRP also had high levels of HuR and low levels of miR-637, mirroring the correlations we found in cultured cells.

As inflammation is now understood to be an underlying etiologic factor in a number of different diseases, it is important to fully understand the mechanisms by which these inflammatory markers are regulated. Unraveling the factors that modulate CRP provides insight for understanding the factors that contribute to the low-grade inflammatory state of aging and will undoubtedly shed light on age-related chronic diseases. The design of primary and secondary prevention therapies and treatment modalities aimed at the process of inflammation is already successfully underway. However, the therapeutic agents mainly target upstream biomarkers. The role of CRP as both a risk factor and a biomarker warrants investigating its regulation by other molecular factors. We have discovered a post-transcriptional mechanism by which the RBP HuR and miRNA miR-637 modulate CRP expression downstream of IL-6 signaling. We have identified HuR and miR-637 as new factors that may play an integral role in the development of the inflammatory state, and may represent new valuable targets in the diagnosis, treatment, or prevention of inflammation and diseases with a major inflammatory component.

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Figure Legends

Fig 1. HuR binds to the *CRP* 3′ UTR. (A) *Left*, HuR immunoprecipitates from HepG2 cells were analyzed by immunoblotting. HuR (arrowhead), immunoglobulin heavy chain (HC) and light chain (LC), and molecular weight (MW) markers are indicated. (B) RIP followed by RT-qPCR analysis was used to measure the enrichment of *CRP* mRNA in HuR IP compared to IgG IP in HepG2 cell lysates, normalized to *GAPDH* mRNA levels in each IP reaction. (C) *Top*, schematic of the *CRP* 3'UTR and the different biotinylated RNAs synthesized for use in biotin pulldown analysis are shown as Full length, a, b, c and d. *Bottom*, the indicated biotinylated *CRP* RNAs or control *GAPDH* 3'UTR were incubated with HeLa cell lysates and HuR was detected by immunoblotting with anti-HuR antibodies. (D) Schematic of the *CRP* 3'UTR dual luciferase reporters. (E) HeLa cells transfected with the luciferase constructs were analyzed by RIP and RT-qPCR analysis to measure the enrichment of Renilla luciferase (*RL*) mRNA in HuR IPs compared to that in IgG IPs. *RL* mRNA levels in each IP were normalized to firefly luciferase (*FL*) mRNA levels and to *GAPDH*. (F) After transfecting HeLa cells with Ctrl or HuR siRNAs, cells were then transfected with either psiCHECK2 or the psiCHECK2-*CRP* (3'UTR) reporters as indicated. The ratio of RL to FL (RL/FL) was normalized to the parent vector (psiCHECK2) and then normalized to luciferase activity in Ctrl siRNA-transfected cells. (F) Cells were transfected as in (E) and the ratio of *RL* mRNA/*FL* mRNA for each reporter construct was quantified by RT-qPCR. Histograms represent the mean + SEM from three independent experiments. *$p<0.05$, **$p<0.01$ compared to Ctrl siRNA by Student's t-test.

Fig 2. HuR promotes *CRP* mRNA stability and translation. (A) *Left*, 48 hrs after transfection of HeLa and HepG2 cells with either Ctrl siRNA or HuR siRNA, *CRP* mRNA levels were measured by RT-qPCR analysis. *Right*, transfected HepG2 cells or HeLa cells were assessed by immunoblotting using anti-CRP, anti-HuR and anti-β-actin antibodies as a loading control. CRP protein levels were quantified from immunoblots and normalized to the levels of actin. Histograms represent the mean + SEM from three independent experiments. *$p<0.05$, **$p<0.01$ compared to Ctrl siRNA by Student's t-test. (B) HeLa cells transfected as in (A) were treated with actinomycin D (2 μg/ml), and RNA was isolated at the times indicated. The levels of *CRP* mRNA and *GAPDH* mRNA levels were assessed by RT-qPCR and normalized to *18S* rRNA. The half-lives ($t_{1/2}$) of *CRP* and *GAPDH* mRNAs were quantified by measuring the time needed for the transcript levels to reach 50% of their original abundance at time 0 hr. (C) HeLa cells transfected with either Ctrl siRNA or HuR siRNA were fractionated into cytoplasmic extracts through sucrose gradients. The arrow indicates the direction of sedimentation. Small (40S) and large (60S) ribosomal subunits and monosomes (80S) in fractions 2–4, and progressively larger polysomes from low to high molecular weight (LMW and HMW, respectively) in fractions 6–12 are shown in the left panel. The distribution (%) of *CRP* and *GAPDH* mRNAs was quantified by RT-qPCR analysis of RNA isolated from 12 gradient fractions.

Fig 3. IL-6-mediated upregulation of CRP is dependent on HuR. (A) After IL-6 (50 ng/ml) treatment for 15 min, RNP IP was performed using HuR or IgG antibodies. *CRP* mRNA was measured by RT-qPCR analysis. (B) HepG2 cells were transfected with either Ctrl siRNA or HuR siRNA and treated with or without IL-6 for 24 hrs. The mRNA and protein levels of CRP and HuR were examined by RT–qPCR (B) and immunoblotting (C). CRP protein levels were quantified from immunoblots and normalized to β-actin control. The histograms represent the mean + SEM from three independent experiments. *$p<0.05$, **$p<0.01$ by Student's t-test.

578 **Fig 4.** miR-637 interacts with *CRP* mRNA. (A) 48 hrs after transfection with miR-Ctrl or with miR-637,
579 the levels of miR-637 (left) and *CRP* mRNA (right) were measured by RT-qPCR analysis or (B) protein
580 levels were assessed by immunoblotting with anti-CRP and anti-β-actin antibodies, as a loading control.
581 CRP protein levels were quantified from immunoblots and normalized to β-actin. (C) HeLa cells were
582 transfected with the antisense (AS) miR-637 or control miRNA. After 72 hrs, the levels of miR-637 (left)
583 and *CRP* mRNA (right) were measured by RT-qPCR analysis or (D) the protein level of CRP and control
584 β-actin were analyzed by immunoblotting. CRP protein levels were quantified from immunoblots and
585 normalized to β-actin. (E) Schematic of psiCHECK2-*CRP* dual luciferase reporters. Red bar, predicted
586 miR-637 binding site. (F) 24 hrs after transfecting HeLa cells with miR-637, cells were transfected with
587 different *CRP* 3'UTR luciferase reporter plasmids and luciferase activity was measured (RL/FL). The
588 histograms represent the mean + SEM from three independent experiments. *p<0.05, **p<0.01,
589 ***p<0.001 by Student's t-test.
590
591 **Fig. 5.** HuR and miR-637 regulate CRP expression competitively. (A) Ctrl siRNA or HuR siRNA and
592 either biotinylated miR-637 (bio-miR-637) or control (bio-miR-Ctrl) were transfected into HeLa cells for
593 24 hrs. Biotinylated miRNAs were precipitated using streptavidin beads. Enrichment of *CRP* mRNA was
594 assessed by RT-qPCR. (B) 24 hrs after transfecting HeLa cells with miR-Ctrl or miR-637, RIP followed
595 by RT-qPCR analysis was used to measure the enrichment of *CRP* mRNA in HuR IP compared to IgG IP.
596 (C) Effects of silencing HuR together with miR-637 on CRP expression. HeLa cells were co-transfected
597 with HuR siRNA and miR-637; 48 hrs later, lysates were assessed for the level of *CRP* mRNA by RT-
598 qPCR analysis (left) and HuR, CRP and loading control β-actin by immunoblotting analysis (right). CRP
599 protein levels were quantified from immunoblots and normalized to β-actin levels. (D) 48 hrs after HeLa
600 cells were co-transfected with Flag-HuR and miR-637, *CRP* mRNA levels were measured by RT-qPCR
601 and normalized to *GAPDH*. (E) HepG2 cells were transfected with HuR siRNA or miR-637 and treated
602 with IL-6 (50 ng/ml) for 24 hrs or left untreated. The *CRP* mRNA levels were examined by RT–qPCR.

603 The histograms show the mean +SEM from three independent experiments. *p<0.05, **p<0.01,
604 ***p<0.001 by Student's t-test in (A-D) and one-way ANOVA and Tukey's post hoc test (E).
605
606 **Fig 6.** Individuals with high CRP have high levels of HuR and low levels of miR-637. (A, B) RNA was
607 isolated from PBMCs of individuals with either low (≤3 mg/L) or high (≥20 mg/L) circulating CRP levels
608 (n=15/group). The levels of *HuR* mRNA and miR-637 were quantified using RT-qPCR. *HuR* mRNA
609 levels were normalized to *HPRT1* mRNA and *UBC* mRNA levels. miR-637 levels were normalized to
610 snoRNAs (*SNU24*, *SNU49* and *U47*). (C) Serum protein levels of IL-6 were quantified from the same
611 individuals as in (A and B). Means in the graph are indicated by a bar. **p<0.01, *** p<0.001 by Mann-
612 Whitney *U* test.

FIG 1

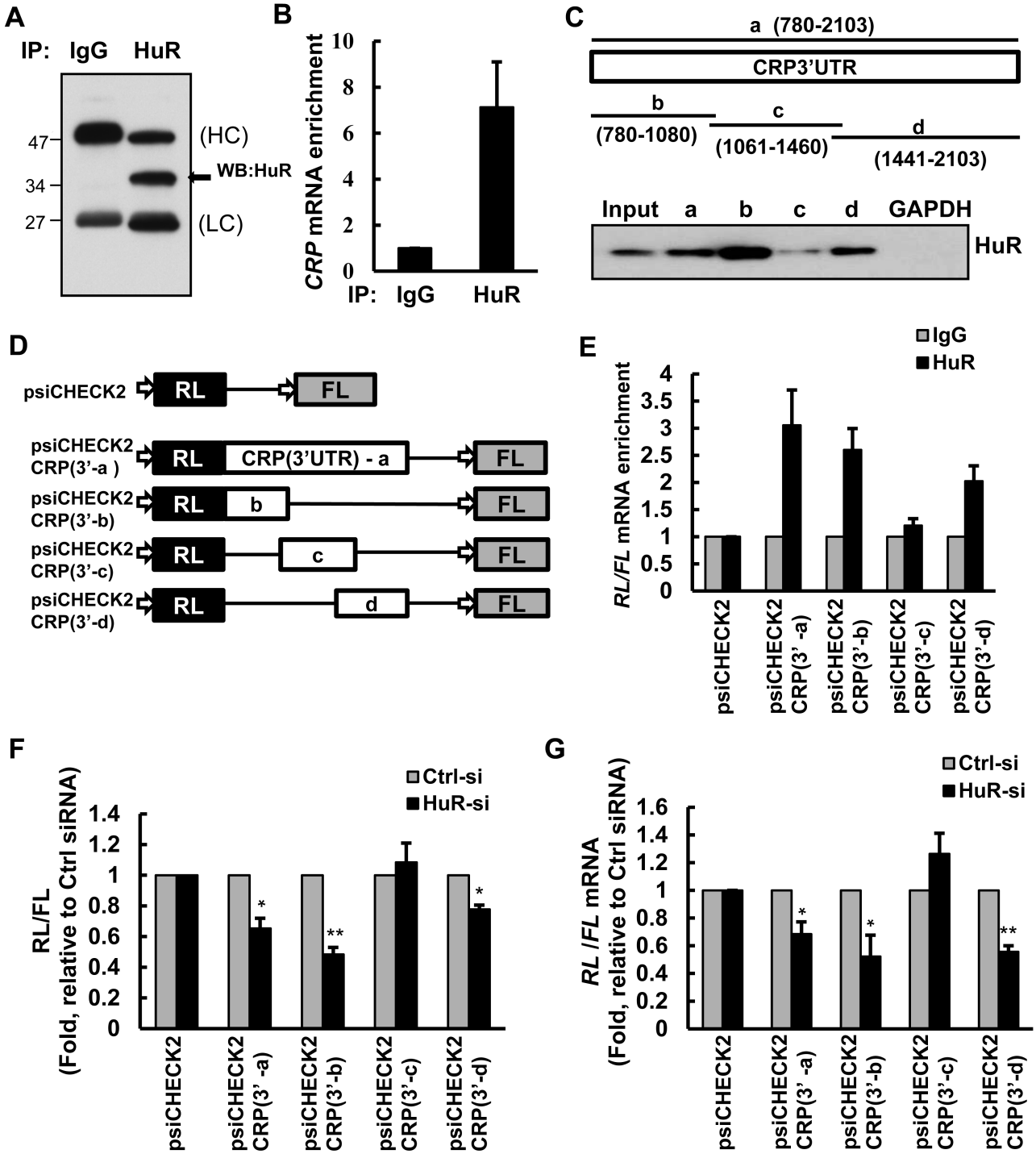

FIG 2

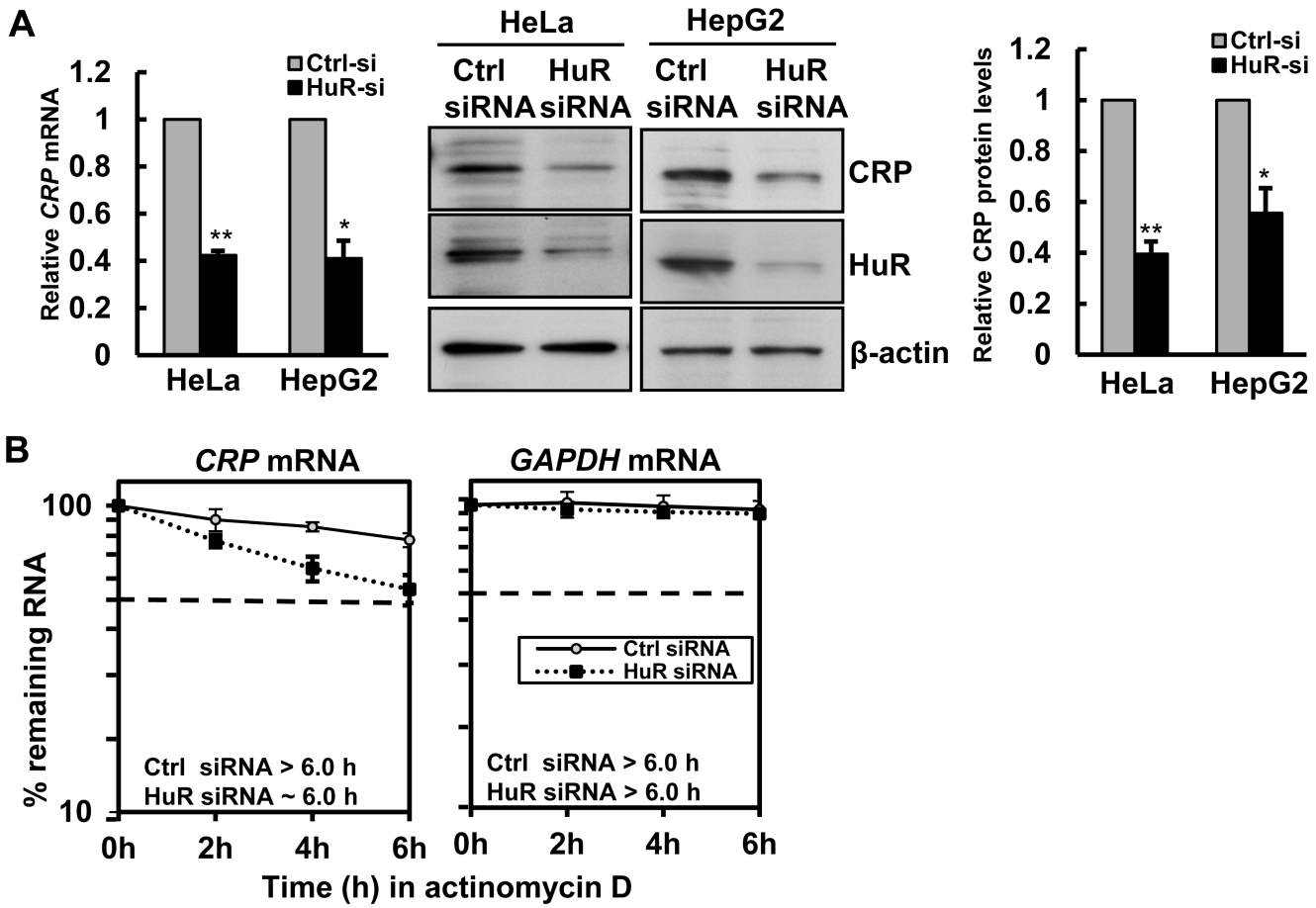

FIG 3

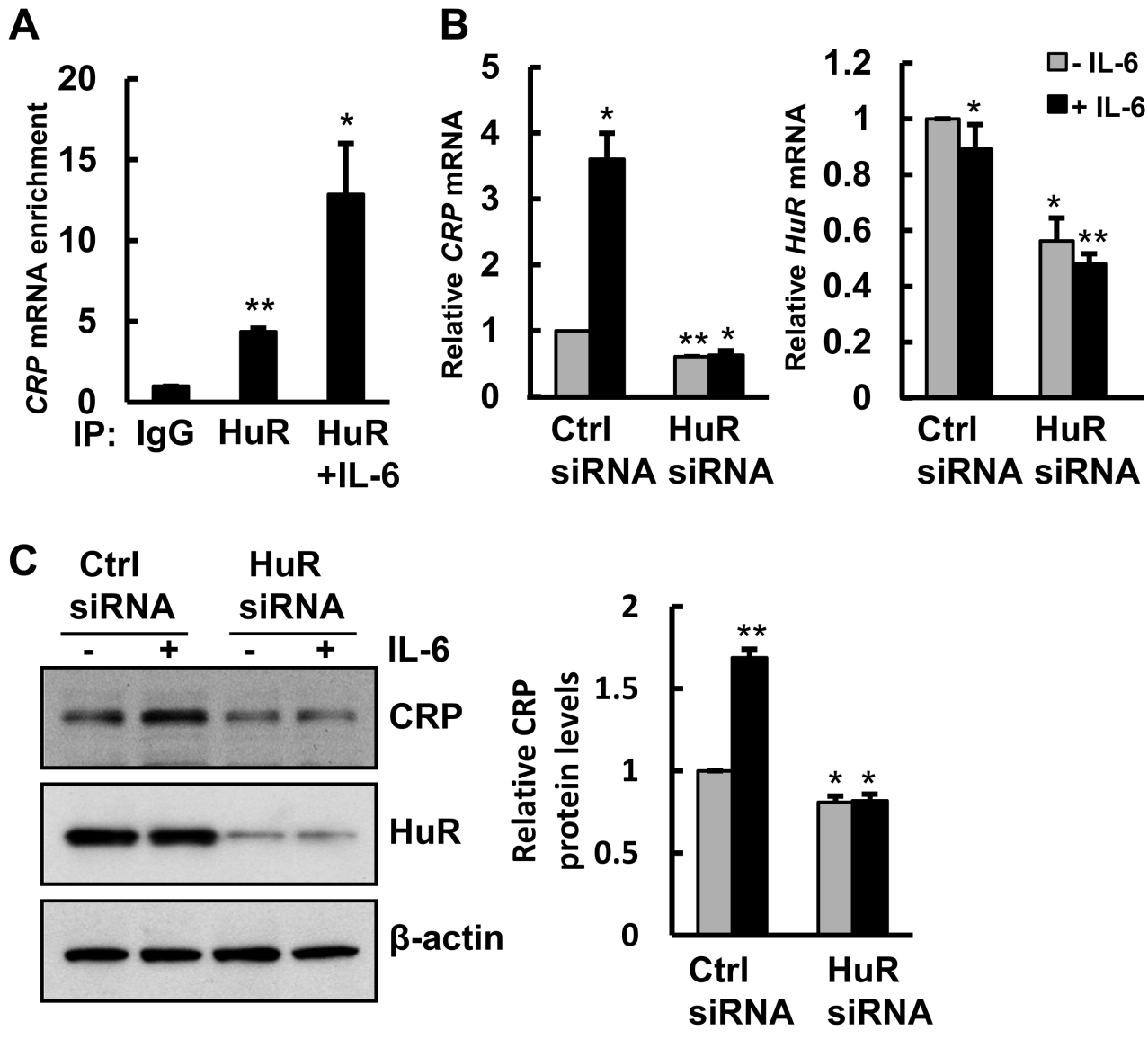

FIG 4

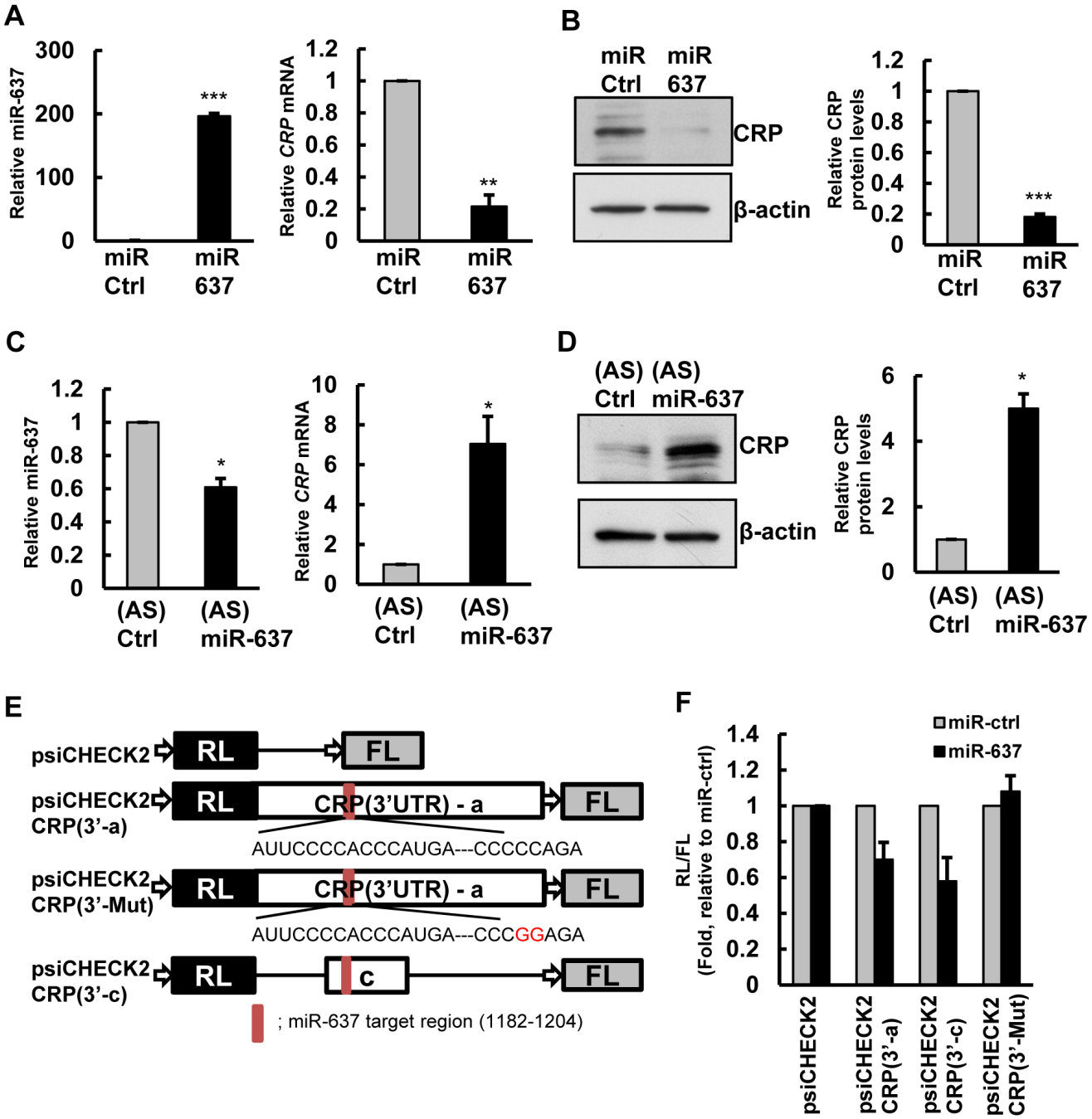

FIG 5

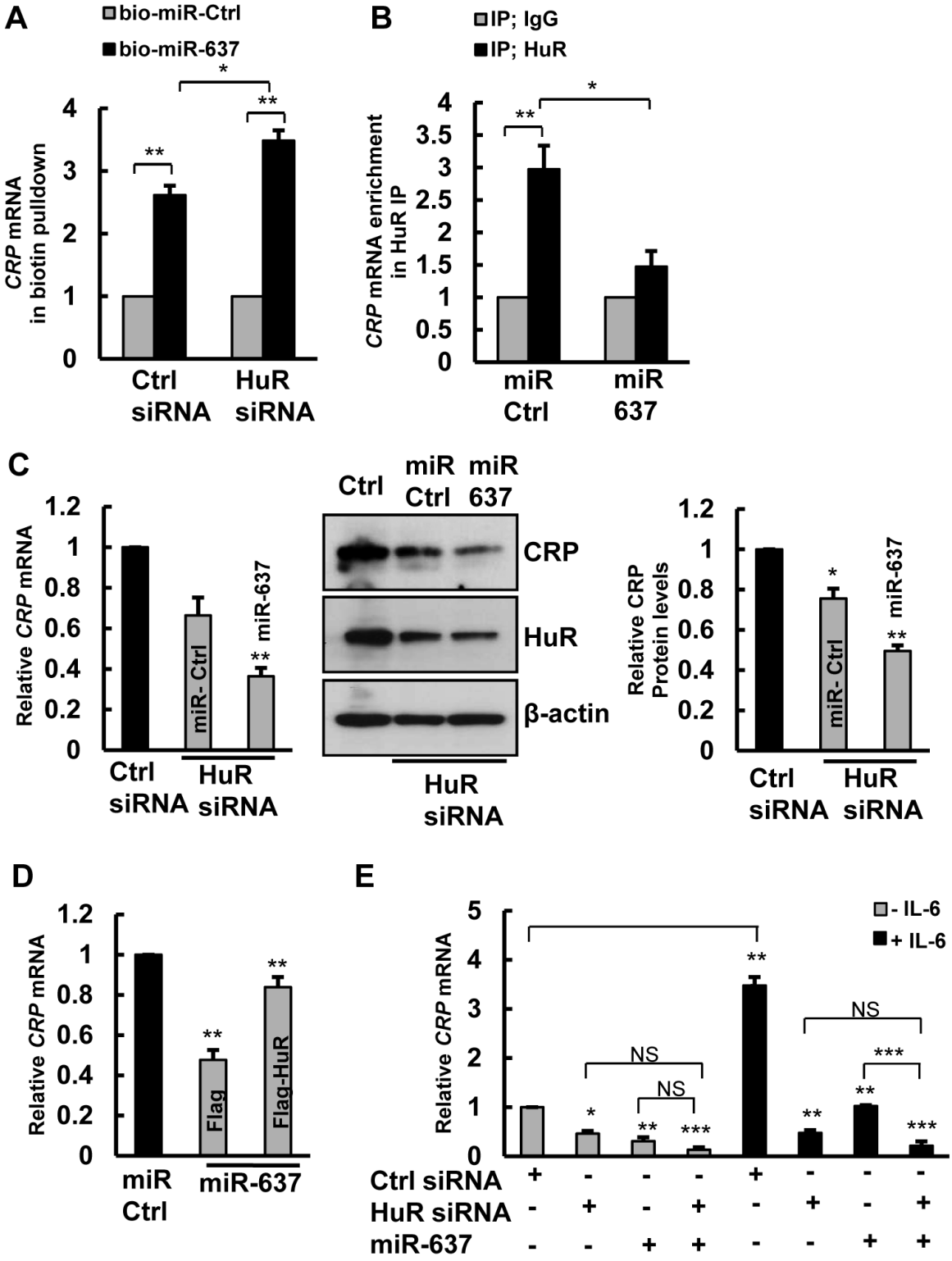

FIG 6