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Posttranscriptional Regulation of the Inflammatory Marker C-Reactive Protein by the RNA-Binding Protein HuR and MicroRNA 637

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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 posttranscriptionally by RNA-binding proteins (RBPs) 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 interleukin-6 (IL-6) increased binding of HuR to *CRP* mRNA, and conversely, HuR was required for IL-6-mediated upregulation of CRP expression. In addition, we identified microRNA 637 (miR-637) as a microRNA that potently inhibited CRP expression in competition with HuR. Taken together, we have uncovered an important posttranscriptional mechanism that modulates the 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.

Inflammatory processes and their inherent regulatory controls are critical for the immune response to injury and pathogens throughout the life span. 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 state of low-grade, sterile inflammation that occurs with age and is characterized by elevations of serum concentrations of proinflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), as well as the acute-phase reactant C-reactive protein (CRP) (1). Given the incidence, morbidity, and mortality of inflammation-based chronic disease, proinflammatory molecules, including CRP, are avidly studied.

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 TNF-α (3, 4). It is well established that circulating levels of CRP and IL-6 are correlated in humans (5, 6). CRP 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 TNF-α 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.

Several studies have focused on the transcriptional regulation of CRP. The *CRP* promoter contains consensus sequences for the transcription factors STAT3 and C/EBPβ, which 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 the proximal C/EBP site (15, 16). IL-1β, 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 posttranscriptional mechanisms that regulate CRP expression.

Mammalian cells regulate gene expression robustly via posttranscriptional mechanisms controlled by RNA-binding proteins (RBPs) 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. Human antigen R (HuR) is a ubiquitously expressed RBP belonging to the Hu/Elav family that modulates the stability, translation, and lo-

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calization 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 immune and stress responses (24, 25). HuR modulates inflammatory responses by promoting the expression of proinflammatory proteins such as COX-2, IL-8, transforming growth factor β (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 noncoding RNAs that modulate gene expression posttranscriptionally by binding to target mRNAs bearing regions of partial complementarity to 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 posttranscriptional 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 miRNA 637 (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 minimal essential medium (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 the vehicle control (phosphate-buffered saline [PBS] and dimethyl sulfoxide [DMSO], respectively). Control small interfering RNA (siRNA) and HuR siRNA (5′-AAGAGGCAATTACCAGT TTCA-3′) were obtained from Qiagen. The pre-miRNA precursor control, the miR-637 and anti-miRNA inhibitor controls, and miR-637 were purchased from Ambion. All siRNAs, miRNAs, and plasmids were transfected by using either Lipofectamine-2000 (Invitrogen) or Lipofectamine-RNAiMAX (Invitrogen). RNA and protein were isolated from the cells 48 h after transfection.

Ribonucleoprotein immunoprecipitation assay. For ribonucleoprotein (RNP) immunoprecipitation (RIP), HepG2 cells or HeLa cells (24 h after transfection with miR-637) (see Fig. 5B) were lysed in a solution containing 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 incubated with 0.1% SDS–0.5 mg/ml proteinase K for 15 min at 55°C. The RNA from the immunoprecipitation (IP) samples was extracted by using acidic phenol, precipitated in isopropanol, and analyzed by reverse transcription and quantitative real-time PCR (RT-qPCR).

RNA isolation and RT-qPCR analysis. Total RNA was isolated from cells by using TRIzol (Invitrogen) according to the manufacturer's instructions. After RT using random hexamers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen), the abundance of transcripts was assessed by qPCR analysis using 2× SYBR green master mix (Applied Biosystems). A QuantiMir RT kit (System Biosciences) was used for cDNA synthesis for miRNAs, U6 snRNA, and snoRNAs. RT-qPCR analysis was performed on an Applied Biosystems model 7500 real-time PCR machine. The following primer pairs were used (forward and reverse, respectively, in each case): AGACATGTCGAGGAAGGCTTTT and TCG AGGACAGTTCCGTGTAGAA for *CRP*, GTGACATCGGGAGAAC GAAT and GCGGTCACGTAGTTCACAAA for *ELAVL1* (HuR), GCTC CTCCTGTTCGACAGTCA and ACCTTCCCCATGGTGTCTGA for the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), CCCTATC AACTTTCGATGGTAGTCG and CCAATGGATCCTCGTTAAAGG ATTT for 18S, AGATGGTCAAGGTCGCAAGCT and GGGCATATCCT ACAACAAACTTGTC for *HPRT1*, ATTTGGGTCGCGGTTCTTG and TG CCTTGACATTCTCGATGGT for *UBC*, TACAAGTACCTCACCGCT TGGT and TGATCTTGTCTTGGTGCTCGTA for the *Renilla* luciferase gene (*RL*), and CTAAGAAGGGCCTGCAGAAGAT and AAGCCCTGGT AGTCGGTCTTAG for the firefly luciferase gene (*FL*). To measure the abundance of miRNAs, the following forward primers were used: GCGA TAACTGACGAAGACTAC for RNU49, TTAAACCACCAAGATCG CTGA for RNU24, GATATCACTGTAAAACCGTTCC for U47, ACTGG GGGCTTTCGGGCTCTGCGT for miR-637, and CACCACGTTTATAC GCCGGTG for U6. The universal primer supplied by the QuantMir RT kit was used as the reverse primer. For peripheral blood mononuclear cell (PBMC) analysis, HuR expression was normalized to the average *HPRT* and *UBC* expression levels by using gene-specific primers. The miR-637 expression levels were normalized to the average expression levels of snoRNAs RNU24, RNU49, and U47.

Biotin pulldown analysis. *CRP* 3′-UTR fragments a, b, c, and d were amplified from the psiCHECK2 luciferase vectors containing the respective 3′ UTRs by using primers that contained the T7 RNA polymerase promoter sequence (5′-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3′) (35). After purification of the template PCR products, biotinylated transcripts were synthesized by using the MaxiScript T7 kit (Ambion). Whole-cell lysates were incubated with 1 μg of purified biotinylated transcripts for 1 h at room temperature, and complexes were then isolated with streptavidin-coupled Dynabeads M-280 (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 primer pairs were used: (T7)AGCTGTGGGTCCTGAAGGTA and AAGTAAACAGGGGCTTTATT for fragment a, (T7)AGCTGTGGGTC CTGAAGGTA and AGAAATTATCTCCAAGATCT for fragment b, (T7)G ATAATTTCTTACCTCACAT and ATTTATACCTAGTGCTTCAT for fragment c, (T7)ATGAAGCACTAGGTATAAAT and AAGTAAACAGGGGCT TTATT for fragment d, and (T7)CCTCAACGACCACTTTGTCA and GGT TGAGCACAGGGTACTTTATT for the *GAPDH* 3′-UTR fragment.

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 by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. For Fig. 1E, 24 h 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 h at 30°C. HuR binding to the ectopic luciferase transcripts was determined by RT-qPCR analysis using primers specific for the *RL* and *FL* mRNAs. HeLa cells were transfected with 100 ng of the indicated 3′-UTR luciferase reporter constructs and transfected again 24 h later with the control and HuR siRNAs or miR-637. Twenty-four hours later, RL and FL activities were measured by 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.

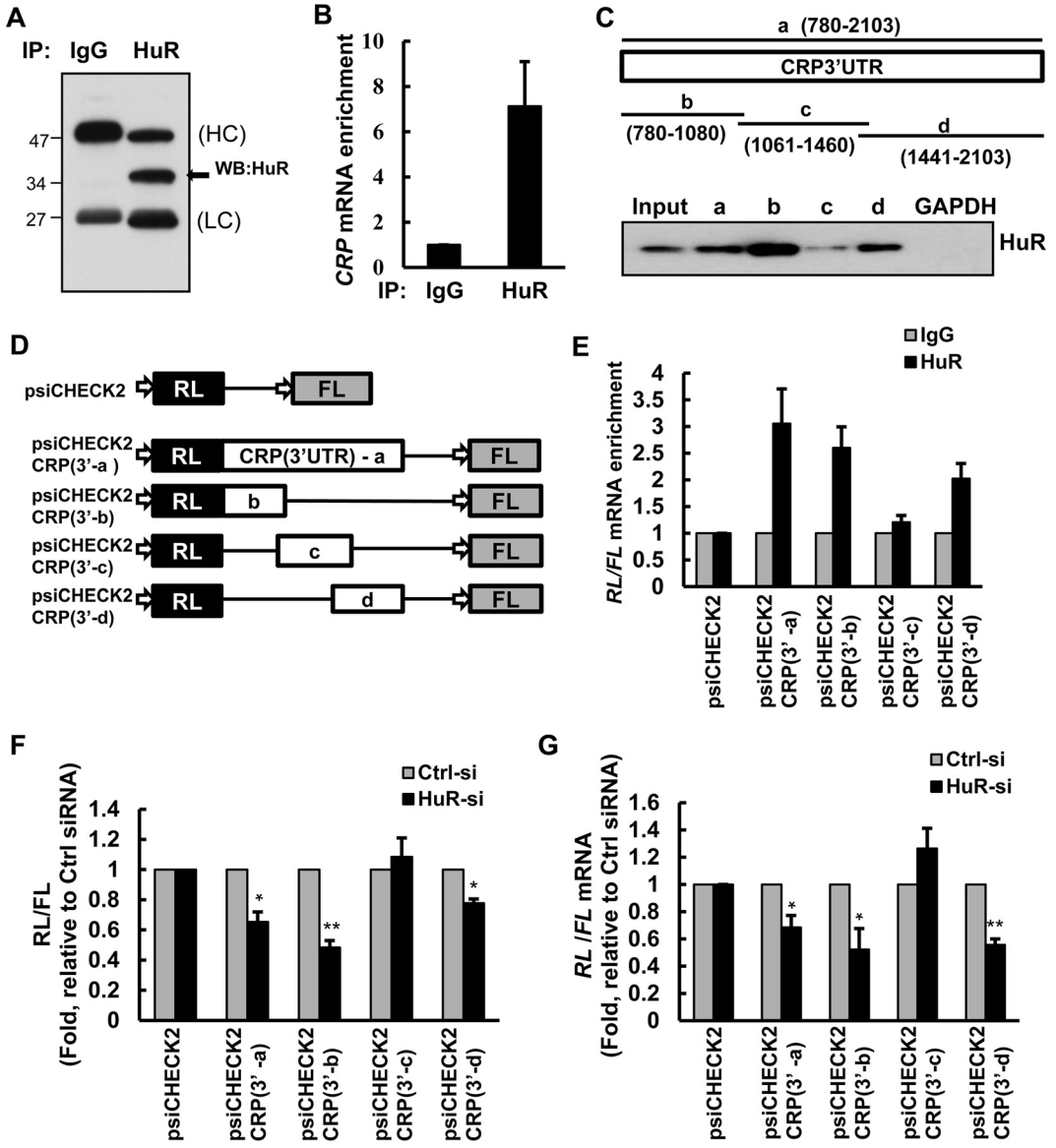

FIG 1 HuR binds to the *CRP* 3′ UTR. (A) HuR immunoprecipitates from HepG2 cells were analyzed by immunoblotting. HuR (arrowhead), the immunoglobulin heavy chain (HC) and light chain (LC), and molecular weight markers are indicated. WB, Western blotting. (B) RIP followed by RT-qPCR analysis was used to measure the enrichment of *CRP* mRNA in HuR IP compared to IgG IP in HepG2 cell lysates, normalized to *GAPDH* mRNA levels for each IP reaction. (C, top) Schematic of the *CRP* 3′ UTR and the different biotinylated RNAs synthesized for use in biotin pulldown analyses (full length and fragments a, b, c, and d). (Bottom) The indicated biotinylated *CRP* RNAs or the control *GAPDH* 3′ UTR was 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 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* mRNA levels. (F) After transfection of HeLa cells with control (Ctrl) or HuR siRNAs, cells were transfected with either psiCHECK2 or the psiCHECK2-*CRP* (3′-UTR) reporters, as indicated. The ratio of RL to FL was normalized to the value for the parent vector (psiCHECK2) and then normalized to luciferase activity in control siRNA-transfected cells. (G) Cells were transfected as described for panel E, and the ratio of *RL* mRNA/*FL* mRNA for each reporter construct was quantified by RT-qPCR. Histograms represent the means and standard errors of the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared to control siRNA, as determined by Student's *t* test).

Polysome analysis. For polysome analysis, 48 h after transfection of HeLa cells with control 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 to 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 hours after transfection of HeLa cells with biotinylated miR-637 or with biotinylated control miRNA from *Caenorhabditis elegans* cel-miR-67 (biot-miR-637 and biot-Ctrl-miR, respectively; both from GE Healthcare Dharmacon), HeLa cells were lysed in a solution containing 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 a 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 bovine serum albumin (BSA) (1 mg/ml) overnight at 4°C. Cytoplasmic lysates were added to the beads, and the beads were 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 by using TRIzol (Invitrogen) as described above and analyzed by RT-qPCR.

Western blot analysis. Cells were washed twice with 1× cold PBS and lysed by using 2× Laemmli sample buffer. The cell lysates were fractionated by SDS-PAGE, transferred onto membranes, and analyzed by using primary antibodies that recognized HuR (Santa Cruz Biotechnology), CRP (Millipore), or the 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 subcohort of women with either low (≤3 mg/liter) or high (≥20 mg/liter) high-sensitivity CRP (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. Fifteen white and 15 African American females with an average age (± standard deviation) of 49.7 ± 8.1 years were used for this study. RNA was isolated from PBMCs by using TRIzol (Invitrogen) according to the manufacturer's instructions. Serum IL-6 levels were quantified by using Searchlight protein arrays from Aushon Biosystems (Billerica, MA).

RESULTS

The RNA-binding protein HuR interacts with the 3′ UTR of *CRP* mRNA. To investigate whether *CRP* mRNA was associated with HuR, we performed ribonucleoprotein (RNP) immunoprecipitation (RIP) assays with 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 the isolation of RNA in 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 (~>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) 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 a negative-control transcript, the 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 express *Renilla* luciferase (RL) from constructs lacking or containing the *CRP* 3′ UTR (psiCHECK2 and psiCHECK2-*CRP* 3′UTR, respectively). These plasmids also contain firefly luciferase (FL), which served as an internal transfection control. We used RIP assays to test the binding of HuR to the ectopically expressed luciferase transcripts. As shown, HuR selectively associated with *RL* mRNAs containing *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 (regions b and d) (Fig. 1C), we first analyzed the luciferase reporter constructs. The ratio of RL activity/FL activity from each transfected reporter plasmid indicated that silencing of 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. 1F). 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 affects the 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.

HuR promotes CRP 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 RNAs (siRNAs). Silencing of 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 shown in Fig. 1G, we investigated whether 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 by RT-qPCR, using 18S rRNA levels for normalization. We found that silencing of HuR selectively decreased the $t_{1/2}$ of *CRP* mRNA to ~6 h, compared to its half-life in control siRNA-transfected cells, which was far higher 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 to 7) and high-molecular-weight (HMW) (fractions 8 to 10) polysomes. RNA was extracted from each of the 12 fractions obtained, 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 of HuR (Fig. 2C). The distribution of the housekeeping *GAPDH* mRNA was the same between the two groups, indicating that silencing of 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 pro-

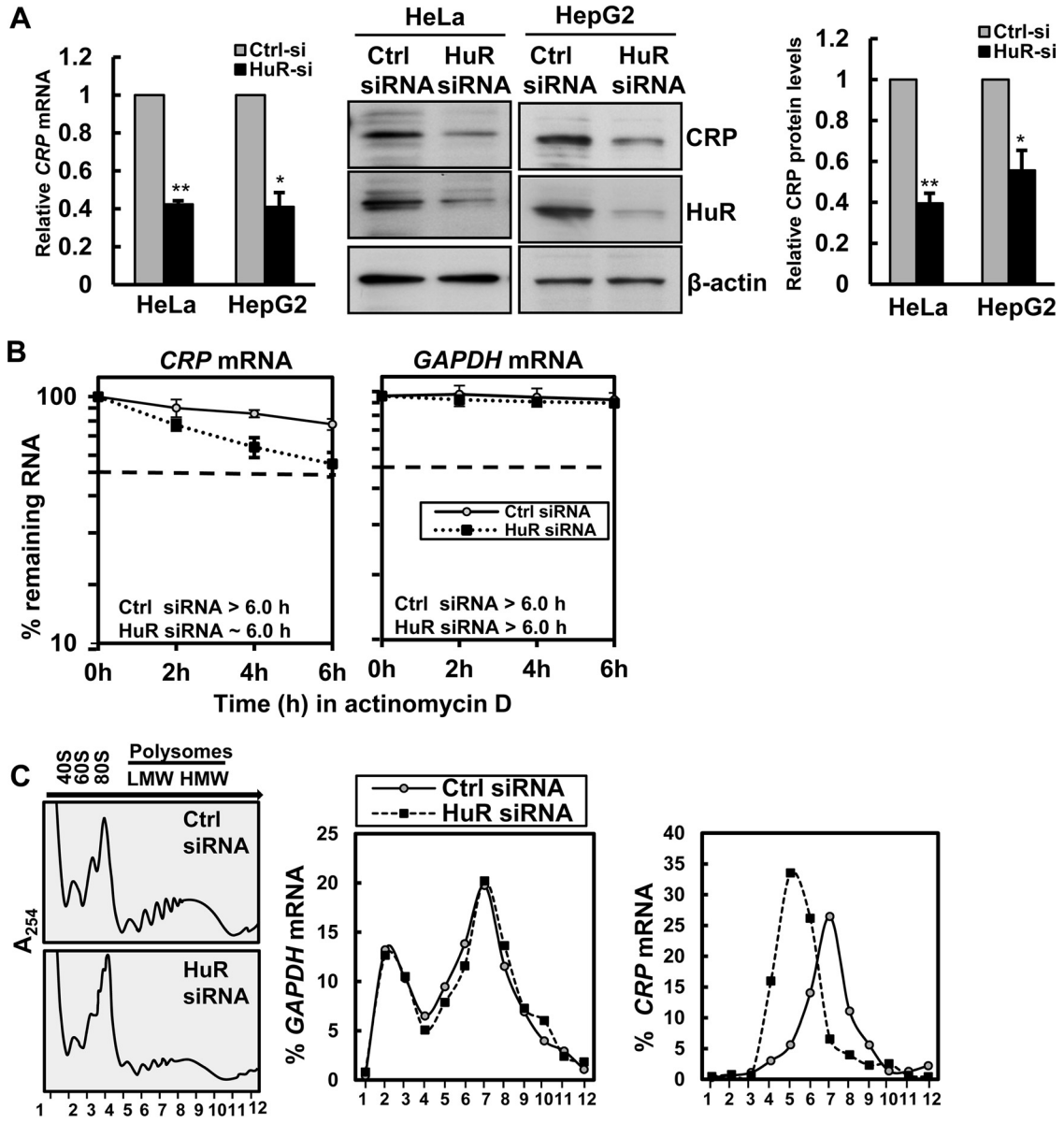

FIG 2 HuR promotes *CRP* mRNA stability and translation. (A, left) Forty-eight hours after transfection of HeLa and HepG2 cells with either control siRNA or HuR siRNA, *CRP* mRNA levels were measured by RT-qPCR analysis. (Right) Transfected HepG2 cells or HeLa cells were assessed by immunoblotting using 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 means and standard errors of the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (compared to control siRNA, as determined by Student's *t* test). (B) HeLa cells transfected as described for panel A were treated with actinomycin D (2 μg/ml), and RNA was isolated at the times indicated. The *CRP* mRNA and *GAPDH* mRNA levels were assessed by RT-qPCR and normalized to 18S rRNA levels. The $t_{1/2}$ values for *CRP* and *GAPDH* mRNAs were quantified by measuring the time needed for the transcript levels to reach 50% of their original abundance at time zero. (C) HeLa cells transfected with either control 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 to 4 and progressively larger polysomes from low to high molecular weights in fractions 6 to 12 are shown at the left. The distribution (percent) of *CRP* and *GAPDH* mRNAs was quantified by RT-qPCR analysis of RNA isolated from 12 gradient fractions.

posed that the age-associated increase in IL-6 levels 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, previously reported data showed that IL-6 can upregulate CRP levels (14). Given this relationship, we hypothesized that HuR may contribute to the increased *CRP* mRNA expression levels in response to IL-6. To test this idea, we treated HepG2 cells with IL-6 and then performed a HuR RIP assay. This analysis revealed that more HuR was 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 C). HuR silencing significantly decreased *CRP* mRNA and protein levels in the presence of IL-6. In contrast, HuR mRNA and protein levels were not

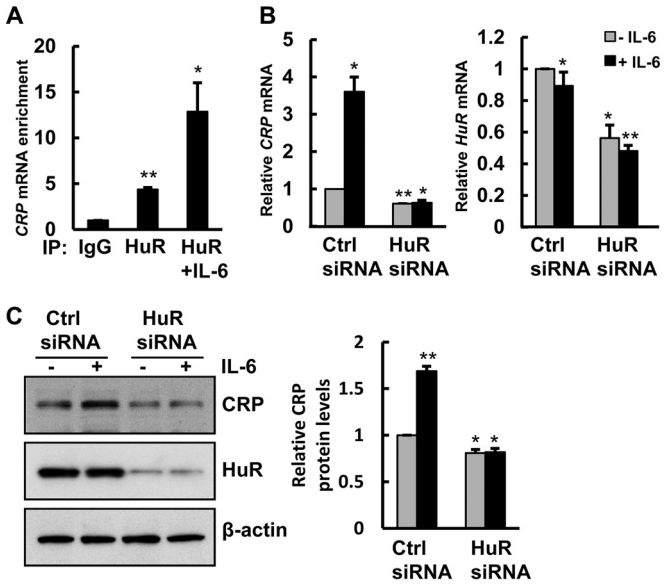

FIG 3 IL-6-mediated upregulation of CRP is dependent on HuR. (A) After IL-6 (50 ng/ml) treatment for 15 min, RIP analysis was performed by using HuR or IgG antibodies. *CRP* mRNA levels were measured by RT-qPCR analysis. (B and C) HepG2 cells were transfected with either control siRNA or HuR siRNA and treated with or without IL-6 for 24 h. 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 values for the β-actin control. The histograms represent the means and standard errors of the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (by Student's *t* test).

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 abundances (Fig. 4A and B), indicating that CRP levels can be modulated by miR-637. In contrast, decreasing the levels of endogenous miR-637 by transfection with an miR-637 antagomir (anti-miR-637) increased both *CRP* mRNA and protein expression levels (Fig. 4C and D). Similar results were also observed by using a locked nucleic acid (LNA) (LNA-anti-miR-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 the *CRP* 3′-c fragment, which contains the predicted miR-637 site (Fig. 4E and F), but this effect was abolished when the miR-637 site was mutated (Fig. 4F). These results suggest that miR-637 reduces the expression levels of CRP by interacting with the *CRP* 3′ UTR.

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

Next, we examined the effect of HuR silencing on miR-637-induced repression of CRP expression. Silencing of HuR additively repressed the miR-637-mediated repression of CRP expression, as indicated by decreases in the levels of *CRP* mRNA and protein in miR-637-overexpressing cells (Fig. 5C). In contrast, ectopic HuR overexpression inhibited the miR-637-mediated repression of CRP expression (Fig. 5D). In addition, we investigated whether HuR and miR-637 competed to regulate CRP expression in response to IL-6 treatment. As shown in Fig. 5E, overexpression of miR-637 or silencing of HuR decreased *CRP* mRNA levels following IL-6 treatment. Simultaneous miR-637 overexpression and HuR silencing in cells had an additive effect on decreasing the CRP abundance in response to IL-6 (Fig. 5E). These findings suggest that HuR and miR-637 competitively regulate *CRP* mRNA.

Individuals with high CRP levels have high levels of HuR and low levels of miR-637. In order to test whether HuR and miR-637 may affect CRP levels *in vivo*, we obtained peripheral blood mononuclear cells from participants in the HANDLS study who have either low (≤3 mg/liter) or high (≥20 mg/liter) circulating levels of hsCRP. HuR mRNA levels were higher and miR-637 levels were lower in individuals with high hsCRP levels, consistent with our *in vitro* data showing that HuR positively and miR-637 negatively regulated CRP expression (Fig. 6A and B). 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 posttranscriptional mechanisms has not been reported. Here, we found that HuR and miR-637 play key roles in the posttranscriptional 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 posttranscriptional 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 inflammation (26, 27). These data indicate that HuR might be valuable as a therapeutic target for acute or chronic inflammatory

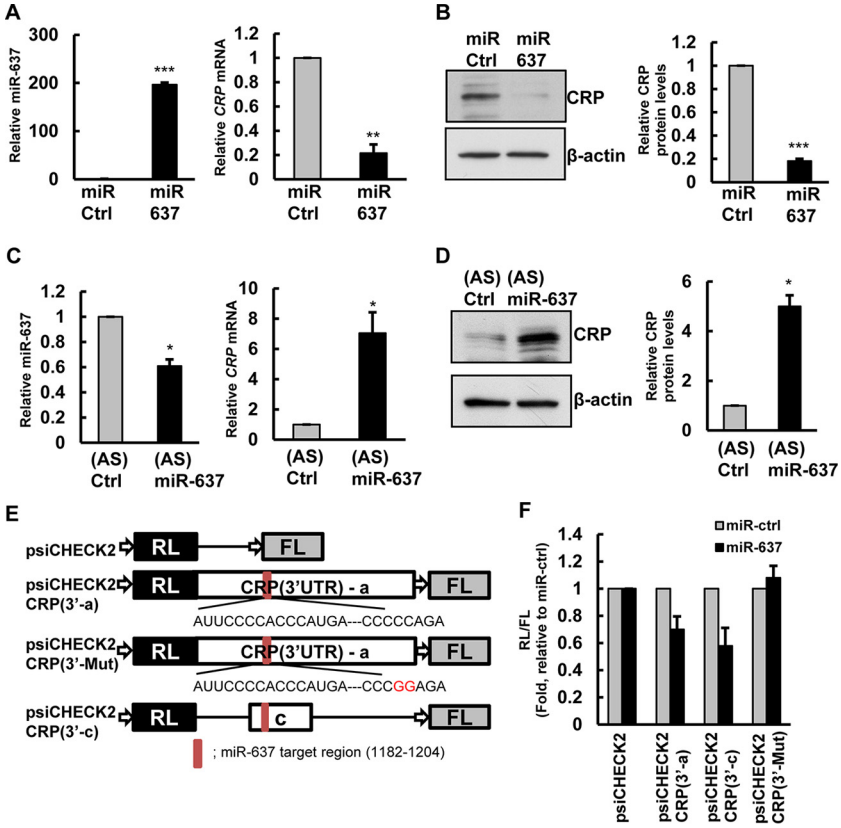

FIG 4 miR-637 interacts with *CRP* mRNA. (A and B) Forty-eight hours after transfection with control miRNA (miR-Ctrl) or with miR-637, the levels of miR-637 (left) and *CRP* mRNA (right) were measured by RT-qPCR analysis (A), or protein levels were assessed by immunoblotting with anti-CRP and anti-β-actin antibodies, as a loading control (B). CRP protein levels were quantified from immunoblots and normalized to values for β-actin. (C and D) HeLa cells were transfected with antisense (AS) miR-637 or control miRNA. After 72 h, the levels of miR-637 (left) and *CRP* mRNA (right) were measured by RT-qPCR analysis (C), or the protein levels of CRP and control β-actin were analyzed by immunoblotting (D). CRP protein levels were quantified from immunoblots and normalized to values for β-actin. (E) Schematic of psiCHECK2-*CRP* dual-luciferase reporters. Red bar, predicted miR-637 binding site. (F) Twenty-four hours after transfection of HeLa cells with miR-637, cells were transfected with different *CRP* 3′-UTR luciferase reporter plasmids, and luciferase activity was measured (RL/FL ratio). The histograms represent the means and standard errors of the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (by Student's *t* test).

diseases. Currently, anti-inflammatory agents under investigation are directed at the IL-1, TNF-α, and IL-6 pathways. Canakinumab and low-dose methotrexate lead to reductions in IL-6 and CRP levels (10); however, our findings that HuR is essential for IL-6-mediated upregulation of CRP suggest that HuR may be a promising target for the design of therapeutic agents to reduce CRP levels in CVD, diabetes mellitus, and other inflammatory diseases. 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 the modulation of HuR function.

We also found that miR-637 is able to inhibit the expression of CRP. miRNAs play a significant role in posttranscriptional gene regulation predominately by acting as repressors (40). miR-637, a primate-specific miRNA, was first identified in 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 levels were reported for four hepatocellular carcinoma cell lines; overexpression of miR-637 inhibited growth and enhanced apoptosis by suppressing the 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 decrease the levels of CRP in at-risk individuals.

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 regions spanning positions 780 to 1080 and 1441 to 2103 of the *CRP* 3′ UTR, while the target site of miR-637 was located at the sequence spanning positions 1182 to 1204, directly 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 data from other reports showing that HuR and miRNAs operate in opposite directions

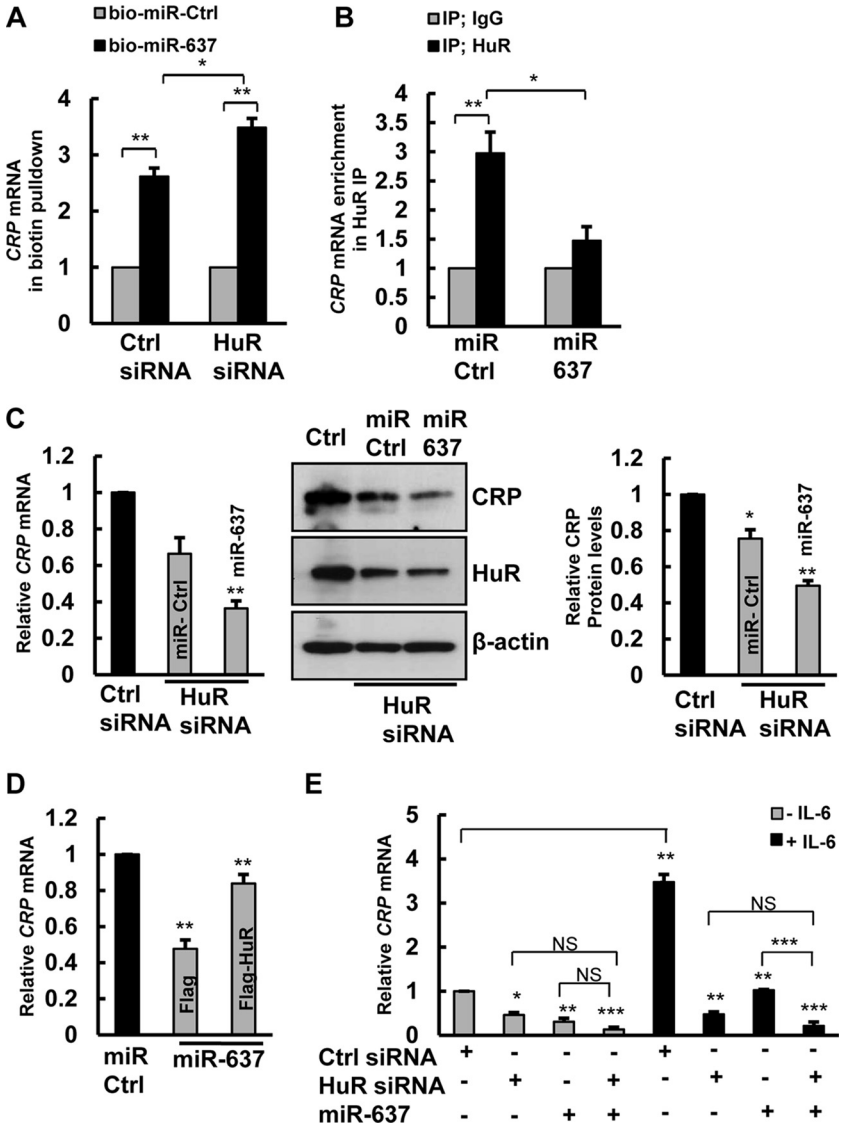

FIG 5 HuR and miR-637 regulate CRP expression competitively. (A) Control siRNA or HuR siRNA and either biotinylated miR-637 (bio-miR-637) or the control (bio-miR-Ctrl) were transfected into HeLa cells for 24 h. Biotinylated miRNAs were precipitated by using streptavidin beads. Enrichment of *CRP* mRNA was assessed by RT-qPCR. (B) Twenty-four hours after transfection of HeLa cells with miR-Ctrl or miR-637, RIP followed by RT-qPCR analysis was used to measure the enrichment of *CRP* mRNA in HuR IP compared to IgG IP. (C) Effects of silencing of HuR together with miR-637 on CRP expression. HeLa cells were cotransfected with HuR siRNA and miR-637; 48 h later, lysates were assessed for the level of *CRP* mRNA by RT-qPCR analysis (left) and the levels of HuR, CRP, and the loading control β-actin by immunoblot analysis (right). CRP protein levels were quantified from immunoblots and normalized to β-actin levels. (D) Forty-eight hours after HeLa cells were cotransfected with Flag-HuR and miR-637, *CRP* mRNA levels were measured by RT-qPCR and normalized to *GAPDH* mRNA levels. (E) HepG2 cells were transfected with HuR siRNA or miR-637 and then treated with IL-6 (50 ng/ml) for 24 h or left untreated. The *CRP* mRNA levels were examined by RT-qPCR. The histograms show the means and standard errors of the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant (as determined by Student's *t* test [A to D] and by one-way analysis of variance and Tukey's *post hoc* test [E]).

(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 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 conformational 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 levels, 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 that we found with 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

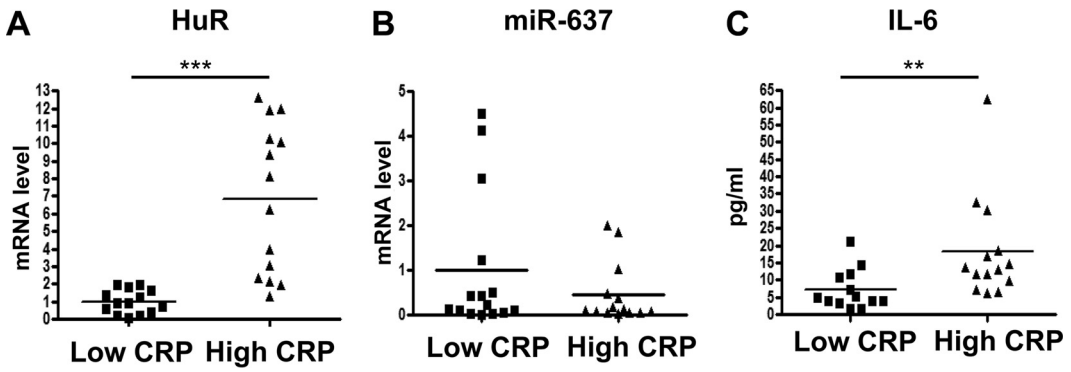

FIG 6 Individuals with high CRP levels have high levels of HuR and low levels of miR-637. (A and B) RNA was isolated from PBMCs of individuals with either low (≤3 mg/liter) or high (≥20 mg/liter) circulating CRP levels ($n = 15$/group). The levels of *HuR* mRNA and miR-637 were quantified by using RT-qPCR. *HuR* mRNA levels were normalized to *HPRT1* mRNA and *UBC* mRNA levels. miR-637 levels were normalized to snoRNA levels (*SNU24*, *SNU49*, and *U47*). (C) Serum protein levels of IL-6 from the same individuals as those for panels A and B were quantified. Means in the graph are indicated by bars. **, $P < 0.01$; ***, $P < 0.001$ (by a Mann-Whitney U test).

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 under way. However, therapeutic agents target mainly upstream biomarkers. The role of CRP as both a risk factor and a biomarker warrants investigation of its regulation by other molecular factors. We have discovered a posttranscriptional mechanism by which the RBP HuR and the 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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